The Regulation of Programmed and Pathological Cell Death in *C. elegans*

by

Brendan D. Galvin

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Signature of author_____

Brendan D. Galvin
September 18, 2006

Certified by_____

H. Robert Horvitz
Professor of Biology
Thesis Supervisor

Accepted by_____

Stephen P. Bell
Chairman of the Graduate Committee
Department of Biology

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Abstract

Programmed cell death, or apoptosis, is important in the development and homeostasis of metazoans. In the nematode *C. elegans*, four genes, *egl-1*, *ced-9*, *ced-4*, and *ced-3*, constitute the core pathway acting in all somatic programmed cell deaths. This pathway is evolutionarily conserved in humans. The BH3-only protein EGL-1 is transcriptionally upregulated in cells fated to undergo programmed cell death, and EGL-1 blocks cell-death inhibition by the cell-death regulator CED-9, a Bcl-2 family member. The binding of EGL-1 to CED-9 releases the Apaf-1-like adaptor protein CED-4 from CED-9, so that CED-4 can activate the caspase CED-3, a protease that is the effector of programmed cell death. In this thesis, I describe three projects, each of which examines one aspect of *C. elegans* cell death.

From screens for mutations that increase cell death in a sensitized genetic background, I identified a gene that protects cells from programmed cell death. This gene, *spk-1*, encodes a homolog of SR protein kinases, which regulate alternative splicing. Previous work has shown that *ced-4* pre-mRNA is alternatively spliced to generate two transcripts that function oppositely in cell death. I found that *spk-1* regulates *ced-4* transcript splicing, thereby influencing the amount of programmed cell death that occurs.

From a screen for genes that promote programmed cell death, I isolated a mutation in a conserved non-coding element in the transcriptionally regulated cell-death activator gene *egl-1*. This element regulates the deaths of specific cells in the *C. elegans* ventral nervous system. I found a novel *C. elegans* transcription factor, Y38C9A.1, that binds this element and might function to regulate *egl-1* transcription and programmed cell death in the ventral nervous system.

In addition to the programmed cell deaths that occur in *C. elegans*, pathological death of specific cells can be caused by mutations in some genes. I characterized two genes, *lin-24* and *lin-33*, that can mutate to cause the inappropriate death of specific hypodermal blast cells. One of these genes, *lin-24*, contains a domain similar to that found in some bacterial toxins. By morphological and genetic criteria, I show that the *lin-24*- and *lin-33*-mediated deaths are unlike previously characterized necrotic and apoptotic cell deaths in *C. elegans*. These deaths require some of the genes responsible for engulfing the corpses generated by programmed cell death, even though the deaths do not require the core genes of the genetic pathway of programmed cell death.

Thesis Advisor: H. Robert Horvitz
Title: Professor of Biology

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TABLE OF CONTENTS

Chapter One

Introduction

I. INTRODUCTION

Initially it seems counterintuitive that cells would posses the ability to kill themselves. However, considering that damaged, infected, or unnecessary cells might harm an organism, the ability to eliminate such cells in a safe way is clearly important. One of the most intuitive examples of a type of cell that should be eliminated are cells in the immune system that recognize self-antigens; such cells can destroy healthy tissues if allowed to persist in the body (FADEEL and ORRENIUS 2005).

The process of cellular elimination, which functions in many different processes, has long fascinated researchers. In 1842, Carl Vogt published a paper that many cite as the first to describe the cell-death process that would later become known as programmed cell death or apoptosis. In this manuscript, Vogt described cell deaths that occurred in toads as they underwent metamorphosis (CLARKE and CLARKE 1996). Over the next century additional papers reported descriptions of similar phenomena in other systems, ranging from ovarian follicles to neurons in the chick embryo (CLARKE and CLARKE 1996). In 1965, Lockshin and Williams proposed the term programmed cell death to describe the deaths they observed during insect development (LOCKSHIN and WILLIAMS 1965). Kerr, Wyllie, and Currie introduced the term apoptosis in 1972 to describe cellular death that occurs with a specific set of ultrastructural features (KERR *et al.* 1972).

Apoptosis is the efficient process of eliminating cells in a manner that prevents the release of cellular contents in order to prevent the consequent deleterious effects such as activation of an inflammatory response. Apoptosis functions in many aspects of human development, from sculpting tissues (*e.g.* removing the webbing between fingers) to eliminating excess neurons in the central nervous system (JACOBSON *et al.* 1997). When the

normal functions of apoptosis do not work properly, a wide range of consequences can result. For example, cancerous cells have frequently lost the ability to undergo apoptosis in response to uncontrolled growth. Conversely, several neurodegenerative disorders are marked by, and may result from, the inappropriate activation of too much apoptosis (FADEEL and ORRENIUS 2005).

Although the phenomenon of programmed cell death has been observed and written about for more than 150 years, confirmation that this process is genetically controlled is only twenty years old, and the identification of the genes that regulate this process is still very much an ongoing process. Genetic, cellular, and biochemical studies have all contributed significantly to our current understanding of programmed cell death (DANIAL and KORSMEYER 2004).

The identification of three genes in the nematode *Caenorhabditis elegans*, *ced-3*, *ced-4*, and *ced-9*, defined the basic components of programmed cell death or apoptosis that function in all systems (HORVITZ 2003). In this introduction I will give an overview of the current state of research of programmed cell death in *C. elegans* and how it relates to our understanding of apoptosis in mammals. I will discuss how cells decide to live or die, how execution of programmed cell death occurs once a cell has made this decision, and how cell corpses are eliminated. I will also mention a few types of cell deaths that occur in *C. elegans* that are morphologically and genetically distinct from programmed cell death. Finally, I will summarize mechanisms that function in mammalian or insect apoptosis but have not been described in *C. elegans*.

II. DEVELOPMENTAL PROGRAMMED CELL DEATH IN *C. ELEGANS*

A. The History of Genetic Studies of Programmed Cell Death in *C. elegans*

C. elegans is the small nematode that Sydney Brenner wisely selected for genetic characterization (BRENNER 1974). Early in the development of *C. elegans* as a system for genetic research, John Sulston determined the cell lineage of the ventral nerve cord (SULSTON 1976). Bob Horvitz and others joined Sulston in this pioneering effort and determined the complete lineage of all cells in this nematode (KIMBLE and HIRSH 1979; SULSTON and HORVITZ 1977; SULSTON *et al.* 1983). The pattern of somatic cell division is almost identical in every individual worm, such that the origin and identity of every cell from fertilization to adulthood is known. The pattern of cell divisions has been drawn up into a complete lineage diagram for *C. elegans*. This lineage map has proven to be a great resource in studies of developmental biology.

Sulston and Horvitz observed that some of the cells generated in the post-embryonic cell lineage were rapidly eliminated in a highly-stereotyped fashion (SULSTON and HORVITZ 1977). One hundred and thirty-one of the 1,030 somatic cells that are generated in the *C. elegans* hermaphrodite undergo this stereotyped elimination; of these, 105 are neurons (SULSTON and HORVITZ 1977; SULSTON *et al.* 1983). The genetic foundations that Sydney Brenner established for this organism suggested the possibility that it might be possible to isolate mutations perturbing this pattern of programmed cell death.

In an effort to identify genes that control the cell lineage of the ventral nerve cord, John Sulston identified an allele of a gene, *nuc-1* (*nuc*, abnormal nuclease), that prevented the degradation of DNA of cells that died by programmed cell death (SULSTON 1976). Next, Ed Hedgecock isolated *ced-1* and *ced-2* (*ced*, cell death abnormal) mutant alleles that blocked the phagocytosis of dead cells by neighboring cells (HEDGECOCK *et al.* 1983).

Although it would be many years before any of these genes were molecularly identified, their isolation was a significant contribution to this field of research. The long-term study of programmed cell death in Bob Horvitz's laboratory led to the identification of the principal genes that function in apoptosis in all animals (HORVITZ 2003). Important to the success of *C. elegans*'s role in defining the basics of programmed cell death is that, unlike most other animals, *C. elegans* are viable in the absence of programmed cell death (ELLIS and HORVITZ 1986). This trait has greatly facilitated much of the progress towards understanding of programmed cell death that has been made using this organism.

Since the identification of *nuc-1*, hundreds of alleles of genes that alter programmed cell death have been isolated; for example in the Horvitz laboratory alone, more than 200 alleles of the gene *ced-3* have been identified as of this writing. Figure 1 shows a schematic of how the best understood of these genes fit into our current model of programmed cell death.

B. The Four Genes *ced-3*, *ced-4*, *ced-9*, and *egl-1* Define a Core Programmed Cell Death Pathway that is Conserved in Most Animals

1. ced-3 *encodes a cysteine protease*

One of the first programmed cell death genes in *C. elegans* to be molecularly identified is *ced-3*, and its identification may be the most important. In a screen to identify suppressors of the persistent programmed cell death corpses observed in mutant *ced-1* animals, *ced-3* alleles that eliminated these corpses were identified (ELLIS and HORVITZ 1986). Close inspection of these mutant animals showed that almost none of the cells that normally died during *C. elegans* development died in these mutants; rather, these cells

survived and showed signs of differentiation (ELLIS and HORVITZ 1986). The cells that survive in *ced-3* mutant worms have not been found to subsequently divide and often adopt a cell fate similar to that of their sister cell. These inappropriately surviving cells can, in some cases, even functionally replace their sister cells (AVERY and HORVITZ 1987).

Years later, *ced-3* was cloned and found to be similar to the mammalian protease interleukin-1β-converting enzyme (ICE) (YUAN *et al.* 1993). The discovery that CED-3 was a protease provided the first mechanistic insight into apoptosis. CED-3 and ICE were the founding members of the cysteine proteases, or caspases (cysteine aspartate-specific protease), that have been shown to function as key effectors of apoptosis in all systems (DEGTEREV *et al.* 2003). Although *ced-3* is the only caspase known to function in *C. elegans*, three other caspase-like genes have been identified and remain to be genetically characterized (SHAHAM 1998). *ced-3* is required in cells fated to undergo programmed cell death, demonstrating that this gene acts cell-autonomously in a process of cellular suicide (YUAN and HORVITZ 1990). When overexpressed in cells that do not normally die in *C. elegans*, *ced-3* can cause their deaths by a process that shares morphological and genetic features with normal programmed cell deaths (SHAHAM and HORVITZ 1996b).

Caspases such as CED-3 are synthesized as inactive zymogens and become active only when they are cleaved, usually through autocleavage or by other proteases, particularly other caspases. The cleavage liberates two subunits, *e. g.* p20 and p10 (RAMAGE *et al.* 1995; YAMIN *et al.* 1996), which heterodimerize to form an active caspase complex composed of two p20 subunits and two p10 subunits (WALKER *et al.* 1994).

In mammals and *D. melanogaster*, the apoptotic caspases can be divided into two classes, the initiator caspases and the effector caspases. The distinction between initiator and

effector caspases does not appear relevant to cell death in *C. elegans*, in which only the caspase CED-3 has been shown to act. The initiator caspases, which resemble CED-3, are characterized by a long prodomain that can function to mediate protein-protein interactions with upstream regulatory molecules (DEGTEREV *et al.* 2003). These caspases generally function by activating the effector caspases, which are characterized by their short prodomains. Once activated, these effector caspases cleave substrates directly involved in the process of apoptosis. Initiator caspases possess substrate preferences that make them better suited to the cleavage and activation of effector caspases than to the cleavage of downstream substrates (THORNBERRY *et al.* 1997). There are numerous mammalian caspases; some of them, such as caspases 3, 6, 7, 8, 9, and 10, have well-established roles in programmed cell death (SADOWSKI-DEBBING *et al.* 2002). In all systems, the final stage in the regulation of apoptosis appears to be the determination of the level of caspase activity.

2. ced-4 *encodes an adaptor protein that is similar to Apaf-1 and activates CED-3*

As a direct result of the *ced-1* suppression screen that identified alleles of *ced-3*, screens were performed that recovered a mutant allele of *ced-4* (ELLIS and HORVITZ 1986). Unlike the molecular identification of *ced-3*, the cloning of *ced-4* failed to immediately provide insight into its function (YUAN and HORVITZ 1992). Like *ced-3*, *ced-4* is required for essentially all programmed cell deaths and acts cell-autonomously (YUAN and HORVITZ 1990). Genetic analysis suggests that *ced-4* functions upstream of *ced-3*. Specifically, overexpression of *ced-4* in the presence of *ced-3* causes robust cell killing, whereas without *ced-3*, this overexpression of *ced-4* does not kill efficiently. Additionally, overexpression of *ced-3* without *ced-4* does kill (SHAHAM and HORVITZ 1996b). Biochemical experiments

using various assays suggested that CED-4 binds to CED-3 and facilitates its activation (CHINNAIYAN *et al.* 1997; SESHAGIRI and MILLER 1997; WU *et al.* 1997; YANG *et al.* 1998). CED-4 has a caspase activation and recruitment domain (CARD) and an AAA ATPase domain. The CARD domain allows CED-4 to interact with and activate CED-3. The function of the putative ATPase domain remains to be established.

Analysis of the *ced-4* cDNAs of the genomic loci of *ced-4* and its homolog in the closely related nematodes *C. briggsae* and *C. vulgaris* led to the discovery that there are at least two splice variants of *ced-4*: the principal splice variant, *ced-4S*, comprising ~90% of observed messages, and an alternate splice variant, *ced-4L*, containing an extra 72 nucleotides within the coding sequence (SHAHAM and HORVITZ 1996a). The two *ced-4* splice variants appear to function oppositely in programmed cell death: the short variant *ced-4S* promotes programmed cell death, whereas the long variant *ced-4L* may promote cell survival (SHAHAM and HORVITZ 1996a). In Chapter 2, I present the characterization of the *spk-1* gene, which is a modifier of *ced-4* killing activity and a regulator of the alternative splicing of *ced-4 in vivo*.

The best-studied mammalian homolog of CED-4 was identified from a labor-intensive biochemical purification in the laboratory of Xiaodong Wang. This approach led to the identification of three fractions that were required for Caspase-3 cleavage, a hallmark of mammalian apoptosis, in a cell-free system. One of these fractions contained the CED-4 homolog Apaf-1 (ZOU *et al.* 1997). Like CED-4, Apaf-1 binds to a caspase (Caspase-9, called Apaf-3 in this biochemical purification scheme) through interaction with its long prodomain, thereby promoting its activation (LI *et al.* 1997). Apaf-1-deficient mice have reduced apoptosis in the brain, hyperproliferation of neuronal cells, and their cells are

resistant to numerous apoptotic stimuli (CECCONI *et al.* 1998; YOSHIDA *et al.* 1998). However, much of the cell death that occurs in the immune system proceeds normally in Apaf-1-deficient mice. Apaf-1 shares two of three domains with CED-4: the CARD domain and the ATPase domain. The third domain, which exists in Apaf-1 but not in CED-4, mediates interactions with cytochrome *c* (ACEHAN *et al.* 2002). The release of the mitochondrial protein cytochrome *c* into the cytoplasm is an important triggering step in many forms of programmed cell death in mammals, but its role in programmed cell death in *C. elegans* has not been examined (LI *et al.* 1997).

3. ced-9 *encodes a protective protein that is similar to the oncoprotein Bcl-2*

In a screen to identify mutations that cause cell survival in *C. elegans*, a gain-of-function allele of *ced-9* was identified, *ced-9(n1950*gf*)* (HENGARTNER *et al.* 1992). This allele, like loss-of-function alleles of *ced-3* and *ced-4*, prevents apoptosis in essentially all somatic cells that normally undergo programmed cell death (HENGARTNER *et al.* 1992). Molecular identification of *ced-9* showed that it encodes a protein similar to the proto-oncoprotein Bcl-2 (HENGARTNER and HORVITZ 1994b). In some follicular lymphomas, Bcl-2 is misexpressed in B cells as the result of a chromosomal translocation (BAKHSHI *et al.* 1985; CLEARY *et al.* 1986; TSUJIMOTO and CROCE 1986). Overexpression of Bcl-2 can protect cells against apoptosis (HOCKENBERY *et al.* 1990; VAUX *et al.* 1988; VAUX *et al.* 1992). The observation that Bcl-2 could functionally replace CED-9 in *C. elegans* provided the first molecular evidence for the existence of a programmed cell death pathway conserved from nematodes to mammals (HENGARTNER and HORVITZ 1994b).

Loss-of-function alleles of *ced-9* cause cells that normally survive to die in a process dependent on the cell-death genes *ced-3* and *ced-4* (HENGARTNER *et al.* 1992). Since elimination of either *ced-3* or *ced-4* prevents the increased death observed in *ced-9(lf)* (loss of function) animals, *ced-9* was proposed to act genetically upstream of *ced-4* and *ced-3* (HENGARTNER *et al.* 1992). Similar to Bcl-2 overexpression in mammalian systems, overexpression of *ced-9* can block normally-occurring programmed cell deaths in *C. elegans* (HENGARTNER and HORVITZ 1994b). *ced-9* also has a cell-killing function that can be observed in genetically sensitized backgrounds (HENGARTNER and HORVITZ 1994a).

Of all the gene families that function in mammalian programmed cell death, the Bcl-2 family is the most complicated. The family members that behave most like CED-9 and Bcl-2 (*i. e.* they are anti-apoptotic), which include Bcl-x_L, Bcl-w, Mcl-1 and A1, generally have four domains found in Bcl-2, known as the Bcl-2 Homology (BH) domains (KUWANA and NEWMEYER 2003). The other main family of Bcl-2 homologs, the pro-apoptotic family, is discussed in the next section.

The observation that many of the Bcl-2 family members are localized to the mitochondria contributed to the hypothesis that mitochondria function in apoptosis (DANIAL and KORSMEYER 2004; HOCKENBERY *et al.* 1990). At the mitochondria, members of the Bcl-2 family may form channels or otherwise regulate the release of apoptosis-promoting factors from the mitochondria into the cytoplasm. The identification of cytochrome *c*, normally found between the inner and outer membranes of the mitochondria, as an important regulator of apoptosis further implicated mitochondria in apoptosis (LI *et al.* 1997). Bcl-2 family members may directly mediate release of cytochrome *c* (KUWANA and NEWMEYER 2003). Several additional functions for mitochondria in apoptosis have been described and

numerous apoptotic mitochondrial associated-proteins have been described, but many remain the subject of debate and none is as well established as cytochrome *c* (KUWANA and NEWMEYER 2003). In mammals, the apoptotic pathway that involves the release of cytochrome *c* from the mitochondria is usually described as the 'intrinsic' pathway, to differentiate it from an 'extrinsic' pathway, which involves extracellular pro-apoptotic ligands and does not necessarily act through the mitochondria. The intrinsic pathway is very important in developmental cell deaths and is the pathway that regulates stress-induced apoptosis such as that caused by DNA damage or the withdrawal of cytokines.

4. egl-1 *encodes a pro-apoptotic BH-3-only protein*

The most upstream gene that functions in the execution of programmed cell death was the last of the execution genes to be molecularly identified. A cell-specific gain-of-function allele of this gene, *egl-1* (*egl*, egg-laying abnormal), was originally identified in a screen for animals that failed to properly lay eggs (TRENT *et al.* 1983). This failure to lay eggs results from the mutant animals' lack of the HSNs (HSN, hermaphrodite-specific neuron), which control egg laying. The inappropriate HSN deaths and the egg-laying defect in this mutant were completely suppressed by loss of *ced-3* function, suggesting that the inappropriate activation of the programmed cell death pathway in the HSNs was causing the inappropriate death of these cells (ELLIS and HORVITZ 1986). When intragenic suppressors of this dominant *egl-1* allele were isolated, a remarkable observation was made: not only were the revertants no longer egg-laying defective, but the HSN neurons of the revertant hermaphrodites no longer died, and essentially none of the 131 somatic developmental programmed cell deaths that occur in wild-type hermaphrodites

occurred in the revertant strains (CONRADT and HORVITZ 1998). Genetic analysis suggested that *egl-1* was functioning upstream of all the execution genes (*ced-3*, *ced-4*, and *ced-9*) to regulate cell death. Further analysis showed that *egl-1* is likely transcribed only in cells developmentally fated to die and that the misexpression of EGL-1 can cause cells to die with morphological and genetic characteristics of programmed cell death.

Molecular identification established that *egl-1* encodes a BH3-only protein. This family of proteins is defined by the possession of only one of the four Bcl-2 homology domains. Mammalian BH3-only proteins are all pro-apoptotic and regulated in numerous ways, including transcription (WILLIS and ADAMS 2005). A second gene encoding a *C. elegans* BH3-only protein, *ced-13*, has been identified and might play a secondary role in *C. elegans* cell death, specifically in the germline (SCHUMACHER *et al.* 2005). The pro-apoptotic BH3-only family in mammals includes the proteins Bim, Bad, Bid, Bik, Bmf, Puma, Noxa, and Hrk, which are generally activated in response to specific types of cellular damage. In addition to transcriptional control analogous to that seen in *C. elegans* (Puma and Noxa (HAN *et al.* 2001; NAKANO and VOUSDEN 2001; ODA *et al.* 2000; YU *et al.* 2001)), mechanisms that regulate the activity of mammalian BH3-only proteins include cleavage (Bid (LI *et al.* 1998; LUO *et al.* 1998)), phosphorylation (Bad (ZHA *et al.* 1996)), and sequestration to components of the cytoskeleton (Bmf (PUTHALAKATH *et al.* 2001) and Bim (PUTHALAKATH *et al.* 1999)).

C. A Molecular Model For the Function of CED-3, CED-4, CED-9, and EGL-1

A combination of cell biological, biochemical, and structural experiments has contributed to a compelling model for how the protein products of *egl-1*, *ced-9*, *ced-4*, and

ced-3 function in programmed cell death. In *C. elegans*, CED-9 is permanently localized to the mitochondria (CHEN *et al.* 2000), where it binds a dimer of CED-4 molecules (YAN *et al.* 2005). In cells that are fated to die, newly transcribed and translated EGL-1 binds to a surface on CED-9, causing a conformational change that releases the CED-4 dimer from CED-9 and the mitochondria. Upon release, CED-4 assembles into a complex containing four CED-4 molecules (YAN *et al.* 2005) and localizes to the nuclear membrane (CHEN *et al.* 2000). This tetramer of CED-4 molecules binds CED-3 and promotes the autoproteolytic activation of CED-3 (YAN *et al.* 2005). This activation of the protease activity of CED-3 causes cell death. The gain-of-function mutation *ced-9(n1950)* alters the interaction surface with EGL-1 and prevents its binding, thus preventing liberation of CED-4 and cell death (YAN *et al.* 2004). The protective CED-4L is believed to protect by preventing the formation of the CED-4 tetramers and the consequent activation of CED-3 (YAN *et al.* 2005).

Although counterparts of each of these components exists and functions in mammalian cell death, aspects of the molecular mechanisms of programmed cell death might differ between worms and mammals. BH3-only proteins in mammals do interact with Bcl-2 family members, but they probably regulate the release of mitochondrial proteins such as cytochrome *c* (KUWANA and NEWMEYER 2003). Unlike in *C. elegans*, mammalian Bcl-2 family members are likely not to interact with Apaf-1 and thus do not activate Apaf-1 by disassociation. Instead, Bcl-2 family members are thought to prevent the release of cytochrome *c* from mitochondria and thereby prevent cytochrome *c* from stimulating the aggregation of Apaf-1 and subsequent Caspase-9 activation.

D. Morphology of Cells Undergoing Programmed Cell Death in *C. elegans*

Cell death in *C. elegans* can be easily observed using Nomarski differential-interference contrast microscopy. Cells progress through several morphological phases as they are dying, and the ultrastructures of these phases have been described (ROBERTSON and THOMSON 1982). At the time of its generation, a cell that is destined to die is usually smaller than its sister and, even before cell division is complete, is sometimes recognized by neighboring engulfing cells. After a period of decreased refractility with little change in cellular architecture, the nucleus appears increasingly refractile when observed by Nomarski. At this stage, electron microscopy (EM) shows that the cytoplasm begins to shrink, the nuclear membranes dilate, and chromatin aggregates begin to form in the nucleus. Next, the entire cell appears refractile and adopts the classic button-like appearance seen with Nomarski optics. Ultrastructurally the nucleus becomes dense, very little cytoplasm is observed, and there is an increase in whorling of internal and plasma membranes. Finally the cell shrinks and disappears, and traces of the corpse can be observed by EM in the engulfing cells. This entire process takes less than an hour in the wild type, but can take several hours in mutants partially compromised in their cell death machinery. Several of these features, including condensation of cytoplasm and chromatin aggregation, are observed in mammalian apoptosis, but others, such as membrane whorls, are not.

E. Engulfment of Programmed Cell Death Corpses

Following the death of cells and the generation of corpses, the corpses are engulfed by neighboring cells. The first genes shown to function in the engulfment of programmed cell death corpses, *ced-1* and *ced-2*, were isolated by Ed Hedgecock in a genetic screen using Nomarski microscopy to identify animals with any abnormality (HEDGECOCK *et al.* 1983). In

animals lacking either *ced-1* or *ced-2* function, unlike in wild-type animals, the cell corpses that were generated by developmental cell death often persist. Whereas in wild-type animals the cell corpses would rapidly disappear, usually within 30 minutes of their generation, the cell corpses of engulfment-defective mutants can persist for hours. This original screen was followed up by additional screens and characterization in the Horvitz laboratory, in which alleles of six additional engulfment genes were isolated (ELLIS and HORVITZ 1991; YU *et al.* 2006; ZHOU *et al.* 2001a).

The eight engulfment genes fall into two partially redundant pathways required for programmed cell death corpse removal: the *ced-1*, *-6*, *-7* and *dyn-1* (dynamin) pathway and the *ced-2*, *-5*, *-10*, *-12* pathway. I will refer to the former as the *ced-1* pathway and the latter as the *ced-2* pathway for the remainder of this chapter. Animals lacking the function of two genes within either pathway do not cause the appearance of more persistent cell corpses than those visible in animals with a single mutation. However, in animals lacking the function of cell corpse engulfment genes from each of the two pathways, many more cell corpses persist than in animals with either of the two mutations on their own (ELLIS *et al.* 1991; GUMIENNY *et al.* 2001; WU *et al.* 2001; YU *et al.* 2006; ZHOU *et al.* 2001a). Careful analysis of which cell corpses persisted in animals with specific alleles suggested that the two pathways are not responsible for engulfing different subsets of cells, but rather are each providing functions partially required for all cell corpse engulfment events (ELLIS *et al.* 1991).

Recent evidence indicates that the engulfment genes function not only in cell corpse removal but also in cell killing (REDDIEN *et al.* 2001). Removal of a single engulfment gene alone can very weakly promote the survival of cells that normally die. In a sensitized genetic background, such as in the presence of a weak *ced-3* loss-of-function allele that by itself

promotes only a small amount of cell survival, elimination of any engulfment gene greatly increases the amount of survival (REDDIEN and HORVITZ 2004). It is believed that, as the cell begins to die, it generates surface markers that neighboring cells recognize; these neighboring cells then begin to engulfment the dying cell and assist in promoting the cell's death.

1. The ced-1, ced-6, ced-7, dyn-1 *pathway functions in recognizing and labeling corpses*

The mechanism of action of the genes that function in the *ced-1* pathway is not entirely clear, despite the fact that some of these genes are homologous to mammalian proteins with known functions. The genes *ced-1*, *-6*, and *-7* appear to function only in the removal of programmed cell death corpses, as no additional abnormalities are associated with mutations in these genes. *ced-1* encodes a protein that is a receptor containing sixteen copies of an atypical EGF-like repeat in its extracellular domain (ZHOU *et al.* 2001b). The short intracellular domain of CED-1 contains tyrosines that might be phosphorylated to regulate the binding of SH2 and PTB (phospho-tyrosine binding) domain containing proteins (ZHOU *et al.* 2001b). The mammalian protein that most closely resembles CED-1 is mEGF10, a mouse protein of unknown function that is related to receptors that have been shown to function in mediating the phagocytosis of apoptotic cells in cell culture assays (MANGAHAS and ZHOU 2005). Analysis using GFP-tagged versions of CED-1 suggests that CED-1 aggregates around cell corpses during their engulfment, suggesting that CED-1 functions in recognizing the cell corpse (ZHOU *et al.* 2001b). Targeted expression suggests that *ced-1* functions in cells responsible for engulfment (rather than in cells that die). Identification of the ligand that CED-1 recognizes on the corpse remains an important unanswered question in

programmed cell death.

ced-6 encodes an adaptor protein containing a PTB domain that has been shown to be capable of interacting with CED-1 (LIU and HENGARTNER 1998; SU *et al.* 2002). CED-6 also contains both a leucine zipper and a proline rich C-terminus, each of which may mediate protein-protein interactions. A mammalian homolog of CED-6 (hCED-6, also called GULP) (LIU and HENGARTNER 1999; SMITS *et al.* 1999), which was identified on the basis of its homology to CED-6, can promote the engulfment of apoptotic cells in mammalian cell culture when overexpressed. Like *ced-1*, *ced-6* is thought to function in cells responsible for engulfment. Because *ced-6* is not required for the clustering of CED-1::GFP in response to apoptotic cells, *ced-6* might act downstream of *ced-1* (ZHOU *et al.* 2001b).

CED-7 is an ATP-binding cassette (ABC) transporter (WU and HORVITZ 1998a). ABC transporters comprise a large family of proteins that actively transport a wide variety of substances (including lipids, sugars, and proteins) across membranes (KLEIN *et al.* 1999). Unlike *ced-1* and *ced-6*, *ced-7* is thought to function in both the engulfing cells and the dying cells, although the significance of this result remains unclear (WU and HORVITZ 1998a). Elimination of *ced-7*, but not the elimination of *ced-6* or other engulfment genes, prevents the accumulation of CED-1::GFP around cell corpses (ZHOU *et al.* 2001b). Because *ced-7* is required in the dying cells and is homologous to ABC transporters, *ced-7* may be responsible for placing a signal that promotes recognition by CED-1 on the surface of the cell corpse. Although such a signal has not been identified, one strong candidate molecule is phosphatidylserine (MANGAHAS and ZHOU 2005).

Recent work has identified a fourth member of this pathway, *dyn-1*. *dyn-1* encodes a large GTPase homologous to dynamin, which in other systems plays well-established roles in

vesicle trafficking (YU *et al.* 2006). Unlike the other genes in the *ced-1* pathway, *dyn-1* is essential. EM analyses suggest that inactivation of *dyn-1* prevents the fusion of intracellular vesicles to the plasma membrane at the site of corpse engulfment. The *dyn-1* work suggests a model in which CED-1 responds to the presence of a cell corpse by accumulating on the surface of the cell next to the cell corpse. The CED-1 proteins then send intracellular signals, likely via CED-6, to recruit intracellular vesicles. This recruitment of vesicles to the plasma membrane is regulated by DYN-1 and allows for extension of the cell membrane around the corpse and engulfment.

2. The proteins CED-2, CED-5, CED-10, and CED-12 are similar to mammalian proteins that regulate cytoskeletal reorganization

Unlike the members of the *ced-1* pathway, whose mechanisms of action are somewhat unclear, each member of the *ced-2* pathway has a clearly identifiable mammalian homolog that functions in cytoskeletal reorganization. Cytoskeletal reorganization is required for many different cellular processes, including cell migration, cell process extension, and phagocytosis. In *C. elegans*, *ced-2*, *-5*, *-10*, and *-12* also function in distal tip cell migration, highlighting their involvement in other processes in *C. elegans* that require cytoskeletal reorganization.

CED-10 is most similar to mammalian Rac1 (REDDIEN and HORVITZ 2000), which is a member of the Ras GTPase superfamily that includes Rho, Rac, and Cdc42. Each of these proteins has a well-established role in cytoskeletal reorganization (JAFFE and HALL 2005). CED-5 is most similar to DOCK180 and is a member of the CDM protein (CED-5, mammalian DOCK180 and *Drosophila* Myoblast city) family (WU and HORVITZ 1998b).

CED-12 is the founding member of a family that includes ELMO1 (GUMIENNY *et al.* 2001; WU *et al.* 2001; ZHOU *et al.* 2001a). CED-5 and CED-12 are thought to function together to act as an atypical guanine nucleotide exchange factor that activates Rac by exchanging GDP for GTP (BRUGNERA *et al.* 2002). Finally, CED-2 is an adaptor protein containing SH2 and SH3 domains and is most similar to mammalian CrkII (REDDIEN and HORVITZ 2000).

The involvement of CED-2 in engulfment might suggest that there is an upstream receptor protein that becomes phosphorylated and binds the adaptor CED-2, thereby promoting CED-2 binding to the CED-5/CED-12 guanine nucleotide exchange factor. Then, CED-5/CED-12 would activate CED-10/Rac1, which promotes cytoskeletal rearrangement. The identity of the receptor and the signal that activate this pathway remains elusive. At one point in my graduate studies, I attempted to knock down the function of each of a majority of predicted *C. elegans* receptor tyrosine kinases using RNAi and screened for engulfment defects. I failed to identify any dsRNAs that caused persistence of cell corpses, however, and thus the receptor functioning in this pathway remains elusive (B. D. GALVIN and H. R. HORVITZ unpublished observations).

The original genetic characterization of this pathway suggested that *ced-10* was likely to function only in a pathway with *ced-2*, *-5*, and *-12*. More recent evidence, including the observation that over-expression of *ced-10* can bypass the block in mutants defective in either the *ced-1* pathway or the *ced-2* pathway but not of mutants lacking function of both pathways, suggests that *ced-10* may also act downstream of the *ced-1* pathway (KINCHEN *et al.* 2005). The fact that *ced-10* is essential for viability of *C. elegans* has made genetic analysis more difficult. Thus, most double-mutant analyses of *ced-10* have been performed using non-null alleles, limiting the conclusions that could be made. Currently there is not a

consensus about whether or not *ced-10* functions in only the *ced-2* pathway or in both the *ced-1* and *ced-2* pathways.

3. Other genes that have been proposed to function in the engulfment of cell corpses

Additional genes have been suggested to function in the engulfment of programmed cell death corpses in *C. elegans*. These genes encode the homologs the phosphatidylserine receptor and a member of the annexin family of proteins. These genes probably do not play a significant role in the engulfment process, however, and have never been identified in screens for mutations that affect engulfment. Interest in these genes began because a hallmark uniquely present on the outer surface of the cell membranes of apoptotic corpses in mammalian systems is phosphatidylserine (FADOK *et al.* 1992). For years, the correlation between phosphatidylserine exposure and cell death has been explored in great detail. One of the first genes identified that functions to recognize phosphatidylserine in mammalian systems is a protein reported to function in both recognition and engulfment (FADOK *et al.* 2000). The protein encoded by this gene, identified by phage display, was called the phosphatidylserine receptor. New evidence from knockout mice suggests that elimination of this gene has no effect on corpse engulfment (BOSE *et al.* 2004). Additionally, recent evidence suggests that this protein is likely to be localized to the nucleus (CIKALA *et al.* 2004; CUI *et al.* 2004). Similarly, contrary to the claims made in an initial report (WANG *et al.* 2003), the *C. elegans* homolog of the proposed phosphatidylserine receptor, *psr-1*, is unlikely to play a role in engulfment, since no persistent cell corpses are observed in animals in which *psr-1* function has been eliminated (B. D. GALVIN and H. R. HORVITZ unpublished observation).

Some members of the annexin family, like the mammalian homolog of NEX-1, are also capable of binding to phosphatidylserine. Thus there has been interest in investigating whether *C. elegans* annexins affect programmed cell death. However, the gene encoding a member of the annexin family called *nex-1* is, despite a previous report (ARUR *et al.* 2003), also unlikely to function in engulfment in *C. elegans* (D. T. HARRIS and H. R. HORVITZ unpublished observation).

F. Additional Genes that Function in Programmed Cell Death

In addition to the genes that have been shown to function in the execution of programmed cell death (*ced-3*, *ced-4*, *ced-9*, *egl-1*, and *ced-13*) and in the engulfment of cell corpses (*ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10*, *ced-12*, and *dyn-1*), several other genes have been shown to function in programmed cell death. These genes are described below.

1. ced-8 *and* ced-11 *encode proteins that are likely to function at cellular membranes*

In the screen for mutant animals containing persistent cell corpses, alleles of *ced-8* were isolated (ELLIS *et al.* 1991; STANFIELD and HORVITZ 2000). Although originally thought to function in cell corpse engulfment, *ced-8* was later shown to function in the timing or efficient progression of cell death. In animals lacking *ced-8* function, an abundance of cell corpses are visible at a late time point at which few cell corpses are observed in wild-type animals; this observation is most likely a result of the deaths proceeding more slowly (STANFIELD and HORVITZ 2000). CED-8 is most similar to the putative membrane transporter XK. The function of CED-8 remains unknown, but mutations in this gene can, like mutations in *ced-7* and other genes, promote additional survival in a weak *ced-3*

background.

Another transmembrane protein whose function in programmed cell death remains unclear is CED-11. CED-11 encodes a member of the TRP (transient receptor potential) superfamily of cation channels (G. M. STANFIELD and H. R. HORVITZ, personal communication). Members of this family of proteins have been shown to be calcium-permeable and are involved in many processes, ranging from thermosensation to mechanotransduction (RAMSEY *et al.* 2006). Loss-of-function alleles of *ced-11* were originally identified as suppressors of the persistent cell corpse phenotype of *ced-5* because they change the appearance of the corpses such that they no longer have the classic refractile button-like appearance observed using Nomarski microscopy. This is the only gene currently known to affect the morphology of the cell corpse.

2. Nucleases function in programmed cell death to degrade the DNA of the dying cell

The first mutation found to alter the process of programmed cell death was in the gene *nuc-1* (SULSTON 1976). In animals lacking *nuc-1* function, the DNA of engulfed dead cells is not properly degraded. *nuc-1* encodes a protein similar to mammalian DNaseII (WU *et al.* 2000). The degradation of DNA during programmed cell death in *C. elegans* is less well understood than in other systems (NAGATA 2005). One of the hallmarks of apoptotic cells in mammalian systems is the presence of TUNEL-positive (Terminal deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling-positive) DNA in cells that have died through a caspase-dependent process (GAVRIELI *et al.* 1992). In *C. elegans*, TUNEL-reactive DNA can be detected in dying cells only during a brief period of time during the death process (WU *et al.* 2000). Analysis of loss-of-function alleles of *nuc-1* suggests that one of

the functions of NUC-1 is to convert TUNEL-reactive DNA to TUNEL-negative DNA, such that in *nuc-1(lf)* animals, TUNEL-reactive 3' hydroxyl ends of DNA fragments accumulate in cell corpses (WU *et al.* 2000). This result suggests that at least one additional nuclease functions upstream of NUC-1 to generate the TUNEL-reactive DNA in the first place. NUC-1 does not function only in the degradation of DNA in programmed cell death, because bacterial DNA in the gut also goes undigested in *nuc-1(lf)* animals. In mammalian systems, the NUC-1 homolog DNaseII is clearly responsible for degrading DNA from apoptotic cells, because in DNaseII-null mice, the macrophages that engulf many apoptotic corpses contain undigested DNA (KAWANE *et al.* 2003; KRIESER *et al.* 2002).

One of the interesting unexplored observations concerning the cell death pathway is that, in animals lacking *ced-1* and *nuc-1*, none of the TUNEL-reactive DNA that is normally observed in *nuc-1(lf)* animals can be detected (WU *et al.* 2000). Loss of *ced-7* also suppresses the formation of TUNEL-reactive DNA in cell corpses, but not to the same degree as does loss of *ced-1*. These results suggest that *ced-1* and to a lesser extent *ced-7* are required to activate the process that leads to the formation of TUNEL-reactive DNA. This activity further distinguishes members of the *ced-1* pathway from the *ced-2* pathway.

A survey of additional DNase-like genes encoded in the *C. elegans* genome led to the suggestion that seven additional nucleases function in some aspects of cell corpse DNA degradation and cell death execution (PARRISH and XUE 2003). This characterization of nucleases was performed using RNAi and awaits further confirmation using loss-of-function alleles. A loss-of-function allele of the gene encoding a homolog of a mitochondria endonuclease (LI *et al.* 2001), which may function in mammalian apoptosis, has also been reported to perturb DNA degradation (PARRISH *et al.* 2001). Although a mutation that maps

to the general location of this gene, *cps-6*, has been isolated in a screen, no DNA sequence change can be found in the genomic locus and thus further characterization of this gene is required. The identity of the remaining critical players in DNA degradation and in particular the nucleases that act to generate TUNEL-reactive DNA ends remains an important open question, as no candidates have been isolated thus far in *C. elegans*.

3. A possible new role for mitochondria in programmed cell death in C. elegans

Recent work has focused greater attention on the role that the mitochondria play in programmed cell death in *C. elegans*. Although much of the mammalian research into programmed cell death has focused on mitochondria, until fairly recently the role of mitochondria in cell death in *C. elegans* has been limited to the localization of the execution proteins CED-9 and CED-4 (CHEN *et al.* 2000). In *C. elegans*, CED-9 is constitutively localized to mitochondria and, in the absence of EGL-1, sequesters CED-4 to the mitochondria and prevents it from activating CED-3. As previously discussed, although the release of cytochrome *c* from mitochondria is a critical step in the activation of apoptosis in mammals, as yet no role has been established for cytochrome *c* in cell death in *C. elegans*. In mammals, mitochondria have been observed to fragment during apoptosis, and interference with this process has been shown to block apoptosis (FRANK *et al.* 2001; KARBOWSKI and YOULE 2003). Evidence has recently been reported that a similar phenomenon occurs in programmed cell death in *C. elegans*. This mitochondrial fragmentation can be induced by EGL-1 in a CED-9-dependent fashion and requires the activity of the dynamin-related protein DRP-1, a protein whose homologs act in mitochondrial fragmentation in mammalian cells (JAGASIA *et al.* 2005). Overexpression of a dominant-negative *drp-1* construct

promotes inappropriate cell survival, whereas overexpression of wild-type *drp-1* can induce cell death. These results suggest that the mitochondria may play a significant role in programmed cell death in *C. elegans*.

III. THE REGULATION OF THE PROGRAMMED CELL DEATHS OF SPECIFIC CELLS IN *C. elegans*

Although many of the core genes that function in all programmed cell deaths are known, the regulation of this process in specific cells is less well understood. Only nine genes that contribute to the regulation of seven programmed cell deaths have been described, from among 152 somatic cell deaths that occur either in the male or in the hermaphrodite. In *C. elegans*, the decision of a cell to live or die depends on whether it expresses *egl-1*. Thus, this discussion of the regulation of cell death in *C. elegans* will focus on the regulation of *egl-1* transcription.

A. Four Genes Contribute to the Regulation of the Deaths of the NSM Sister Cells

Two of the 131 cells that die during hermaphrodite development are the sisters of the serotonergic neurosecretory motor (NSM) neurons. In a screen to identify mutations that cause the inappropriate survival of the sisters of the NSMs, alleles of two genes were isolated. Loss-of-function mutations in *ces-2* (*ces*, cell death specification) prevent the deaths of the NSM sister neurons, and a gain-of-function mutation in *ces-1* prevents these deaths and the deaths of the sisters of the I2 neurons (ELLIS and HORVITZ 1991). The model for how these genes function in wild-type animals is that CES-1 is a repressor of *egl-1* transcription, and CES-2 is a repressor of *ces-1* transcription (METZSTEIN *et al.* 1996).

Specifically, CES-2 binds to an intronic element in *ces-1* and prevents its transcription. In *ces-1(gf)* mutant animals, the DNA element to which CES-2 normally binds is mutated such that CES-2 can no longer bind and *ces-1* is likely expressed. CES-1 then prevents death by binding to a regulatory site in *egl-1* and preventing its transcription (THELLMANN *et al.* 2003). Loss of *ces-2* results in a failure to repress *ces-1* expression and thus similarly promotes survival. This model is supported by the observation that loss of *ces-1* completely suppresses the survival observed in *ces-2(lf)* animals. The products of two other genes, *hlh-2* and *hlh-3* (*hlh*, helix loop helix), promote transcription of *egl-1* in the NSM sister cells by binding to sites that overlap with the CES-1 binding site (THELLMANN *et al.* 2003).

Based on the studies of *ces-1* and *ces-2* in *C. elegans*, the mammalian genes SLUG and HLF (hepatic leukemia factor), respectively, were shown to regulate programmed cell death in mammalian cells. HLF had been previously identified because a translocation that causes a chimeric version of HLF fused to the E2A gene is associated with acute lymphoblastic leukemia (HUNGER *et al.* 1992; INABA *et al.* 1992). The translocation found in leukemic cells leads to the production of a protein that is a fusion of the DNA-binding domain of HLF to the activation domain of E2A. This HLF-E2A chimera binds to SLUG and activates its expression, leading to a reduction in the amount of apoptosis in developing blood cells, and contributing to the development of leukemia (INUKAI *et al.* 1999). The mechanism by which SLUG over-expression leads to a reduction in apoptosis is that SLUG directly binds to and represses PUMA, a BH3-only gene (*i.e.* a homolog of *egl-1* in *C. elegans*) (WU *et al.* 2005).

B. Expression of and Killing by *egl-1* in the HSNs is Inhibited by the Binding of TRA-1 in Hermaphrodites

The regulation of cell survival of the HSNs, two cells that die in males and survive in the hermaphrodite, has been described (CONRADT and HORVITZ 1999). Since these cells control egg laying, elimination of the HSNs leads to an egg-laying defect. As mentioned above, gain-of-function alleles of *egl-1* were identified in screens for animals that do not properly lay eggs (TRENT *et al.* 1983). In these dominant alleles of *egl-1*, the binding site for TRA-1, a protein that can function as a transcriptional repressor of *egl-1* in hermaphrodites, is mutated and prevents binding (CONRADT and HORVITZ 1999). TRA-1 (*tra*, sexual transformer) is the most downstream gene product that functions in *C. elegans* sex determination. TRA-1 normally binds to the site altered by these *egl-1* gain-of-function mutations and prevents transcription of *egl-1* in hermaphrodite HSNs. Thus, in animals in which the TRA-1 binding site in *egl-1* has been mutated, *egl-1* transcription is no longer inhibited and the HSNs inappropriately die.

The transcriptional activators that function to promote the transcription of *egl-1* in the absence of this repression have not been conclusively identified. A screen for mutations that suppress the death of the HSNs in *egl-1(gf)* hermaphrodites led to the identification of two genes, *eor-1* and *eor-2*, that contribute to death of the HSNs (HOEPPNER *et al.* 2004). EOR-1 is a putative transcription factor containing zinc finger domains, and EOR-2 is a novel conserved protein. The protein products of these genes may directly bind and contribute to the activation of *egl-1* transcription.

C. The Programmed Death of a Specific Ventral Cord Neuron is Controlled by the Homeodomain Transcription Factors MAB-5 and CEH-20

The Hox genes *mab-5* and *ceh-20* function together to regulate the death of the P11.aaap cell (a neuron in the posterior ventral nerve cord) by binding to the *egl-1* locus (LIU *et al.* 2006). Loss of either *mab-5* or *ceh-20*, which are both expressed in this cell, prevents this death and specifically prevents *egl-1* transcription. Transgenic *egl-1* constructs containing mutations that perturb the binding of MAB-5 and CEH-20 fail to rescue the inappropriate survival of this cell in *egl-1* rescue assays. This direct control of *egl-1* expression by Hox genes demonstrates a direct link between these important developmental genes and the control of programmed cell death.

D. An Evolutionarily Conserved Enhancer Element is Required for the Death of the VC-like Neurons

In a screen to identify additional genes that regulate programmed cell death, a non-coding mutation in *egl-1* was identified. This element is specifically required for the death of six neurons - the VC-like neurons - in the ventral nerve cord of *C. elegans* hermaphrodite. The characterization of this element and the identification of proteins that may bind to this element to regulate these deaths are presented in Chapter 3 of this thesis.

E. *egl-1* Does Not Regulate Programmed Cell Death in the Germline

Although the execution genes *ced-3*, *ced-4*, and *ced-9* all function in both somatic and germline cell death, two observations suggest that programmed cell death in the germline is regulated differently. First, a gain-of-function mutation in *ced-9(n1950)*, which prevents the

deaths of essentially all somatic cells programmed to die, has no affect on germ cells (GUMIENNY *et al.* 1999). Second, loss of *egl-1* does not promote survival in the germline (CONRADT and HORVITZ 1998). Structural analysis of CED-9 and EGL-1 has shown that the *ced-9(n1950)* mutation perturbs the ability of BH3-only proteins such as EGL-1 to interact with CED-9 (YAN *et al.* 2005). What controls germline deaths upstream of *ced-9* is unknown. Although the regulation of normal death in the germline is not understood, germ cell death in response to toxic insults such as DNA may to be regulated by several genes. One of these genes (*cep-1*, which is similar to p53) may induce *egl-1* transcription and death in response to DNA damage (DERRY *et al.* 2001; SCHUMACHER *et al.* 2001). However, *egl-1* does not regulate the normal programmed cell deaths that occur in the germline during development in the absence of DNA damage.

F. The Programmed Deaths of Several Male-Specific Cells are Regulated Differently

Nearly all of the somatic programmed cell deaths that occur in *C. elegans* are believed to utilize the *egl-1*, *ced-9*, *ced-4*, and *ced-3* genes. Two cell deaths that occur in males may be exceptions. One of these deaths appears to be determined stochastically, such that one member of an equipotent pair of cells, B.alapaav and B.arapaav, always dies and the other survives (SULSTON *et al.* 1980). Death of either B.alapaav or B.arapaav requires the engulfment genes *ced-1* and *ced-2* (HEDGECOCK *et al.* 1983), which contribute only very weakly to most cell deaths in *C. elegans*. The complete dependence of this death on engulfment is highlighted by the fact that ablation of the P12.pa cell, which normally engulfs the cell corpse generated in this cell death, causes this pair of cells both to survive (SULSTON and WHITE 1980). This linkage of programmed cell death to the cell-cell signaling that must

be occurring among these cells to ensure that only one of these two cells survives is a novel phenomenon, and further study of these deaths might provide important insights into the regulation of programmed cell death. Additionally, the complete dependence of this death on engulfment should be explored.

The second of the uniquely regulated male-specific deaths occurs in the linker cell, whose migration leads the extension of the developing gonad in the male (KIMBLE and HIRSH 1979). This cell dies by a process that morphologically resembles programmed cell death but is not dependent on genes known to function in programmed cell death (ELLIS and HORVITZ 1986) (M. C. ABRAHAM and S. SHAHAM, unpublished observations).

IV. PATHOLOGICAL DEATHS OF SPECIFIC CELLS

In addition to the programmed cell deaths that occur during development and in the adult germline, there are several pathologic cell deaths that occur in *C. elegans* as a result of mutations in specific genes. These deaths are not a part of normal development. Most of the mutations that cause these deaths have been isolated in screens for specific phenotypes, such as touch-insensitivity or failure to possess a functional vulva. These deaths are described below.

A. Channel Hyperactivation Can Cause the Necrotic Deaths of Neurons

In a screen to identify worms that were defective in the avoidance response to gentle touch, several dominantly acting alleles of *mec-4* (*mec*, mechanosensory abnormal), referred to as *mec-4(d)*, were isolated that cause the inappropriate death of six neurons (CHALFIE and AU 1989; CHALFIE and SULSTON 1981). Similarly, an allele of the gene *deg-1 (deg*,

degeneration of certain neurons) also causes neuronal death and was isolated in a screen for animals defective in a different touch response (CHALFIE and WOLINSKY 1990). Both of these genes encode homologs of subunits of DEG/ENaC (degenerin/epithelial Na^+ channel) ion channels, and gain-of-function mutations in each of these genes, which cause inappropriate channel activity and death, affect homologous residues (CHALFIE and WOLINSKY 1990; DRISCOLL and CHALFIE 1991). MEC-10, UNC-8, and UNC-105 are also subunits of DEG/ENaC channels and can be mutated to cause the death of specific cells (HUANG and CHALFIE 1994; LIU *et al.* 1996; TAVERNARAKIS *et al.* 1997).

DEG/ENaC channels are heteromeric transmembrane complexes that function in processes ranging from sensory perception to electrolyte balance (MANO and DRISCOLL 1999). The gain-of-function mutations cause the deaths of the specific neurons in which these channels are expressed by hyperactivating the channels and causing increased ion influx that is ultimately toxic. Loss-of-function alleles of *mec-4*, *deg-1*, and others do not cause cell death. Other genes can also mutate to cause necrotic deaths. For example, gain-of-function mutations in *deg-3*, which encodes a subunit of a nicotinic acetylcholine receptor, and transgenic expression of activated $G\alpha_s$ (BERGER *et al.* 1998; KORSWAGEN *et al.* 1997), can cause deaths that are morphologically very similar to the *mec-4(d)* and *deg-1(d)*-induced cell deaths.

These cell deaths induced by channel hyperactivation are both genetically and morphologically distinct from programmed cell deaths. Unlike programmed cell deaths, which form compacted cell corpses, the neurons that die swell to several times their normal diameter before disappearing. Ultrastructurally, electron-dense membranous whorls are observed, followed by a swelling and degradation of organelles, distortion of the nucleus, and

the appearance of large vacuoles (HALL *et al.* 1997). These deaths resemble mammalian cellular necrosis, and for this reasons these types of channel hyperactivation-induced cell death in *C. elegans* are normally referred to as being necrotic cell deaths (WALKER *et al.* 1988). Genetic analysis established that these deaths are independent of the canonical programmed cell death pathway, as *ced-3*, *ced-4*, *ced-9*, and *egl-1* are not required for these deaths (CHUNG *et al.* 2000). Although mutations in the engulfment genes slow the disappearance of the dying necrotic cells, they do not contribute to the cell-killing process in necrotic cell death (CHUNG *et al.* 2000). Calcium appears to function in these necrotic deaths, because mutations in calreticulin, which regulates intracellular calcium, and other genes that function in calcium regulation can suppress the deaths of these cells (XU *et al.* 2001). Finally, these deaths also require the activity of calcium-activated calpain proteases and aspartyl proteases (SYNTICHAKI *et al.* 2002).

B. Rare Mutations in *lin-24*, *lin-33*, and *pvl-5* Can Cause Pn.p Cell Death

In a screen to identify genes that affect vulval cell lineages, alleles of two genes, *lin-24* and *lin-33*, were identified that caused deaths morphologically distinct from both programmed and necrotic deaths (FERGUSON and HORVITZ 1985). The mutations in *lin-24* and *lin-33* cause the death of the Pn.p hypodermal blast cells, three of which normally give rise to the vulva; thus these deaths result in vulvaless animals. These deaths are characterized in Chapter 4 of this thesis.

A mutation in the uncloned gene *pvl-5* can also cause the death of the Pn.p cells (JOSHI and EISENMANN 2004). A single allele of *pvl-5* was isolated in a screen for abnormally shaped vulvas. The Pn.p deaths seen in *pvl-5* mutants require *ced-3* and *ced-9*

but do not require *ced-4*. This unique genetic profile distinguishes these deaths from traditional programmed cell deaths, necrotic cell deaths, and the Pn.p cell deaths observed in *lin-24* and *lin-33* mutants. The authors of the *pvl-5* work suggest that *pvl-5* may normally protect against *ced-4*-independent death in these cells.

V. PROGRAMMED CELL DEATH REGULATORY MECHANISMS DESCRIBED IN INSECTS OR MAMMALS BUT NOT IN NEMATODES

The discoveries concerning *C. elegans* cell death described above have pioneered the genetic analysis of programmed cell death and laid the foundations of much of the pathway. Genetic, cellular, and biochemical studies of *D. melanogaster* and of mammalian systems have complemented these studies (CASHIO *et al.* 2005; DANIAL and KORSMEYER 2004). Three cell death regulatory mechanisms discovered in *D. melanogaster* and in mammalian cells play significant roles in programmed cell death but have not been described as functioning in *C. elegans* cell death. These three mechanisms are described below.

A. Receptor-mediated Caspase Activation in Mammals

Programmed cell death may be more complex in vertebrates than in *C. elegans*. In addition to the larger number of genes involved in the regulation of apoptosis, caspases in mammals can be activated by mechanisms that do not appear to function in *C. elegans*. For example, mammalian caspases can be activated by transmembrane receptors. The Fas/APO-1/CD95 receptor, a member of the tumor necrosis factor (TNF) superfamily, has a well-characterized role in direct activation of caspases, bypassing the action of the mammalian EGL-1, CED-9, and CED-4 homologs. In the late 1980s, two groups identified

antibodies that could induce apoptosis of specific cells (TRAUTH *et al.* 1989; YONEHARA *et al.* 1989). Cloning of the genes that encode the antigens of these antibodies led to the identification of the Fas receptor and established that it was similar to the TNF receptors (ITOH *et al.* 1991; OEHM *et al.* 1992). This discovery was significant because it had been previously observed that adding TNF to tumors caused cell death (BEUTLER and CERAMI 1986; CARSWELL *et al.* 1975; OLD 1985). Soon after the cloning of the Fas receptor, the Fas ligand was identified (SUDA *et al.* 1993). Around the same time, Shigekazu Nagata and colleagues identified mutations in both Fas and the Fas ligand that could lead to lymphoproliferative autoimmune disorders in mice (TAKAHASHI *et al.* 1994; WATANABE-FUKUNAGA *et al.* 1992). These observations suggested an important role for Fas and Fas-ligand in negative selection of autoreactive T cells in the thymus.

Discoveries made in many laboratories have contributed to description of the mechanism by which Fas induces apoptosis. Activation of Fas by the Fas ligand induces formation of the death-inducing signaling complex (DISC), the complex of proteins consisting of Fas, an adaptor protein, and a caspase. Upon Fas ligand binding to its receptor FADD (Fas-associated death domain containing protein), an adaptor protein, binds to the death domain in the C-terminus of Fas and recruits one of two initiator caspases, caspase-8 or caspase-10 (KISCHKEL *et al.* 1995). Upon oligomerization, caspase-8 (or caspase-10) becomes activated by proximity-driven cleavage and then cleaves and activates caspase-3, an effector caspase. This receptor-activated apoptotic pathway is usually referred to as the extrinsic pathway and can activate effector caspases independently of the mitochondria and the Bcl-2 superfamily by engaging death receptors at the cell membrane. The extrinsic pathway can also interface with the mitochondrial, or intrinsic, pathway through

caspase-8-mediated cleavage of Bid, a BH3-only protein. Cleavage of Bid releases tBid (truncated Bid), which can then translocate to the mitochondria and induce the release of cytochrome *c*, leading to further activation of the apoptotic pathway (LI *et al.* 1998).

B. Direct Activation of an Apoptotic DNase by Caspase Cleavage of its Inhibitor

Although not essential for apoptosis, the degradation in DNA of apoptotic cells is one of the most commonly assayed markers of this process (NAGATA 2005). In mammalian cells, DNA degradation is, in part, mediated by the caspase-activated DNase (CAD, also called DFF40) (ENARI *et al.* 1998; LIU *et al.* 1997; SAKAHIRA *et al.* 1998), which does not have an identifiable homolog in *C. elegans*. This DNase generates double strand breaks with a hydroxyl group at the 3′ end (WIDLAK *et al.* 2000). This hydroxyl group acts as a substrate for terminal deoxynucleotidyl transferase (TdT), which is commonly used to detect apoptotic cells in the TUNEL-labeling protocol. For CAD to be functional, it needs to be synthesized in the presence of its partner ICAD (inhibitor of CAD), which acts as a chaperone and ensures proper folding of CAD. ICAD also binds and inhibits the activity of mature, folded CAD (SAKAHIRA *et al.* 2000). Upon activation of caspase-3, ICAD is cleaved and active CAD is liberated to cleave chromosomal DNA in the nucleus. Although a gene encoding this enzyme has not been found in the *C. elegans* genome, a DNase (like CAD) that generates TUNEL-reactive DNA must function in programmed cell death in *C. elegans*, because TUNEL-reactive DNA is generated and accumulates in animals lacking the nuclease *nuc-1*.

C. Inhibitors of Apoptosis (IAPs) and IAP Inhibitors Act To Regulate Cell Death in *D. melanogaster* and in Mammals

In *D. melanogaster*, the main mechanism by which programmed cell death is regulated appears to be different from that found in *C. elegans*. Observations by Hermann Steller and colleagues sparked extensive research into the genetic regulation of programmed cell death in *D. melanogaster*. Their studies indicated that in *D. melanogaster*, as in *C. elegans*, a large number of cells die during embryonic development. These cell deaths ultrastructurally resembled those observed in *C. elegans* and in mammalian systems (ABRAMS *et al.* 1993). It was found in this work that the vital die acridine orange specifically labels apoptotic cells in *D. melanogaster* embryos (ABRAMS *et al.* 1993). The screening of a deficiency collection for embryos that lacked acridine orange staining led to the identification of four overlapping deficiencies that eliminated all death (WHITE *et al.* 1994). The deficiencies removed three genes (Reaper, Hid, and Grim) that act in developmental deaths.

Both transcriptional and posttranscriptional regulation of Reaper, Hid, and Grim are central to the control of apoptosis in *D. melanogaster* (CASHIO *et al.* 2005). The proteins encoded by these genes are thought to induce death by inhibiting the function of Diap1 (*Drosophila* inhibitor of apoptosis). Loss of Diap1 causes lethality and a great increase in apoptosis (GOYAL *et al.* 2000; LISI *et al.* 2000; WANG *et al.* 1999). IAPs, like Diap, bind to the active sites of caspases and block their activity by sequestration or by promoting their degradation (CASHIO *et al.* 2005). Reaper, Hid, and Grim function both by promoting IAP degradation and by binding IAPs more efficiently than do caspases (CASHIO *et al.* 2005). Mammals have proteins (namely, SMAC/DIABLO and OMI/HTRA2) that are distantly related to and function similarly to Reaper, Hid, and Grim, and these proteins may be important activators of apoptosis in mammals (VAUX and SILKE 2003).

VI. CONCLUSION

In *C. elegans*, the major determinant of whether most cells will undergo programmed cell death is whether or not *egl-1* is expressed. If *egl-1* is expressed, it inhibits the anti-apoptotic function of CED-9, releasing the adaptor protein CED-4 to activate the caspase CED-3. Mammalian systems possess homologs of EGL-1, CED-9, CED-4, and CED-3, and the genetic and molecular interactions between the components have important similarities to those found in *C. elegans*. However, mammalian systems also have pathways that activate caspases directly, by bypassing activities similar to those provided in *C. elegans* by *egl-1* and *ced-9*. One such pathway is the Fas/Fas ligand pathway, described above. This Fas/Fas ligand system is specific to mammals; *D. melanogaster* does not seem to have any clear homologs of Fas, and although it does possess homologs of CED-9, CED-4, and CED-3, it does not appear to have BH3-only proteins similar to EGL-1. Thus, the decision of cells to undergo programmed cell death in *D. melanogaster* is based neither on expression of an EGL-1-like protein nor on the activity of a Fas-like pathway, but instead on expression of the IAP regulators, Reaper, Hid, and Grim. Although IAP homologs exist in *C. elegans* and in mammals, they do not play an obvious role in programmed cell death in *C. elegans* (FRASER *et al.* 1999; SPELIOTES *et al.* 2000) (E. K. SPELIOTES and H. R. HORVITZ unpublished observations) and the role they play in mammalian apoptosis is unclear (VAUX and SILKE 2003).

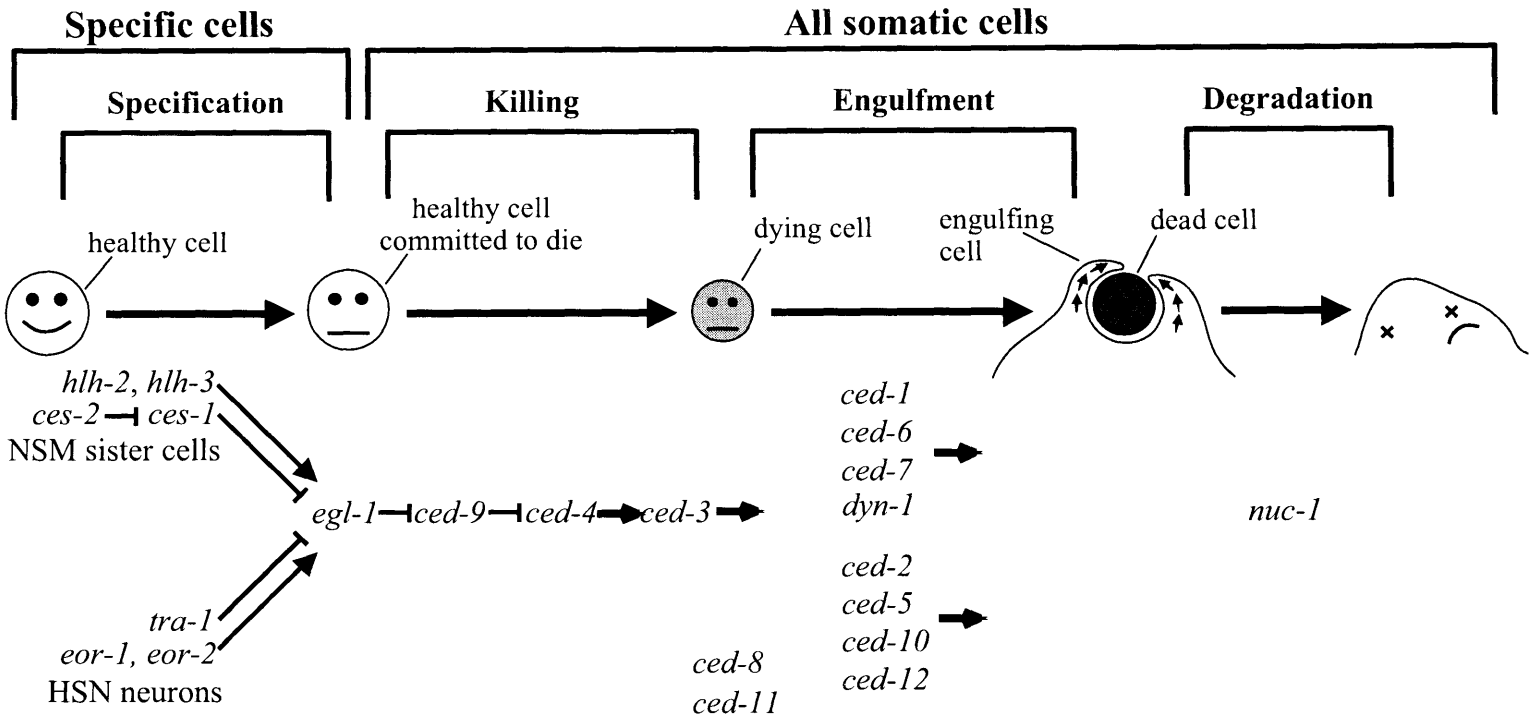
Although the details of upstream regulation may differ among *C. elegans*, *D. melanogaster*, and mammals, the regulation of programmed cell death in all systems comes down to the activation of caspases. In general, especially in *C. elegans* and mammals, a single conserved mechanism is used to directly regulate this process: an adapter protein

forms a platform that can bind caspases and activate them. These platforms can be assembled in different ways, but they all accomplish the same task: bringing pro-caspase molecules close together such that they cleave and activate one another. The conservation of the proteins and the fact that critical discoveries have been made in each system highlights the importance of these parallel investigations. From future studies in each of these systems, we will continue to identify the critical mechanisms for the regulation of apoptosis. Ultimately, therapeutic interventions may be available to correct the negative consequences that can result from the misregulation of this pathway.

In my thesis work, I have investigated the mechanisms of regulation of cell death in *C. elegans*, building on the work described in this introduction. In the following chapters, I describe some of the experiments I have performed. In Chapter 2, I present the characterization of a regulator of *ced-4* splicing isolated in a screen to identify genes that function in programmed cell death. In Chapter 3, I discuss the identification of a tissue-specific enhancer element in *egl-1* and a novel transcription factor that may bind to this element and activate *egl-1* transcription in a specific subset of cells. In Chapter 4, I describe my work characterizing a cytotoxic cell death process that is induced by mutations in two genes and requires engulfment genes, but is distinct from programmed cell death.

FIGURE LEGENDS

Figure 1. A molecular genetic pathway for the genes controlling programmed cell death in *C. elegans*.



hlh-2: E/Daughterless txn factor
hlh-3: Achaete-scute txn factor
ces-1: Snail family txn factor
ces-2: bZIP txn factor
tra-1: Zn-finger txn factor
eor-1: PLZF txn factor
eor-2: Putative txn factor

egl-1: BH3-only protein
ced-9: Bcl2 homolog
ced-4: APAF1 homolog
ced-3: Caspase

ced-8: XK-like
ced-11: Trp channel-like

ced-1: mEGF10 receptor
ced-6: Adaptor protein
ced-7: ABC transporter
dyn-1: Dynamin

ced-2: CrkII homolog
ced-5: DOCK180 homolog
ced-10: Rac GTPase
ced-12: ELMO1 homolog

nuc-1: DNase II

Figure 1

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Chapter Two

The SR Protein Kinase *spk-1* Regulates Programmed Cell Death in *C. elegans* by Controlling the Alternative Splicing of *ced-4* RNA

Brendan D. Galvin and H. Robert Horvitz

SUMMARY

To identify additional genes involved in programmed cell death in *C. elegans*, we performed a genetic screen to isolate mutations that cause an increase in the number of programmed cell deaths. We screened for suppressors of the cell-death defect due to a partial loss-of-function mutation in *ced-4*, which encodes an essential regulator of programmed cell death that promotes death by activating the caspase CED-3. We identified one *ced-4* suppressor, which had a mutation in the gene *spk-1*. The *spk-1* gene encodes a protein that is homologous to SR protein kinases, which are thought to regulate splicing. Previous work suggests that *ced-4* can be alternatively spliced and that the splice variants function oppositely, with the longer transcript (*ced-4L*) inhibiting programmed cell death. *spk-1* appears to promote cell survival by increasing the amount of the protective *ced-4L* splice variant. We conclude that programmed cell death in *C. elegans* is regulated by an alternative splicing event that is controlled by an SR protein kinase.

INTRODUCTION

Multicellular organisms can eliminate cells by a process known as programmed cell death or apoptosis. Apoptosis plays a fundamental role in development and in tissue homeostasis and human health (FADEEL and ORRENIUS 2005; MEIER *et al.* 2000). Notably, improper control of apoptosis is associated with disease. Defects in apoptosis that cause either too much death or too little have been implicated in cancers, neurodegenerative diseases, and autoimmune diseases (FADEEL and ORRENIUS 2005).

During the development of the *C. elegans* hermaphrodite, 1,090 somatic cells are generated by an essentially invariant pattern of divisions (KIMBLE and HIRSH 1979; SULSTON and HORVITZ 1977; SULSTON *et al.* 1983). Of these cells, 131 undergo programmed cell death (SULSTON and HORVITZ 1977; SULSTON *et al.* 1983). At least 20 genes have been identified that regulate either the pattern or the process of programmed cell death in *C. elegans* (LETTRE and HENGARTNER 2006). The core pathway of programmed cell death in *C. elegans* is composed of four genes: *egl-1*, *ced-9*, *ced-4*, and *ced-3*. The cloning of these four genes defined a molecular pathway for programmed cell death later shown to be conserved in vertebrates (CONRADT and HORVITZ 1998; HENGARTNER *et al.* 1992; METZSTEIN *et al.* 1998; YUAN and HORVITZ 1992; YUAN *et al.* 1993).

CED-3 is a defining member of the caspase (cysteine aspartate-specific protease) family (YUAN *et al.* 1993). The identification of *ced-3* as encoding a protease led to the discovery that caspases are required for many apoptotic cell deaths in mammals (DEGTEREV *et al.* 2003). *ced-4* encodes a protein similar to Apaf-1 (apoptotic protease activating factor) (YUAN and HORVITZ 1992; ZOU *et al.* 1997), which facilitates CED-3 activation (CHINNAIYAN *et al.* 1997; SESHAGIRI and MILLER 1997; WU *et al.* 1997; YANG *et al.* 1998).

Cells are protected from programmed cell death by *ced-9* (HENGARTNER *et al.* 1992), which encodes a member of the Bcl-2 family of apoptotic regulators (HENGARTNER and HORVITZ 1994b). Human BCL-2 can block programmed cell death in *C. elegans* and can substitute for *ced-9*, demonstrating a functional conservation (HENGARTNER and HORVITZ 1994b; VAUX *et al.* 1992). Finally, *egl-1* encodes a BH3-only protein (defined as a Bcl-2 family member lacking obvious BH1, BH2, and BH4 domains) that negatively regulates CED-9 in dying cells (CONRADT and HORVITZ 1998). Transcriptional control of *egl-1* is likely to be the major mechanism of the regulation of programmed cell death (CONRADT and HORVITZ 1998). *egl-1*, *ced-9*, *ced-4*, and *ced-3* act within dying cells to control all somatic programmed cell deaths in *C. elegans* (CONRADT and HORVITZ 1998; YUAN and HORVITZ 1990); loss-of-function mutations in *egl-1*, *ced-4*, or *ced-3* or a gain-of-function mutation in *ced-9* prevent essentially all somatic programmed cell deaths (CONRADT and HORVITZ 1998; ELLIS and HORVITZ 1991; HENGARTNER *et al.* 1992).

ced-4 encodes two transcripts, *ced-4L* and *ced-4S*, that appear to have opposing functions (SHAHAM and HORVITZ 1996). Whereas *ced-4S* (the predominant transcript) promotes cell death, *ced-4L* protects cells from death. The observation that *ced-4* has two functionally different splice variants originated from the identification of a DNA element in the *ced-4* genomic locus that was not contained within the originally described *ced-4* cDNA. This element was highly conserved in two *Caenorhabditis* species, *C. briggsae* and *C. vulgaris*, that are distant relatives of *C. elegans* (SHAHAM and HORVITZ 1996). This conservation is significant because only the coding regions and key regulatory elements are likely conserved between these *Caenorhabditis* species. Further studies confirmed that this element was part of an alternative cDNA that encodes a protein that can protect cells against

death. The function of CED-4L was determined by overexpressing this protein in *C. elegans* and observing that, unlike CED-4S, CED-4L protects cells against programmed cell death (SHAHAM and HORVITZ 1996). Recent structural work suggests that CED-4L prevents the formation of the CED-4 tetramer, which is required for activation of CED-3 and the initiation of programmed cell death (YAN *et al.* 2005).

Approximately 2,500 genes in *C. elegans* (about 13% of the genes currently annotated in Wormbase (http://www.wormbase.org/)), are predicted to be alternatively spliced (ZAHLER 2005). Splicing is a highly regulated process that removes intronic fragments of RNA from newly transcribed pre-mRNA molecules. The spliceosome, which is composed of small nuclear ribonucleoprotein particles (snRNPs) and proteins, is the complex responsible for splicing (NILSEN 2003). Two families of proteins that assist the spliceosome in splice-site selection are the heteronuclear ribonucleoproteins (hnRNPs) and the serine-arginine-rich proteins (SR proteins) (BLACK 2003). These proteins are thought to function oppositely; SR proteins bind to exonic splicing enhancer elements and promote the inclusion of exons, whereas hnRNPs bind to exonic splicing silencer elements and suppress the inclusion of exons. The SR proteins are likely regulated by the phosphorylation of serines in the SR domains, which are thought to regulate function by modifying protein-protein interactions (SANFORD *et al.* 2005). SR proteins are required for splicing in cellular extracts and might need to be phosphorylated to function (SANFORD *et al.* 2005). In this study, we describe a role for an SR protein kinase in the alternative splicing of *ced-4* RNA and the regulation of programmed cell death.

RESULTS

A Screen for Genes that Function to Prevent Programmed Cell Death

The study of programmed cell death in *C. elegans* has primarily focused on isolating two types of mutants: those with extra cells and those with either abnormal or persistent cell corpses. To identify genes that protect cells from undergoing programmed cell death, we screened for mutations that increase the number of programmed cell deaths. Specifically, we performed a modifier screen for mutations that suppressed the cell-death defect that is observed in animals with a loss-of-function mutation in *ced-4*. To assay cell death, we examined the ventral nerve cord. During development, 13 neuroblasts -- W, and P1-P12 -- together generate 10 cells that undergo programmed cell death (SULSTON and HORVITZ 1977). We used worms expressing the *lin-11::gfp* reporter *nIs106* to visualize a subset of these deaths: the Pn.aap cells (the posterior daughter of the anterior daughter of the anterior daughter of one of 12 P blast cells) (REDDIEN *et al.* 2001). In the ventral cord of wild-type animals, six Pn.aap cells survive and express *gfp*, whereas the other six undergo programmed cell death (the two anterior-most, P1.aap and P2.aap, and the four posterior-most, P9-P12.aap) (SULSTON and HORVITZ 1977). By contrast, in mutants defective in cell death, such as animals with strong loss-of-function mutations in *ced-3*, five extra Pn.aap cells survive and reliably express *gfp*, generating a total of eleven GFP-positive cells. The most anterior cell, P1.aap, does not always express *gfp* and is thus not scored in this assay (REDDIEN *et al.* 2001). The survival of Pn.aap cells can be easily monitored in strains carrying the *lin-11::gfp* reporter using a fluorescence-equipped dissecting microscope.

To facilitate the isolation of mutations with subtle effects on programmed cell death, we screened for mutations that cause an increase in programmed cell death in a sensitized

genetic background. Worms containing a non-null allele of *ced-4*, such as *n3158*, are partially defective in programmed cell death. The *n3158* mutation is an early missense mutation that changes serine 163 to phenylalanine (B. M. HERSH and H. R. HORVITZ, personal communication). These animals on average have 4.9 extra GFP-positive Pn.aap cells. Although *n3158* thus strongly prevents the programmed cell deaths of the Pn.aap cells, it is an allele of medium strength when assayed for survival of cells fated to die in the anterior pharynx, an independent quantitative assay for programmed cell death (HENGARTNER *et al.* 1992). *ced-4(n3158)* animals have an average of 6.0 extra cells in the anterior pharynx, whereas animals carrying a null allele of *ced-4* have an average of 12.8 extra cells (B. M. HERSH and H. R. HORVITZ, personal communication).

Using the *lin-11::gfp* reporter, we screened for suppressors of the partial loss-of-function *ced-4(n3158)* mutant by looking for mutants with a reduced number of GFP-positive Pn.aap cells (*i.e.*, fewer than eleven) (Figure 1). The screen was performed such that mutations in essential genes could be isolated, because mutations that increase programmed cell death might not produce viable progeny. For example, *ced-9*, which is known to protect against cell death, is an essential gene (HENGARTNER *et al.* 1992). Specifically, we saved siblings of each screen isolate such that animals heterozygous for mutations in an essential gene would be maintained. From a screen of 5,000 mutagenized haploid genomes, one strong suppressor, *n3418*, was isolated. In addition to recessively reducing the number of GFP-positive Pn.aap cells in the *ced-4(n3158)* background, this mutation caused recessive sterility. The decrease in the number of GFP-positive cells in the *ced-4(n3158) n3418* double mutant was not a consequence of a defect in the generation of the Pn.aap cells, since the GFP-positive cells were present in *n3418*; *ced-3(null)* animals

(data not shown). This observation indicates that P2.aap and P9-P12.aap are generated normally but are more likely to undergo programmed cell death in the *ced-4(n3158) n3418* strain than in *ced-4(n3158)* animals. The *ced-3* null allele failed to suppress the sterility conferred by *n3418*, suggesting the sterility was not caused by excessive programmed cell death (data not shown).

n3418 Is a Loss-of-Function Allele of *spk-1*, Which Encodes an SR Protein Kinase

Using a combination of visible phenotypic markers, deficiencies, and polymorphisms, we mapped our suppressor mutation *n3418* to a 90 kb region on linkage group III that contains 24 genes (Figure 2A). We found that both a deletion allele of and injection of dsRNA directed against one of theses genes, *spk-1* (KUROYANAGI *et al.* 2000), caused a sterility resembling that of the *ced-4(n3158)* suppressor *n3418*. We identified a mutation that changes the codon for tryptophan 142 (TGG) to an opal stop codon (TGA) in *spk-1* in *n3418* mutant animals (Figure 2B). A deletion allele of *spk-1*, *ok706*, failed to complement the sterility of *n3418* (data not shown). Based on these observations, we concluded that the sterility of *n3418* was caused by the mutation in *spk-1*. To determine if loss of *spk-1* function also suppressed the defect in programmed cell death caused by *ced-4(n3158)*, we tested the effect of the *spk-1(ok706Δ)* deletion allele in *ced-4(n3158)* animals. *spk-1(ok706Δ)* caused suppression of the cell-death defect in *ced-4(n3158)* animals to a similar extent as did *spk-1(n3418)* (Table 1). Together, these results indicate that both the suppression of *ced-4(n3158)* and the sterility are caused by *n3418*, a loss-of-function mutation in *spk-1*.

spk-1 encodes an SR protein kinase (Figure 2B). The SR protein kinase family regulates alternative splicing by phosphorylating SR proteins, which are thought to be key

regulators of splicing (BLACK 2003). In animals carrying only a loss-of-function allele of *spk-1*, either *n3418* or *ok706Δ*, the wild-type number of Pn.aap cells was observed (Table 1), showing that loss of *spk-1* does not cause ectopic Pn.aap programmed cell death in these cells.

spk-1(n3418) Strongly Suppresses Partial Loss-of-Function Alleles of *ced-4*

To determine if the loss of *spk-1* function specifically increased death in strains homozygous for the allele *ced-4(n3158)*, we tested the ability of *spk-1(n3418)* to suppress additional alleles of *ced-4* (Table 2). *spk-1(n3418)* also suppressed the cell-death defect of animals carrying *ced-4(n3141)*, which is a slightly weaker allele than *ced-4(n3158)*, the allele used in our screen. The allele *n3141* is a missense mutation that changes arginine 53 to lysine (B. M. HERSH and H. R. HORVITZ, personal communication). Thus, *spk-1(n3418)* suppressed two partial loss-of-function alleles of *ced-4*. However, *spk-1(n3418)* did not suppress *ced-4* null alleles. Specifically, *spk-1(n3418)* failed to suppress either the strong missense allele *ced-4(n3040)* or the early nonsense allele *ced-4(n1162)*, both of which cause the complete survival of the Pn.aap cells and of all the cells that normally die in the anterior pharynx (B. M. HERSH and H. R. HORVITZ, personal communication).

To determine if suppression by *spk-1* was specific to the *ced-4* gene, we tested the ability of *spk-1(n3418)* to suppress alleles of three other genes that function in the killing step of programmed cell death. We assayed *ced-3*, *ced-9*, and *egl-1* alleles that cause a cell-death defect similar in strength to that caused by *ced-4(n3158)* and *ced-4(n3141)* in the Pn.aap cells. We observed that *spk-1(n3418)* can weakly suppress the cell-death defect observed in strains homozygous for alleles of *ced-9*, or *ced-3*, but not to the same extent as observed in

ced-4 strains (Table 3). Even the *ced-3* allele *n2427*, which show the strongest interaction with *spk-1(n3418)* of the non-*ced-4* alleles, is only moderately suppressed by *spk-1*. Thus, *spk-1(n3418)* most strongly modifies the cell-death defect of partial loss-of-function alleles of *ced-4*.

spk-1 Regulates the Alternative Splicing of *ced-4*

Since *spk-1* most strongly suppresses *ced-4*, we hypothesized that *spk-1* may have a specific interaction with *ced-4*. This hypothesis is attractive given that SR protein kinases are thought to regulate alternative splicing and *ced-4* is the only cell death-gene known to be regulated by alternative splicing in *C. elegans* (BLACK 2003; SHAHAM and HORVITZ 1996). *ced-4* encodes two different transcripts; the shorter *ced-4* splice product, *ced-4S*, is thought to function in promoting programmed cell death, whereas the longer product, *ced-4L*, is thought to inhibit programmed cell death (SHAHAM and HORVITZ 1996).

To test directly if *spk-1(ok706Δ)* modifies the splicing of *ced-4*, we performed semi-quantitative RT-PCR of *ced-4* using RNA from wild-type and homozygous *spk-1(ok706Δ)* animals. Specifically, a primer set that amplifies both the long and short splice forms of *ced-4* was used to detect *ced-4* transcripts. We observed that animals lacking *spk-1* showed a decreased amount of the *ced-4L* product (Figure 3). Given the anti-apoptotic role of CED-4L, the loss of *spk-1* should lead to an increased level of cell death through the loss of the protective *ced-4L* transcript, as we observed. This is consistent with the observation that animals lacking *spk-1* have increased death in animals containing partial loss-of-function alleles of *ced-4*.

DISCUSSION

From a genetic screen to identify additional regulators of programmed cell death in *C. elegans*, we isolated a mutation in the splicing regulator *spk-1*, which encodes an SR protein kinase. Loss-of-function alleles of *spk-1* increase the amount of death that occurs in strains containing partial loss-of-function alleles of *ced-4* and might also weakly increase death in animals with mutations in *ced-3* or *ced-9*. The increase in death correlates with a decrease in expression of the *ced-4L* transcript in *spk-1* null animals. Previous analysis of the function of *ced-4L* relied largely on overexpression studies, whereas the data presented in this chapter suggest that the alteration of the endogenous levels of *ced-4L* can directly alter the amount of programmed cell death. We conclude that, in *C. elegans*, an SR protein kinase regulates the splicing of *ced-4*. We propose that the regulation of *ced-4* splicing plays a role in the control of programmed cell death.

Given our genetic and molecular data, we suggest a model in which SPK-1 normally promotes the generation of *ced-4L* transcripts that protect cells from dying (Figure 4). The loss of *spk-1* decreases the level of *ced-4L*, thereby increasing the likelihood that cells will die. We suggest that SPK-1 controls the splicing of the fourth exon in *ced-4* by regulating the phosphorylation of an SR protein.

Although there is increased death in the *spk-1(lf) ced-4(lf)* animals, we have not observed ectopic deaths in *spk-1* null animals in otherwise wild-type backgrounds. One possible explanation is that the regulation of *ced-4* splicing is a minor determinant of whether cells live or die. The pathway for programmed cell death in *C. elegans* generally has been described as linear, with EGL-1 promoting death by inhibiting the function of CED-9 (CONRADT and HORVITZ 1998). In the absence of EGL-1, CED-9 prevents CED-4 activation

of the caspase CED-3 (METZSTEIN *et al.* 1998). It has been observed, however, that in *ced-9(null)*; *ced-3* partial loss-of-function animals, cell death occurs in a relatively normal pattern (HENGARTNER and HORVITZ 1994a). A linear model in which EGL-1 functions through CED-9 would predict that in the absence of CED-9, cell death dependent on EGL-1 expression would not occur normally. However, this result shows that there must be additional mechanisms that regulate programmed cell death independently of *egl-1*. Perhaps the alternative splicing of *ced-4* is one such mechanism that contributes to the decision of which cells live or die, but in a wild-type animal, this contribution cannot be detected. In sensitized cells, such as those that usually die but survive because of a partial loss of *ced-4* function, mechanisms that can influence whether cells live or die are exposed. For example, the promotion of cell killing by genes involved in cell-corpse engulfment is best observed in a sensitized background containing a partial loss-of-function allele of *ced-3* (REDDIEN and HORVITZ 2004).

spk-1 is likely required for embryonic development in *C. elegans*, given that RNAi with a high concentration of *spk-1* dsRNA causes embryonic lethality (KUROYANAGI *et al.* 2000). If so, why are *spk-1* null homozygotes viable and sterile? We suggest that these animals are sustained by the SPK-1 provided by their heterozygous mothers. The maternal stores of SPK-1 might be depleted by late development when the deaths of the Pn.aap cells occur. Of the 131 programmed cell deaths, 113 occur before hatching; of the remaining 18, many occur before those of the Pn.aap cells, which die at the end of the first or beginning of the second larval stages (SULSTON and HORVITZ 1977; SULSTON *et al.* 1983). Thus, it may be that the effect that *spk-1* has on increasing death in *ced-4* loss-of-function animals would

not be observed if we assayed the cell death of most other cells, which die before hatching and possibly before the maternal stores of SPK-1 are depleted.

We believe that neither the sterility caused by *spk-1* alleles nor the lethality caused by *spk-1* dsRNA is a consequence of inappropriately increased cell death, as *spk-1; ced-3(null)* doubly mutant animals, in which no cell death has been observed, are still sterile. Thus, *spk-1* seems to be essential for additional processes. SPK-1 is the only protein found in *C. elegans* that is highly similar to mammalian SR protein kinases. SPK-1 can phosphorylate the SR domain of a *C. elegans* SR protein *in vitro* (KUROYANAGI *et al.* 2000). There are eight genes that might encode SR proteins in *C. elegans*. Treatment with dsRNA directed against individual SR genes does not result in an abnormal phenotype, except for *rsp-3* (SR protein), which causes embryonic lethality (KAWANO *et al.* 2000; LONGMAN *et al.* 2000; LONGMAN *et al.* 2001). However, treatment with dsRNA directed against specific combinations of SR genes results in sick or dead worms (KAWANO *et al.* 2000; LONGMAN *et al.* 2000; LONGMAN *et al.* 2001).

To date, *spk-1* and the Drosophila kinase Doa are the only genetically characterized SR protein kinase genes for which mutations have been isolated and shown to affect levels of different splice isoforms of target transcripts. Our work shows that, in *C. elegans*, the SR protein kinase SPK-1 controls the alternative splicing of *ced-4*. Previous work in *Drosophila melanogaster* has shown that the Clk/Sty protein kinase Doa controls the alternative splicing of *dsx* (*doublesex*), which regulates sex determination (DU *et al.* 1998). Clk/Sty kinases are distinct from SR protein kinases like SPK-1, but they do also phosphorylate SR proteins. Reducing the function of *Doa* alters the splicing of *dsx* such that both male and female-specific *dsx* mRNAs are produced in female flies (DU *et al.* 1998). This

misregulation of splicing correlates with a reduction in the level of phosphorylation of RBP1, an SR protein that likely functions in sex determination (DU *et al.* 1998).

ced-4 is the only gene in *C. elegans* thought to produce alternative splice variants with opposing roles in programmed cell death (SHAHAM and HORVITZ 1996). In mammals, however, alternative splice forms have been described for many regulators of apoptosis, although the functional significance of these isoforms remains largely unknown (SCHWERK and SCHULZE-OSTHOFF 2005). For example, the gene *caspase-2/Ich-1*, a homolog of the *C. elegans* gene *ced-3*, produces functionally distinct alternative splice variants (WANG *et al.* 1994). The long *caspase-2* transcript promotes apoptosis, whereas the short transcript protects cells from death. Like that of *C. elegans ced-4*, the alternative splicing of mammalian *caspase-2* is influenced by SR proteins and/or SR protein kinases. Studies in tissue culture of a *caspase-2* minigene, a plasmid construct consisting of a small portion of the genomic locus surrounding the introns of interest, suggest that overexpression of the SR proteins SC35 and ASF/SF2 promotes the skipping of a specific exon and the generation of the proapoptotic isoform *caspase-2L* (JIANG *et al.* 1998). Conversely, overexpression of hnRNP (heteronuclear ribonucleoprotein) A1 promotes inclusion of this exon and the generation of a transcript consistent with the anti-apoptotic *caspase-2S* isoform (JIANG *et al.* 1998).

Whereas these studies of *caspase-2* do present evidence that SR proteins regulate the alternative splicing of programmed cell death genes, they contradict the established roles of SR proteins and hnRNPs in the promotion of exonic inclusion and exclusion, respectively. As described in the introduction, SR proteins normally promote inclusion of exons, whereas hnRNPs play the opposite role. One explanation for this discrepancy might be that SR

proteins are not properly regulated by phosphorylation when they are overexpressed, as they were in the *caspase-2* studies. The genetic studies of *Doa* in *Drosophila* suggest that phosphorylated SR proteins promote the inclusion of exons (DU *et al.* 1998). If we assume that SPK-1 phosphorylates SR proteins in *C. elegans in vivo* as it does *in vitro* (KUROYANAGI *et al.* 2000), then our results are consistent with the *Drosophila* findings that suggest the phosphorylation of SR proteins promotes the inclusion of exons. When *spk-1* is deleted from *C. elegans*, the incorporation of the *ced-4L* exon decreases, just as, in *Drosophila* when *Doa* function is reduced, the levels of incorporation of the female-specific exon of *dsx* decrease.

Although *caspase-2* is the only mammalian cell death gene whose alternative splicing has been shown to be influenced by SR proteins, certainly many other mammalian genes involved in programmed cell death are alternatively spliced. For example *bcl-x*, which encodes a protein homologous to CED-9, is generated from a long splice isoform, Bcl-x_L, which inhibits apoptosis and contains all four of the BH domains found in Bcl-2 (BOISE *et al.* 1993). The protein generated from the shorter isoform, Bcl-x_S, promotes apoptosis and contains only the BH3 and BH4 domains (BOISE *et al.* 1993). Consistent with their proposed functions, Bcl-x_S protein and mRNA are widely expressed and found in tissues that contain cells undergoing apoptosis (BOISE *et al.* 1993; KRAJEWSKI *et al.* 1994).

Although many alternative splice forms of mammalian genes such as *bcl-x* and *caspase-2* that function in apoptosis have been described, little is known about the functions of the alternative splice forms and the mechanisms that regulate the splice site selection of these genes (SCHWERK and SCHULZE-OSTHOFF 2005). Our studies in *C. elegans* establish a robust assay in a genetically tractable system for further investigations into how alternative

splicing is regulated in general, and how in particular alternative splicing interfaces with programmed cell death.

FUTURE DIRECTIONS

The most important question that arises from the identification of SPK-1 as a modifier of *ced-4* RNA splicing is how it functions. Previous work suggests that SR protein kinases regulate splicing by phosphorylating SR proteins. I am currently in the process of obtaining deletion alleles for each of the eight SR proteins found in the *C. elegans* genome. Each of these deletion alleles will be tested for the ability to suppress the cell-death defect of *ced-4(n3158)* animals. Determining genetically if a specific SR protein is responsible for regulating splicing of *ced-4* would be important.

Although isolation of large amounts of RNA from *spk-1(Δ)* animals remains a technical challenge because loss-of-function alleles of *spk-1* confer sterility, I would like to confirm the observations made by RT-PCR using an assay that does not depend upon PCR amplification, such as RNase protection. Additionally, I have generated a CED-4L-specific antibody that might allow me to directly determine if *spk-1* alleles result in a corresponding change of the amount of CED-4L protein.

I would also like to assay the role *spk-1* plays in cell deaths other than those of the specific cells I assayed in this work. Since maternal rescue may preclude the analysis of early developmental deaths, late developmental deaths, such as those that occur in the male tail, might be the best cells to assay.

Finally, it would be interesting to modify the *ced-4* genomic locus by homolgous recombination to make targeted changes to test the function of various portions of the genomic locus. Although no robust system is currently available for homologous recombination in *C. elegans*, when it is, these critical experiments need to be performed. I have spent several months attempting to modify the genomic locus without success.

EXPERIMENTAL PROCEDURES

Strains and General Techniques

Strains were cultured as described by BRENNER (1974) and grown at 20° C. The Bristol strain N2 was used as the wild-type strain, except in multifactor mapping experiments that used the polymorphic wild-type strain CB4856 (WICKS *et al.* 2001). The mutations used are listed below, and are description by RIDDLE (1997) unless otherwise indicated:

LGIII: *ced-4(n3158)* (S. CAMERON and H. R. HORVITZ, unpublished), *ced-4(n3141)* (G. M. STANFIELD and H. R. HORVITZ, unpublished), *ced-4(n1162)*, *ced-4(n3040)* (E. K. SPELIOTES and H. R. HORVITZ, unpublished), *spk-1(n3418)* (this study), *spk-1(ok706)* (*C. elegans* Gene Knockout Consortium), *lon-1(e185)*, *ced-9(n3377)* (P. W. REDDIEN and H. R. HORVITZ, unpublished)

LGIV: *ced-3(n717)*, *ced-3(n2427, n2436)* (HENGARTNER and HORVITZ 1994a)

LGV: *egl-1(n3331)* (REDDIEN *et al.* 2001), *egl-1(n4045)* (B. D. GALVIN and H. R. HORVITZ, unpublished), *nIs96(lin-11::gfp)* (REDDIEN *et al.* 2001)

LGX: *nIs106(lin-11::gfp)* (REDDIEN *et al.* 2001)

The translocation *hT2* containing the integrated transgene *qIs48* was also used (WANG and KIMBLE 2001).

Screen for Suppressors of *ced-4(n3158)*

We mutagenized L4 *ced-4(n3158); nIs106* hermaphrodites with ethyl methanesulfonate (EMS) as described by BRENNER (1974). After allowing the animals to recover, P_0s were transferred one per 5 cm plate. After 3-5 days, 12 F_1 L4 hermaphrodites were individually transferred to 5 cm plates, and F_2 animals on these plates were

subsequently screened for animals with the wild-type number of GFP-positive Pn.aap cells. We screened the progeny of 2500 F_1 animals. Six siblings were picked from any plate on which a suppressor was isolated to ensure that heterozygous siblings were maintained for isolates that were inviable.

Quantitation of Cell Death in the VC-like neurons and the Anterior Pharynx

Pn.aap cells expressing GFP were visualized using a dissecting microscope equipped with fluorescence optics (M^2BIO, Kramer Scientific, Valley Cottage, NY). Animals late in the fourth larval stage were picked using a standard dissecting microscope and transferred to the fluorescence-equipped dissecting microscope for counting of their surviving VC-like neurons.

Cloning of *spk-1*

Using standard deficiency and mapping techniques, we placed the *n3418* mutation between a polymorphism on cosmid F54C8 (snp_F54C8[1]) and a polymorphism on ZK1098 (snp_ZK1098[1]) that exists between *C. elegans* strains N2 and CB4856.

Determination of Mutant Allele Sequences

We used PCR-amplified regions of genomic DNA to determine mutant sequences. For *spk-1(n3418)* we determined the sequence of all exons and splice junctions. Sequences were determined using an ABI Prism 3100 Genetic Analyzer.

Semi-Quantitative RT-PCR Analysis of *ced-4* Transcripts

Sixty-six 9-cm plates of *spk-1(ok706Δ)/hT2[qIs48]* animals were washed into 1 L of liquid growth media. The culture was grown until saturated with gravid adults and harvested prior to starvation or the appearance of dauer larvae. Worms were bleached to collect embryos. The embryos were allowed to hatch for 12 hours in 500 mL of liquid growth media and sorted for non-GFP-positive worms for 4 hours using a COPAS BioSort instrument (Union Biometrica, Holliston, MA) according to manufacturer's instructions. Worms were collected in batches of 50,000 and concentrated by centrifugation. The young larva were flash frozen in liquid nitrogen and stored at -80° C in Trizol (Invitrogen, Carlsbad, CA). The same procedure was performed on the N2 control, strain except the 1 liter culture was started from six 9 cm plates. RNA was isolated from the animals as previously described (PORTMAN 2006), except smaller volumes were used. The purified RNA was DNase treated using amplification grade Deoxyribonuclease I (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT reactions were performed with random hexamers according to the manufacturer's instructions for use with SuperScript III First strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Dilutions of RT-reaction were performed to establish a linear range for semi-quantitative PCR reactions. The sequences of the *ced-4* primers are 5'-ACCTTGCTGATTTCCTCGAA-3' and 5'-AATGAAGTCGGTGATCGAATG-3', and for the loading control *ama-1* (which encodes the large subunit of RNA polymerase II) they are 5'-GATCAGGCGACCTATTTGGA-3' and 5'-TGGAAGAAGAATTCCGATGG-3'.

FIGURE LEGENDS

Figure 1: Screen to Identify Genes that Protect Against Programmed Cell Death

After mutagenizing *ced-4(n3158); nIs106* animals with EMS, we used fluorescence microscopy to examine F_2 individuals for a decreased number of GFP-positive cells.

Figure 2: Molecular Identification of the *ced-4(n3158)* Suppressor *spk-1(n3418)*

(A) Schematic representation of the physical map of the *spk-1(n3418)* region and the gene structure of *spk-1* showing the location of the allele *n3418* and the deletion allele *ok706*Δ. The interval to which *spk-1(n3418)* was mapped contained 24 genes. RNAi against one gene, *B0464.5*, in the interval caused a sterility that resembled that of *spk-1(n3418)* (KUROYANAGI *et al.* 2000). Sequence analysis and genetic tests with the deletion allele *spk-1(ok706)* showed that this identified nonsense mutation caused the observed phenotype.

(B) SPK-1 is similar to mammalian SR Protein Kinases. Human SRPK1 and SRPK2 are compared to SPK-1. The numbers indicate amino acid positions. The red asterisk indicates the location of the *n3418* mutation, which changes tryptophan 142 to a opal stop codon.

Figure 3: *spk-1* Promotes Generation of the Protective *ced-4L* Transcript

This agarose gel stained with ethidium bromide shows products generated by PCR using primers flanking the first three introns of *ced-4* and the sixth intron of the control *ama-1* from wild-type and *spk-1(ok706*Δ*)* RNA, labeled + and Δ respectively at the bottom of the gel (see Experimental Procedures for details). The *ama-1* band in the *spk-1(ok706*Δ*)* is smeared due to an impurity in the gel. 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA) was run in the first and sixth lanes and the 200, 300, 400, and 500 base-pair bands are labeled.

Figure 4: Model for the Regulation of *ced-4* RNA Splicing and Programmed Cell Death by SPK-1

(A) In wild-type animals SPK-1 promotes the generation of the protective *ced-4L* alternative splice variant, which protects against programmed cell death. Loss of SPK-1 decreases the production of *ced-4L*, thereby decreasing protection against programmed cell death.

(B) SPK-1 might promote the use of the *ced-4L* splice site in the *ced-4* pre-mRNA by phosphorylating an SR protein.

Table 1. Loss-of-function Alleles of *spk-1* Suppress the Survival of Extra Pn.aap Cells in *ced-4(n3158)* Animals

Genotype[a]	No. extra Pn.aap cells ± SE (*n*)
Wild type	0 (109)
ced-4(n3158)	4.9 ± 0.04 (50)
ced-4(n3158) spk-1(n3418)	2.0 ± 0.1 (71)
ced-4(n3158) spk-1(ok706Δ)	1.3 ± 0.1 (57)
spk-1(n3418)	0 (56)
spk-1(ok706Δ)	0 (37)

The number of extra Pn.aap cells were counted as described in Experimental Procedures. Because *spk-1(n3418)* and *spk-1(ok706Δ)* cause recessive sterility, strains containing these alleles were derived from heterozygous parents; see Experimental Procedures. *n*, number of animals.

[a] All strains are homozygous for *nIs106*, an integrated *lin-11::gfp* reporter (REDDIEN et al. 2001).

Table 2. *spk-1(n3418)* Suppresses Only the Cell-death Defect of Partial Loss-of-function Alleles of *ced-4*

Genotype[a]	No. extra cells in anterior pharynx ± SE (*n*)	No. extra Pn.aap cells ± SE (*n*)
ced-4(n3141)	3.0 ± 0.4 (>15)[c]	4.6 ± 0.1 (50)
ced-4(n3141) spk-1(n3418)[b]	ND	1.2 ± 0.1 (55)
ced-4(n3158)	6.0 ± 0.7 (>15)[c]	4.9 ± 0.04 (50)
ced-4(n3158) spk-1(n3418)	ND	2.0 ± 0.1 (71)
ced-4(n3040)	11.5 ± 0.4 (>15)[c]	5.0 ± 0 (52)
ced-4(n3040) spk-1(n3418)[b]	ND	5.0 ± 0 (50)
ced-4(n1162)	11.8 ± 0.2 (>15)[c]	4.9 ± 0.03 (50)
ced-4(n1162) spk-1(n3418)[b]	ND	4.9 ± 0.04 (58)

The number of extra Pn.aap cells and number of extra cells in anterior pharynx were counted as described in Experimental Procedures. Because *spk-1(n3418)* and *spk-1(ok706Δ)* cause recessive sterility, strains containing these alleles were derived from heterozygous parents; see Experimental Procedures. *n*, number of animals. ND, not determined.

[a] All strains used to count extra Pn.aap cells are homozygous for *nIs106*, an integrated *lin-11::gfp* reporter (REDDIEN et al. 2001).

[b] *spk-1(n3418)* was *cis*-marked with *lon-1(e185)* in each of these strains to facilitate identification of homozygous animals.

[c] These data were provided by BRAD HERSH and H. R. H. (unpublished observations).

Table 3. *spk-1(n3418)* Weakly Suppresses the Survival of Extra Pn.aap Cells of Partial Loss-of-function Alleles of Some Additional Ced Genes

Genotype[a]	No. extra Pn.aap cells ± SE (*n*)
ced-3(n2427)	3.4 ± 0.2 (50)
spk-1(n3418); ced-3(n2427)[b]	1.2 ± 0.1 (60)
ced-3(n2436)	4.8 ± 0.1 (50)
spk-1(n3418); ced-3(n2436)[b]	4.0 ± 0.1 (52)
ced-9(n3377)	2.3 ± 0.1 (50)
spk-1(n3418) ced-9(n3377)[b]	1.6 ± 0.1 (67)
egl-1(n3331)	4.7 ± 0.1 (50)
spk-1(n3418); egl-1(n3331)[b]	4.5 ± 0.1 (48)
egl-1(n4045)	3.8 ± 0.1 (50)
spk-1(n3418); egl-1(n4045)[b]	3.6 ± 0.1 (60)

The number of extra Pn.aap cells were counted as described in Experimental Procedures. Because *spk-1(n3418)* causes recessive sterility, strains containing this allele were derived from heterozygous parents; see Experimental Procedures. *n*, number of animals.

[a] All strains are homozygous for *nIs106*, an integrated *lin-11::gfp* reporter, except those containing *egl-1(n3331),* which are homozygous for the related reporter *nIs96* (REDDIEN et al.

[b] *spk-1(n3418)* was *cis*-marked with *lon-1(e185)* in each of these strains to facilitate identification of homozygous animals.

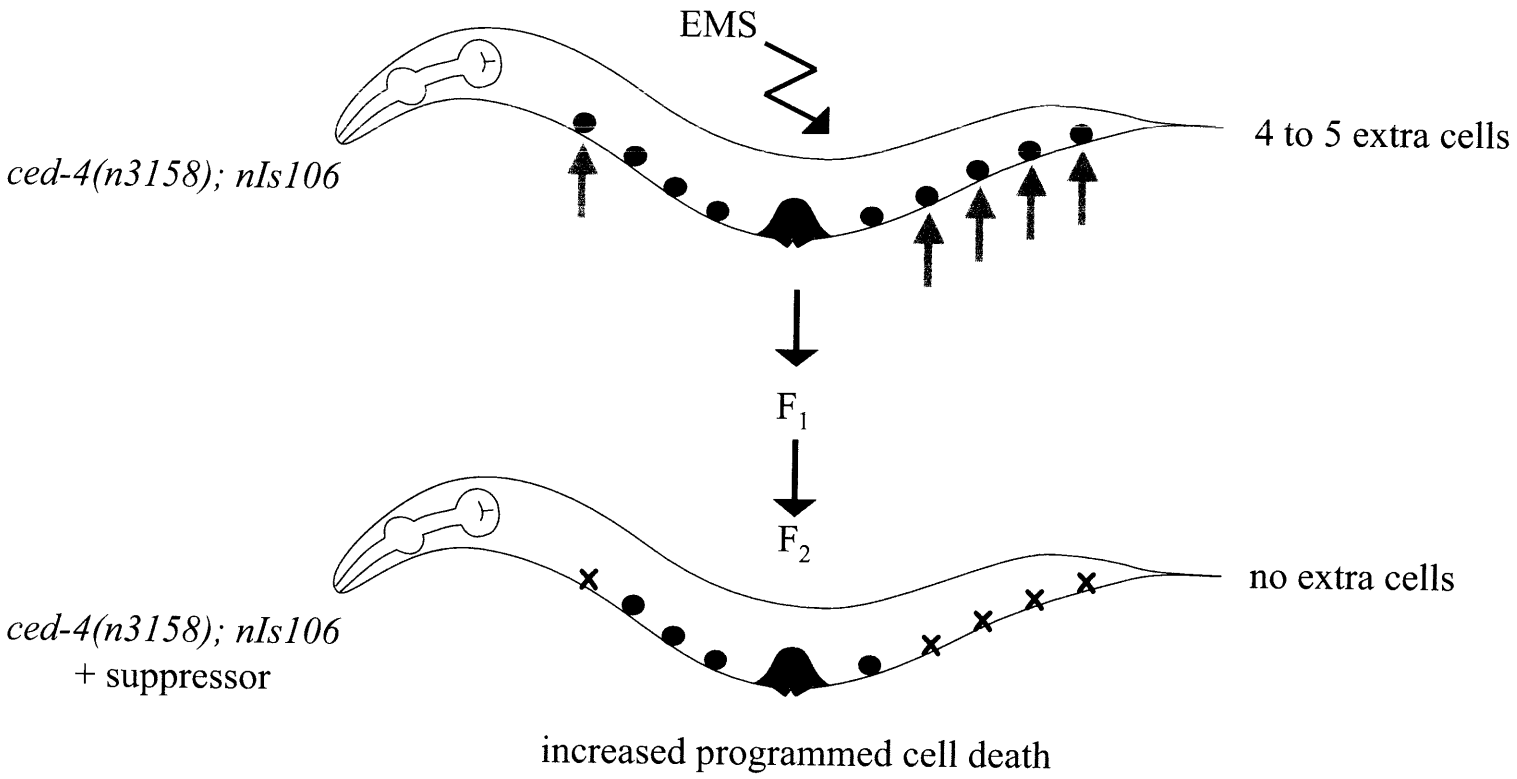
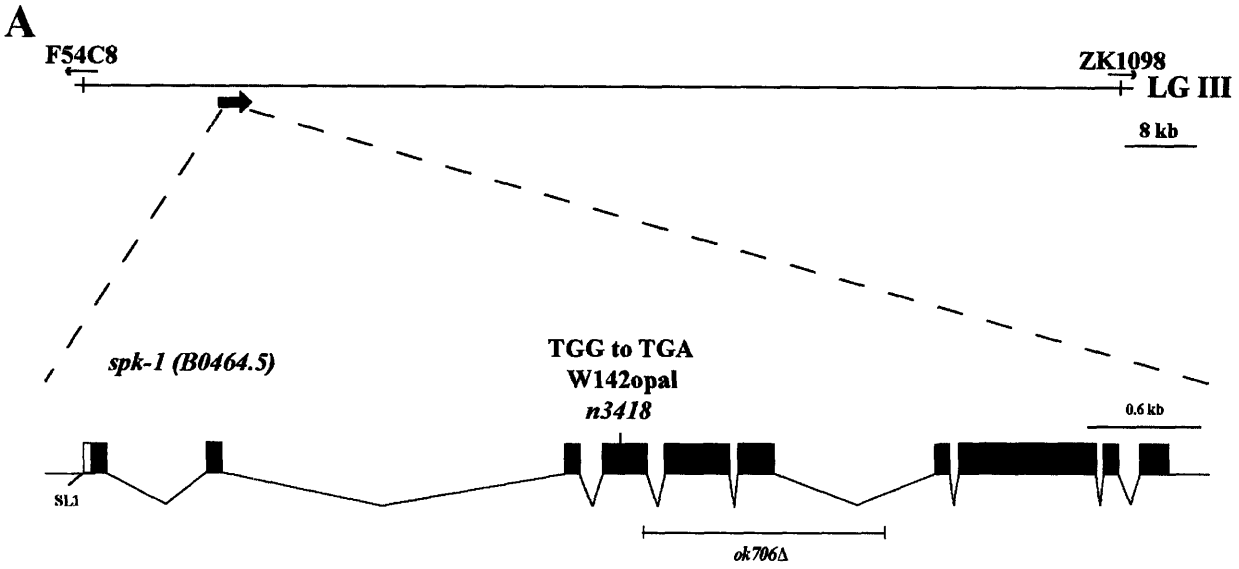

EMS
ced-4(n3158); nIs106
4 to 5 extra cells
F1
F2
ced-4(n3158); nIs106
+ suppressor
no extra cells
increased programmed cell death
= location of extra Pn.aap cell
= location of dead Pn.aap cell

Figure 1


A
F54C8
ZK1098
LG III
8 kb
spk-1 (B0464.5)
TGG to TGA
W142opal
n3418
0.6 kb
SL1
ok706Δ

B

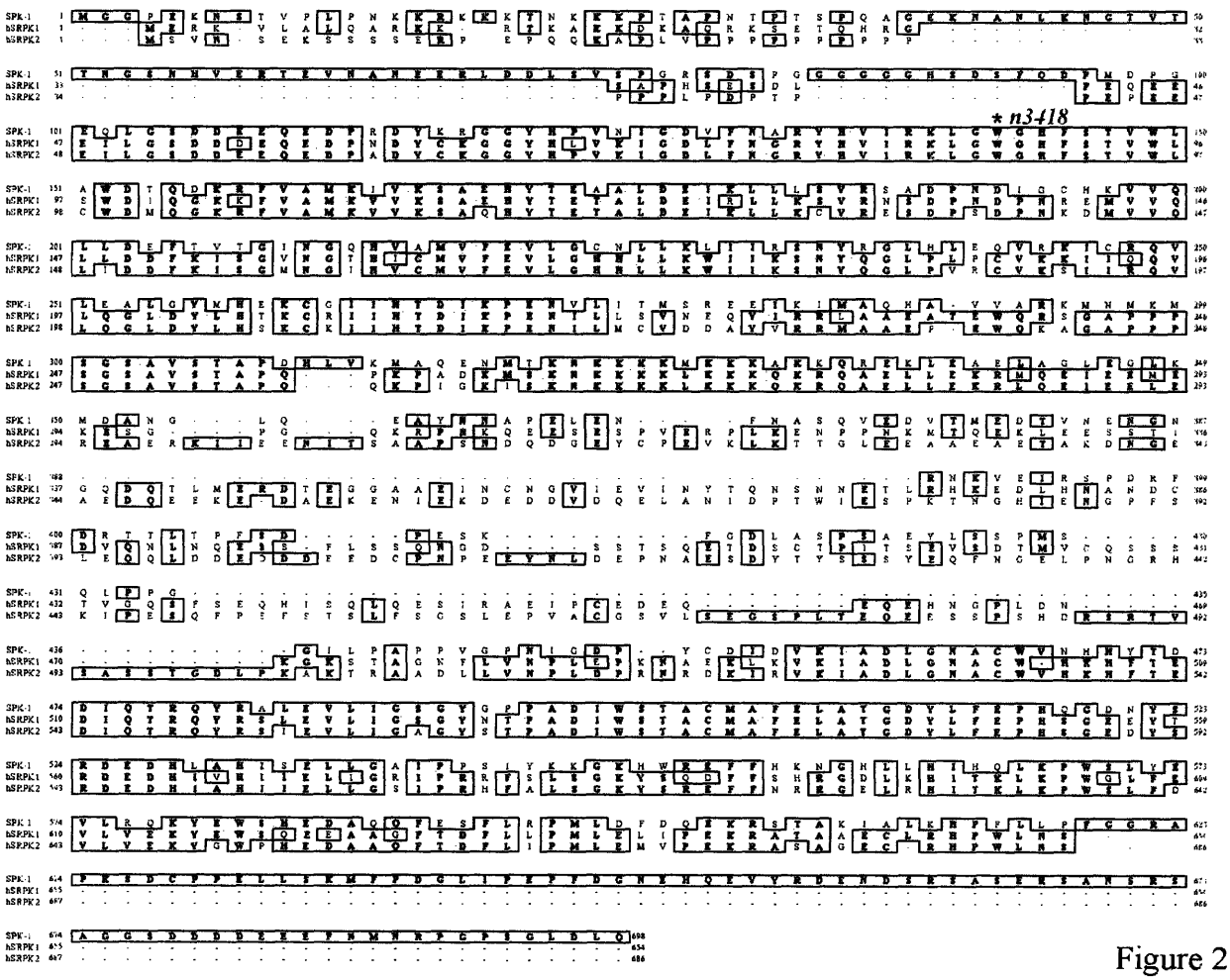

* n3418

Figure 2

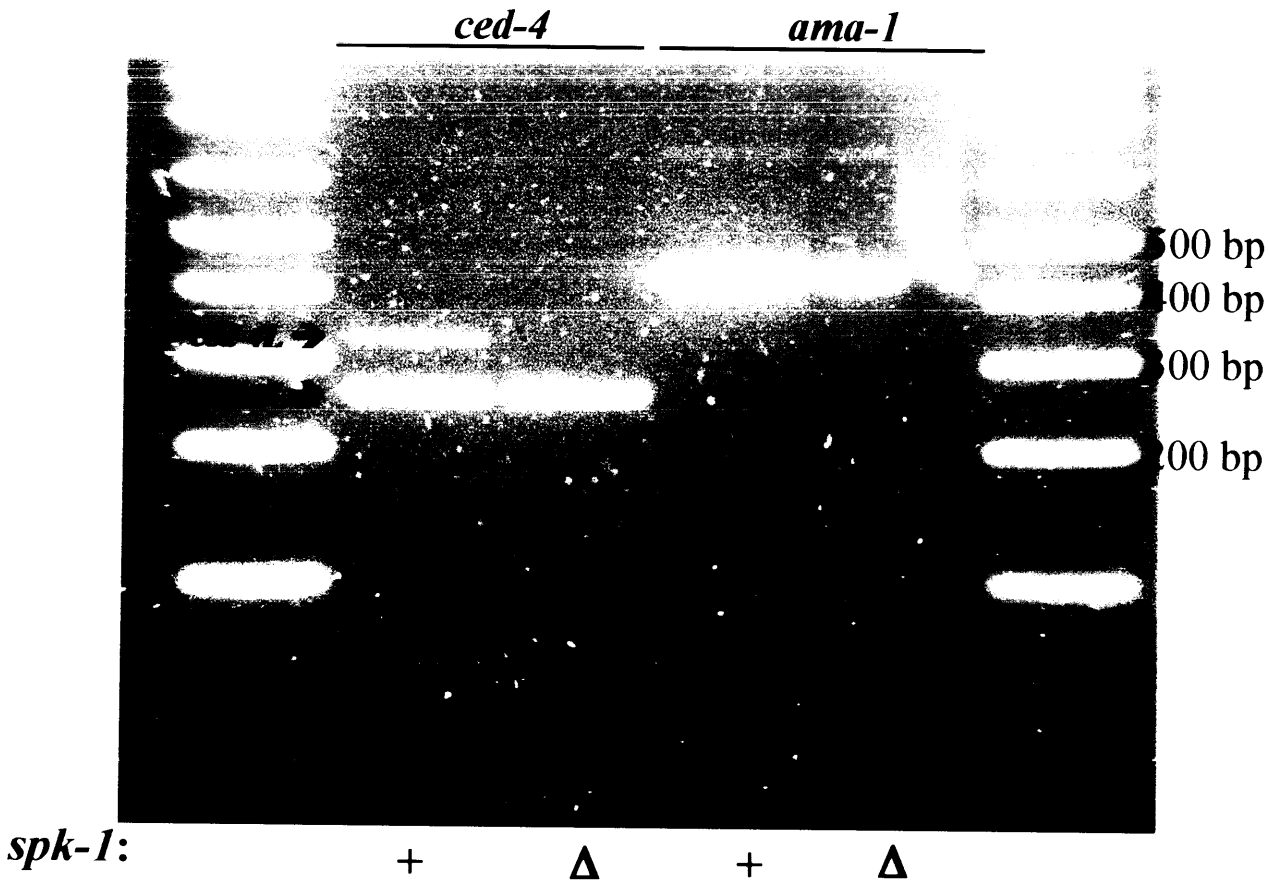


Figure 3

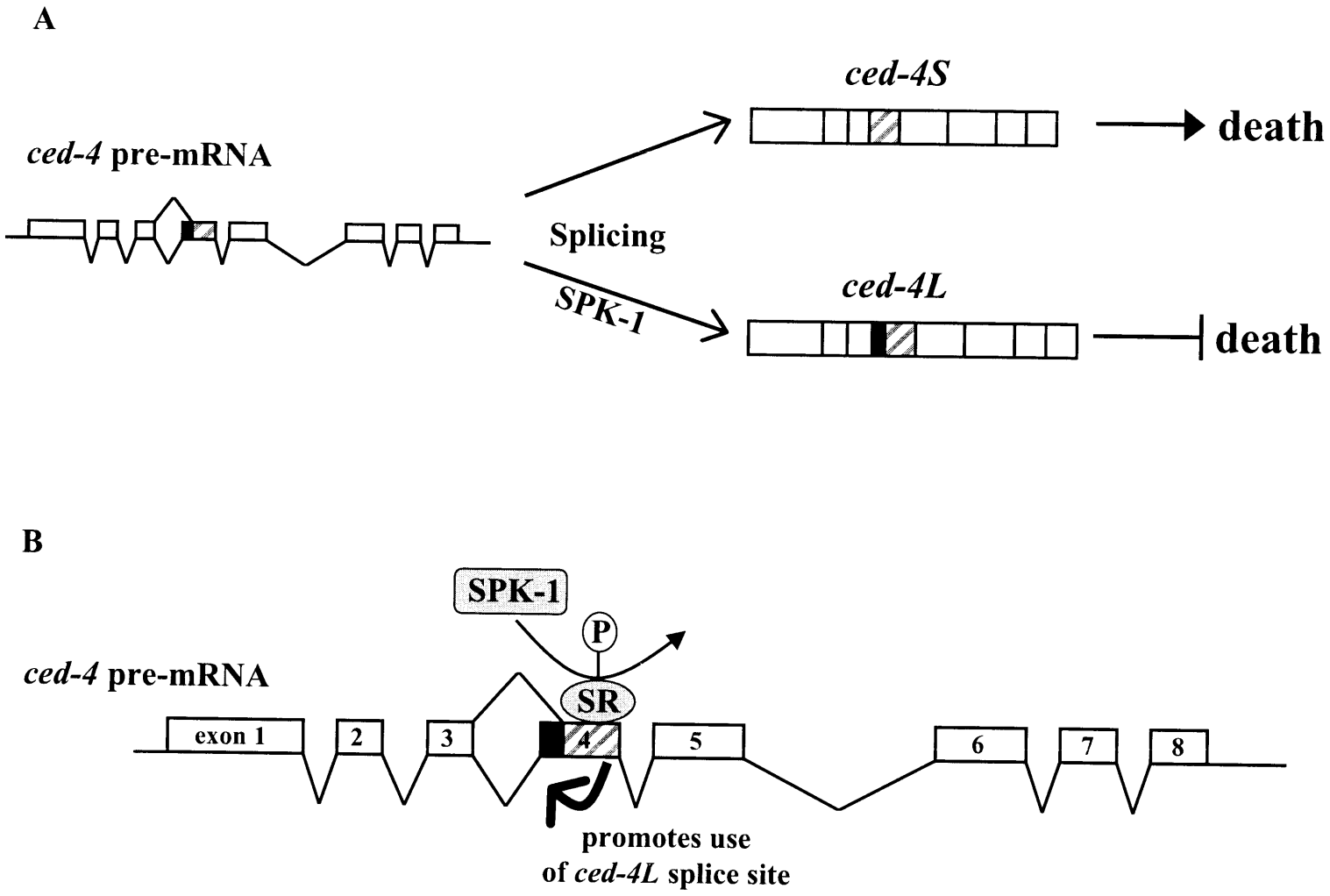

A
ced-4S
ced-4 pre-mRNA
death
Splicing
SPK-1
ced-4L
death
B
SPK-1
P
ced-4 pre-mRNA
SR
exon 1
2
3
4
5
6
7
8
promotes use
of ced-4L splice site

Figure 4

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Chapter Three

Apoptosis of Specific Neuronal Cells in *C. elegans* Requires an Enhancer Element of *egl-1* that is Bound by a Novel Transcriptional Activator

Brendan D. Galvin, Dave T. Harris, Natalia J. Martinez[*], A. J. Marian Walhout[*], and H. Robert Horvitz

I performed the yeast one-hybrid screen in the laboratory of Marian Walhout. Natalia Martinez, a graduate student in the Walhout laboratory, provided me with technical assistance. Dave Harris isolated *egl-1(n4629)* when he was my rotation student.

[*] University of Massachusetts Medical School, Worcester, MA 01605

SUMMARY

The BH3-only killer gene *egl-1* is the most upstream gene that functions in the execution phase of programmed cell death in *C. elegans* and is thought to be expressed only in cells that die. The mechanisms by which *egl-1* gene expression is regulated are known for only a small number of cells that die, including the HSNs, the NSM sister cells, and P11.aaap, a neuron located in the posterior ventral nerve cord. From a screen to identify mutants defective in programmed cell death, we isolated a mutation in *egl-1* that lies in an evolutionarily conserved DNA element four kilobases downstream of the coding sequence. Both this point mutation and a deletion allele that removes this conserved element prevent the deaths of the VC-like ventral cord neurons that normally die in hermaphrodites. We performed a yeast one-hybrid screen to identify proteins capable of binding to this conserved DNA element. We identified three candidate proteins that could strongly interact with a reporter construct containing the wild-type enhancer element; two of these three candidates failed to activate a reporter containing a mutant enhancer element. Analysis of one of these two candidates, *Y38C9A.1*, suggests that it may function to regulate the deaths of the VC-like neurons *in vivo*: both dsRNA directed against this gene and two deletion alleles of this gene can partially prevent the deaths of the VC-like neurons. *Y38C9A.1* encodes a novel protein with no characterized homologs or obvious DNA binding domains. Thus, we have identified both a new regulatory site in *egl-1* that controls expression in specific neuronal cells and a novel protein that may bind and activate transcription at this site.

INTRODUCTION

Programmed cell death, or apoptosis, is the highly regulated process of eliminating cells that are either unnecessary or deleterious. Apoptosis is essential for the proper development and health of most animals. The elimination of a wide variety of cells, including self-reactive immune cells or fibroblasts with DNA damage, prevents the survival of potentially dangerous cells. The misregulation of apoptosis contributes to numerous disease processes, ranging from cancer (associated with decreased apoptosis) to forms of neurodegeneration (associated with increased apoptosis) (FADEEL and ORRENIUS 2005).

During the development of the *C. elegans* hermaphrodite, 1,090 cells are generated, 131 of which are eliminated by programmed cell death (KIMBLE and HIRSH 1979; SULSTON and HORVITZ 1977; SULSTON *et al.* 1983). Many genes have been identified in *C. elegans* that function in this process of programmed cell death (LETTRE and HENGARTNER 2006). Each of these genes can be placed into one of four steps: specification, by which cells become fated to undergo programmed cell death; execution of the death process; engulfment of the dying cell by neighboring cells; and the degradation of the dead cell within the engulfing cell. The conservation of this pathway is highlighted by the fact that related proteins play similar roles in apoptosis in all animals.

The four *C. elegans* genes that function in the execution step in all somatic programmed cell deaths are *egl-1* (*egl*, egg laying defective), *ced-9* (*ced*, cell death defective), *ced-4*, and *ced-3*. *egl-1*, which encodes a BH3-only (Bcl-2 homology region 3) protein, is thought of as the master regulator of programmed cell death, as it is likely to be expressed only in cells that die and is the most upstream gene that functions in the death of all somatic cells (CONRADT and HORVITZ 1998). BH3-only proteins are also key

pro-apoptotic proteins in mammals (WILLIS and ADAMS 2005). EGL-1 inhibits the anti-apoptotic function of the Bcl-2 homolog CED-9 (CONRADT and HORVITZ 1998), which functions to sequester CED-4 (the *C. elegans* Apaf-1 ortholog) to mitochondria (CHEN *et al.* 2000). Binding of EGL-1 to CED-9 liberates CED-4 (CHEN *et al.* 2000; DEL PESO *et al.* 2000; DEL PESO *et al.* 1998; YAN *et al.* 2004), which can then function to activate the caspase (cysteine aspartate-specific protease) CED-3 (CHINNAIYAN *et al.* 1997; SESHAGIRI and MILLER 1997; WU *et al.* 1997; YANG *et al.* 1998). The autoproteolytic activation of CED-3, the most downstream player in the execution step, provides the critical activity required for all programmed cell deaths to occur. Elimination of *egl-1*, *ced-4*, or *ced-3* function, or a gain-of-function mutation in *ced-9*, prevents all somatic programmed cell death (CONRADT and HORVITZ 1998; ELLIS and HORVITZ 1986; HENGARTNER *et al.* 1992).

The current model is that transcriptional regulation of *egl-1* is central to the control of individual cell deaths; that is, cells in which *egl-1* is transcribed die and cells in which *egl-1* is not transcribed survive. We understand the transcriptional regulation of *egl-1* for only a small number of the 152 somatic cells that die either in hermaphrodite or male development. For the two hermaphrodite-specific neurons (HSNs), which control egg laying in the hermaphrodite and die in the male, the sex-specific repression of *egl-1* transcription has been described. In the HSNs, TRA-1, which is the most downstream gene product in the sex determination pathway and is specifically active in hermaphrodites, binds to a regulatory element of *egl-1* and prevents its transcription (CONRADT and HORVITZ 1999). In males, which lack TRA-1 activity, *egl-1* transcription in the HSNs is not repressed, and the HSNs die. In hermaphrodites that contain a mutation disrupting the TRA-1 binding site that regulates *egl-1* expression in the HSNs, the HSNs inappropriately die (CONRADT and

HORVITZ 1999). Given that TRA-1 acts as a repressor of *egl-1* expression in the HSNs, the question of what activates *egl-1* expression in these cells remains. EOR-1, a putative transcription factor containing zinc finger domains, and EOR-2, a novel conserved protein, may function together to promote the death of the HSNs; however, these proteins have not yet been shown to directly regulate *egl-1* transcription (HOEPPNER *et al.* 2004).

The mechanism of the regulation of *egl-1* has also been described for the NSM sister neurons, which are two pharyngeal neurons that die in wild-type animals. The identification of gain-of-function mutations in *ces-1* helped to define *egl-1* regulation in these cells (ELLIS and HORVITZ 1991). CES-1 is a member of the Snail/Slug family of zinc finger transcription factors (METZSTEIN and HORVITZ 1999). The *egl-1* genomic locus contains a site to which CES-1 can bind; a mutation of this site that specifically disrupts CES-1 binding blocks the ability of overexpressed CES-1 to protect the NSM sister neurons from death (THELLMANN *et al.* 2003). This site overlaps with a binding site for two basic helix-loop-helix (bHLH) transcription factors, HLH-2 and HLH-3, which promote *egl-1* transcription in the NSM sisters (THELLMANN *et al.* 2003). The death decision of the NSM sister neurons is thought to be made by the regulation of the two opposing activities of CES-1 on the one hand and HLH-2 and HLH-3 on the other.

The mechanism of activation of *egl-1* transcription in another dying cell has also been studied. Analysis of transgenic animals and biochemical assays suggest that the proteins encoded by the HOX genes *ceh-20* and *mab-5* directly bind to elements controlling *egl-1* expression and activate *egl-1* transcription, thereby regulating the death of P11.aaap, a posteriorly located cell in the ventral nerve cord (LIU *et al.* 2006). This work suggests that HOX genes, which are important developmental genes that regulate body morphology and

cell fate, can directly regulate cell death by binding to sites within the *egl-1* genomic locus and control *egl-1* transcription.

Given the prominent role apoptosis plays in many disease processes, proteins that bind to and regulate *egl-1* expression are good candidates to possess homologs involved in disease processes. For example, this link with disease has already been established for the homologs of the proteins that regulate the death of the NSM sister neurons. In addition to mutations in *ces-1*, mutations in *ces-2* were identified in the genetic screens for mutations that prevent this death. Gain-of-function mutations in *ces-1* and loss-of-function mutations in *ces-2* prevent NSM sister neurons from normally undergoing programmed cell death (ELLIS and HORVITZ 1991; METZSTEIN *et al.* 1996). Based on these studies of *ces-1* and *ces-2*, the mammalian homologs of CES-1 and CES-2 were shown to function in regulate apoptosis in hematopoietic and lymphoid cells. CES-2 is similar to the proto-oncogene HLF (hepatic leukemia factor), a transcriptional repressor (METZSTEIN *et al.* 1996). The oncogenic form of this gene is the result of the chromosomal translocation t(17;19), which fuses the DNA binding domain of HLF to the transactivation domain of E2A (HUNGER *et al.* 1992; INABA *et al.* 1992). This chimeric protein binds to and causes overexpression of the anti-apoptotic CES-1 homologue SLUG, which can prevent the death of the leukemic pro-B cells (INUKAI *et al.* 1999). Recent work suggests that SLUG may directly prevent the expression of a BH3-only gene, a function highly similar to the function of CES-1 in repressing *egl-1* expression in the NSM sister neurons of *C. elegans* (WU *et al.* 2005).

In mammalian systems, the transcriptional regulation of genes that encode BH3-only proteins is one of the major modes by which apoptosis is regulated. Numerous BH3-only proteins are found in mammalian genomes, including Bim, Bad, Bid, Bik, Bmf, PUMA,

Noxa, and Hrk (WILLIS and ADAMS 2005). Redundancy has complicated the genetic analysis of these genes, but some, such as PUMA (p53 upregulated modulator of apoptosis), which is directly regulated by p53, have well-understood roles as transcriptionally-regulated regulators of apoptosis. The transcription factor p53 is a critical tumor suppressor gene that is often mutated in cancers and is an important regulator of apoptosis (HOFSETH *et al.* 2004). In its first intron, PUMA has a p53 binding site, which is required for its radiation-induced transcriptional activation by p53 (YU *et al.* 2001). p53 regulation of *PUMA* appears to be physiologically relevant, as *PUMA*-knockout mice closely resemble *p53*-knockout mice with respect to apoptosis. Both knockout mice are similarly impaired for apoptosis induced by γ-irradiation in thymocytes, oncogenes in MEFs, and DNA damage in neurons, suggesting that PUMA is one of the main transcriptional targets of p53 in the induction of apoptosis (JEFFERS *et al.* 2003; VILLUNGER *et al.* 2003).

Given the importance of BH3-only proteins in regulating apoptosis, we sought to understand the regulation of *egl-1* transcription in a subset of cells whose decision to die was not previously understood. In this work, we identified a conserved enhancer element in *egl-1* that controls the death of specific cells in the ventral cord. Using a yeast one-hybrid screen, we identified the protein Y38C9A.1, which binds to this element and may directly regulate the transcription of *egl-1* in these cells *in vivo*. Deletions of either the *egl-1* non-coding element or *Y38C9A.1* each promote survival in the same cells. Y38C9A.1 may encode a novel DNA-binding protein, as it has no characterized homologs or recognizable domains from any previously identified transcription factors.

RESULTS

Identification of a Mutation in an Evolutionarily Conserved DNA Element in *egl-1*

We performed a screen seeking mutants defective in programmed cell death in *C. elegans* by screening for enhancers of the cell-death defect of animals containing the mutation *n2427* (HENGARTNER and HORVITZ 1994), a weak loss-of-function allele of the caspase gene *ced-3*. To assess the amount of cell death, we used a modified *lin-11::gfp* reporter that is expressed in neurons of the Pn.aap lineage in the ventral cord (REDDIEN and HORVITZ 2004). In the *C. elegans* ventral nerve cord of the hermaphrodite, twelve cells are generated in the lineal position Pn.aap (the posterior daughter of the anterior daughter of the anterior daughter of one of twelve P blast cells); six of those twelve (the two anterior-most, P1.aap and P2.aap, and the four posterior-most, P9-P12.aap) die by programmed cell death (Figure 1A) (SULSTON and HORVITZ 1977). The six Pn.aap cells that survive differentiate into class VC motor neurons, some of which innervate the vulval muscles of the adult hermaphrodite. The modified *lin-11::gfp* reporter *nIs106* expresses in Pn.aap cells, including Pn.aap cells that are normally fated to die but whose deaths have been prevented by mutations blocking programmed cell death (REDDIEN *et al.* 2001). These cells that usually die but whose death is prevented will be called VC-like neurons in this chapter, because they closely resemble the VC motor neurons that survive in wild-type animals (WHITE *et al.* 1991). Five of these VC-like neurons can be reliably scored by assaying expression of *lin-11::gfp* (REDDIEN *et al.* 2001). This reporter is extremely useful in studying programmed cell death, because it provides a measure of programmed cell death that is both quantifiable at the single-animal level and easily scored using a dissecting microscope equipped with fluorescence optics. In

our screen, we isolated *lin-11::gfp* animals possessing an increased number of GFP-positive cells, reflecting a possible defect in programmed cell death.

One mutation we identified in our screen was designated *n4045*. This mutation promoted survival of GFP-positive VC-like neurons that normally undergo programmed cell death (Table 1). This effect on VC-like neuron survival was not dependent on a defect in *ced-3* killing activity, as *n4045* strongly protected these cells when it had been separated from the *ced-3(n2427)* mutation used in the screen (Figure 1, Table 1). We mapped *n4045* to an interval containing the known regulator of programmed cell death *egl-1*. *n4045* failed to complement the Ced phenotype of a loss-of-function allele of *egl-1(n1084 n3082)* in the VC-like neurons (data not shown). A single mutation was found in the strain containing this allele, located approximately four kilobases downstream of the coding region of *egl-1* (Figure 2). This mutation lies in a small region that is highly conserved in sequence and location in the genomes of the two *Caenorhabditis* species with sequenced genomes, *C. briggsae* and *C. remanei* (Figure 2). These three species are distantly related nematodes in which only coding sequences and important regulatory regions are thought to be conserved. We named this DNA element in *egl-1* the VCK (VC-like neuron killing) element.

Isolation of Additional *egl-1* Alleles Mutated in the VCK Element

To identify additional alleles of *egl-1* that might help better define the VCK element, we performed an *egl-1* non-complementation screen using the *lin-11::gfp* reporter. We mated mutagenized males with *egl-1(lf)* hermaphrodites and screened for animals with undead VC-like neurons. In a screen of approximately 13,000 haploid genomes, the single *egl-1* allele *n4629* was isolated (Table 1). *n4629* caused the identical base change identified

in *egl-1(n4045)* (Figure 2). This independent isolation of the identical mutation strongly suggested that this sequence alteration was responsible for the observed phenotype of survival of the VC-like neurons.

At this point we could not distinguish between two models of how this point mutation was behaving: the mutation might disrupt the binding of a transcriptional activator or it might increase binding of a transcriptional repressor, perhaps by creating a regulatory site not normally found in this location. To distinguish between these two models we sought a deletion of the VCK element. A very small deletion of 309 base pairs was isolated from a library of mutagenized *C. elegans*, *n4908*Δ (Figure 2). This deletion was centered on the *n4045* mutation such that approximately 150 base pairs were eliminated on both sides of the point mutation. *n4908*Δ was able to phenocopy the point mutations *n4045* and *n4629*, establishing this site as a binding site for a transcriptional activator. Unlike the two point mutations, the deletion allele completely eliminated the deaths of all VC-like neurons, suggesting that the point mutations do not completely eliminate the binding of the transcriptional activator (Table 1).

The VCK Element Specifically Controls Cell Death of the VC-like Neurons

The mutations in the VCK element of *egl-1* that we isolated strongly prevented the deaths of the VC-like neurons that normally die in wild-type hermaphrodites. It remained to be determined whether this effect was specific to these cells or the consequence of a more general loss of *egl-1* function, affecting the deaths of other cells that normally die in *C. elegans*. Another anatomical region in which cell death is routinely assayed is the anterior pharynx; the cells of this region can readily be counted using Nomarski differential-

interference contrast microscopy (HENGARTNER *et al.* 1992). Sixteen cells of the anterior pharynx, arising from multiple lineages, normally undergo programmed cell death, and the number of these cells that inappropriately survive provides a quantitative assay of possible defects in programmed cell death. In the anterior pharynx, no additional cells were observed in strains containing the *egl-1* mutation *n4045* or the VCK deletion allele *n4908*Δ (Table 2). Additionally, when these alleles were assayed in the presence of a weak *ced-3* allele, *n2427*, which provides a sensitized background in which very weak cell-death defects can be detected (REDDIEN *et al.* 2001), no enhancement of the weak survival caused by *ced-3(n2427)* was observed (Table 2). These findings suggest that the mutations in the VCK element specifically prevent the death of the VC-like neurons. Thus, the VCK enhancer element appears to promote tissue-specific activation of *egl-1* transcription.

A Yeast One-Hybrid Screen Identified Proteins that Bind the VCK element

To identify proteins that bind to the VCK element, we undertook a yeast one-hybrid screen. The yeast-one-hybrid system is conceptually similar to the yeast two-hybrid system that is used for the detection of protein-protein interactions (CHIEN *et al.* 1991). In the yeast one-hybrid system, a single hybrid protein containing a *C. elegans* cDNA fused to a strong, heterologous activation domain is tested for reporter gene activation, which occurs when the hybrid protein interacts with the DNA bait containing the DNA element of interest (LI and HERSKOWITZ 1993). The reporter construct used in these one-hybrid screens contained eight copies of a 31 base-pair element centered on the point mutation found in *egl-1(n4045)* and *egl-1(n4629)* (Figure 3A). Versions of this reporter were generated containing the wild-type sequence and the *n4045* mutant version, both for screening purposes and to determine

whether candidate interactors specifically activate the wild-type reporter but not the mutant reporter. Two types of screens were conducted in parallel. In the first, we tested the ability of approximately two-thirds of the 1,000 predicted transcription factors (DEPLANCKE *et al.* 2004) in the *C. elegans* genome to bind to the VCK element. In the second, we screened a library of most *C. elegans* cDNAs fused to an activation domain (WALHOUT *et al.* 2000) for candidates that bind to the VCK element.

None of the predicted *C. elegans* transcription factors that we tested activated the wild-type or mutant reporters. They were tested on yeast strains containing the *egl-1* reporters by either individually mating yeast strains containing each of the transcription factor fusions or by transformation with a library containing only these transcription factor fusions. Three candidates that successfully retested were identified from the cDNA library screen. *Y38C9A.1* was isolated three times from the cDNA library screen with the wild-type reporter. *B0238.11* was isolated once from the cDNA library screen with the wild-type reporter. The last candidate was defined by a cDNA containing *Y38F11.3* and parts of two adjacent predicted genes (*Y43F11.5* and *Y38F11.2*); this cDNA was recovered three times, once when screening using the wild-type reporter and twice when screening using the mutant reporter.

When each of these isolates was retested against both the mutant and wild-type reporters, both *Y38C9A.1* and *B0238.11* selectively activated the wild-type reporter, whereas *Y43F11.3* activated the mutant and wild-type reporters equally (Figure 3B). The selective activation of the wild-type but not the *n4045* mutant reporters by both *Y38C9A.1* and *B0238.11* was of particular interest, because such a profile might represent genes whose binding to the enhancer element is prevented by the point mutation. Thus, these two genes

are candidates for functioning *in vivo* to regulate *egl-1* transcription in the VC-like neurons that normally undergo programmed cell death. Of the three predicted proteins we found that could interact with the *egl-1* enhancer element in the yeast one-hybrid system, only one, B0238.11, is predicted to contain a recognizable DNA-binding domain, a high mobility group (HMG) box domain (BIANCHI 1995).

Inactivation of *Y38C9A.1* Promotes the Survival of VC-like Neurons

To determine whether the candidates isolated in the yeast one-hybrid screen function to regulate the death of the VC-like neurons, we screened a library of mutagenized *C. elegans* and isolated animals carrying deletions in each of the three candidate interactors (Figure 3C). These deletions were each tested using the modified *lin-11::gfp* reporter *nIs106* to determine whether they promoted survival of the VC-like neurons that normally undergo programmed cell death. The deletion allele *n4909*Δ eliminates *Y43F11.3*, the only predicted gene wholly contained in the yeast one-hybrid screen isolate that also contains part of *Y43F11.5* and *Y43F11.2*. Animals containing this deletion appeared superficially wild-type and did not promote survival of VC-like neurons. *n4904*Δ, which deletes *B0238.11*, causes early larval arrest, consistent with the phenotype we observed by injection of dsRNA directed against this gene (data not shown). None of the small number of animals that developed far enough to express *gfp* from the *lin-11::gfp* reporter had surviving VC-like neurons.

Two deletion alleles of *Y38C9A.1*, *n4799*Δ and *n4917*Δ, as well as injection of dsRNA directed against *Y38C9A.1*, caused several abnormalities. Most significantly, the deletions caused survival of VC-like neurons that normally die. In Figure 4A, a confocal image is shown of a representative animal carrying a deletion in *Y38C9A.1*, and harboring

surviving VC-like neurons. This finding is consistent with a role for *Y38C9A.1* in the control of the death of the VC-like neurons. Additionally, in combination with the *lin-11::gfp* reporter *nIs106*, the *Y38C9A.1* deletion alleles appear to cause an alteration in sex determination, since animals were observed that contained sexual characteristics of both hermaphrodites and males.

DISCUSSION

Transcriptional control of *egl-1*, a BH3-only protein-encoding gene, is the primary mechanism of regulating somatic programmed cell death in *C. elegans* (CONRADT and HORVITZ 1998). Similarly, transcriptional activation of the pro-apoptotic BH3-only proteins is fundamental to the regulation of apoptosis in mammals (WILLIS and ADAMS 2005). Thus an understanding of how *egl-1* is regulated in cell death is critically important. This work has established a mechanism of transcriptional control of *egl-1* in specific cells, the VC-like neurons in the ventral nerve cord, in which the mechanism of *egl-1* activation was previously uncharacterized. By means of a genetic screen, we identified a non-coding regulatory site of *egl-1* required for the death of the VC-like neurons. Genetic analysis suggests that mutations in this VCK element can perturb the binding of a transcriptional activator. We identified three proteins capable of binding to the VCK element in a yeast one-hybrid assay and found that one of these proteins may regulate *egl-1* transcription *in vivo*. This protein product of the *Y38C9A.1* gene has no known DNA-binding domains and thus may contain a previously unidentified DNA-binding domain.

Deletion of *Y38C9A.1* caused abnormalities not observed in strains carrying mutations in the VCK element, demonstrating that *Y38C9A.1* affects processes in addition to *egl-1* activation in the VC-like neurons. The observed survival of the VC-like neurons might be a result of perturbation of sexual identity rather than a defect in the specification of death of the VC-like neurons, as some animals carrying a deletion in *Y38C9A.1* display sexual characteristics of both males and hermaphrodites. Alternatively, *Y38C9A.1* might control VC-like neuron survival and also function in other independent processes in *C. elegans*, possibly explaining why this gene has not been isolated in the numerous screens performed

with this reporter. Significantly, deletion of *Y38C9A.1* did not promote survival of the VC-like neurons as strongly as did mutations in the non-coding *egl-1* regulatory element. This result suggests that Y38C9A.1 is unlikely to be the only activator that binds to this site. It remains to be seen whether two or more of the three candidates isolated in the yeast one-hybrid screen might act redundantly to control VC-like neuron cell death.

Consistent with our studies of *Y38C9A.1*, Previously identified *egl-1* regulators seem to be involved in multiple processes and are not exclusively devoted to regulating *egl-1* transcription to control cell death. TRA-1, which represses *egl-1* transcription in the HSNs, is a master regulator of sex determination (MEYER 2000). Mutations in *eor-1* and *eor-2*, two genes that promote HSN cell death, both cause a range of abnormalities, including incompletely penetrant lethality (HOEPPNER *et al.* 2004). *hlh-2*, which activates transcription of *egl-1* in the NSM sister cells, is essential (KRAUSE *et al.* 1997), as is *ceh-20*, which activates *egl-1* in P11.aaap, a cell near the tail (LIU *et al.* 2006). The partner with which CEH-20 dimerizes to control *egl-1* expression in the P11.aaap cell, MAB-5, is not essential, but animals lacking *mab-5* function show cell lineage defects in the tail region, including the cells whose survival *mab-5* appears to directly control (KENYON 1986). CES-2 is a regulator of excretory canal cell morphogenesis (WANG *et al.* 2006) in addition to controlling *egl-1* expression in the NSM sister cells (METZSTEIN *et al.* 1996; THELLMANN *et al.* 2003). Our work demonstrates that deletions of *Y38C9A.1*, in addition to promoting survival of the VC-like neurons, cause abnormalities in sex determination that lead to animals displaying characteristics of both males and hermaphrodites.

To date, only two transcriptional factors seem to regulate *egl-1* specifically and not affect additional processes: HLH-3, which has a very weak influence on cell death and

appears to act primarily as a cofactor for regulation of *egl-1* expression by the essential gene *hlh-2* (THELLMANN *et al.* 2003); and CES-1, which acts to repress *egl-1* expression in the NSM sisters (THELLMANN *et al.* 2003). It remains highly possible that HLH-3 and CES-1 possess additional functions, as yet unknown, beyond their cell-specific roles in the control of programmed cell death.

Although *egl-1* transcription has been suspected of being the main control mechanism of programmed cell death in *C. elegans*, a limited number of genes that control its expression have been identified. The well understood regulation of *egl-1* in the HSNs was established in a way that is similar to this work, by identifying non-coding mutations in *egl-1* that alter the survival decision of a specific cell (CONRADT and HORVITZ 1999). Mutational analysis of the *egl-1* locus using extrachromosomal arrays in transgenic animals was important for establishing the roles of *hlh-2*, *hlh-3*, *ceh-20*, and *mab-5* in the regulation of *egl-1* transcription. It is very likely that the transcription factors that regulate *egl-1* expression function in many processes in addition to programmed cell death, such that they cannot be identified in genetic screens. This has certainly been the case for most genes that regulate *egl-1* expression studied to date. The genetic approaches used in this manuscript of identifying non-coding regulatory regions in *egl-1*, and then using these regulatory regions to identify proteins that bind to them, may therefore be the most useful method of identifying additional factors that control the survival decisions of specific cells by regulating *egl-1* expression.

FUTURE DIRECTIONS

The current data suggest that genes that regulate *egl-1* expression in a cell-specific fashion might not function solely to regulate *egl-1* expression; rather, loss-of-function mutations in these genes might well cause additional phenotypic defects, making such mutations difficult to isolate in screens. Our work and other related work suggest that an alternative genetic approach to identifying genes that regulate *egl-1* expression is to identify functional regulatory elements of *egl-1* and then identify proteins that bind to these sites by methods such as one-hybrid screens. These regulatory elements can be identified by recovering animals from a genetic screen, most efficiently in an *egl-1* non-complementation screen as demonstrated in this work. Alternatively, one can test mutant strains deleted for genomic regions near to the *egl-1* coding region, an approach this work also validates. In the case of the targeted deletions, it may be possible by means of rescue experiments or by a return to *egl-1* non-complementation screens to identify within a larger deletion the specific regulatory element responsible for cell-specific control of *egl-1* expression. Further understanding of the regulation of *egl-1* expression and the expression of mammalian BH3-only genes is clearly critical to our understanding of programmed cell death, so taking a non-standard approach to isolating *egl-1* transcription factors seems warranted. Approaches such as are described above and those we have used in this work may serve to identify new regulatory regions and the factors that act upon them.

In the yeast one-hybrid screen, I identified two additional genes that remain to be characterized. Given their strong interaction with *egl-1* in the yeast one-hybrid assay, it is possible that these genes regulate *egl-1* expression in cells other than the VC-like neurons. Worms carrying deletions in each of these genes should be examined for other cell-death

defects. Recent data suggest that the *egl-1* deletion allele *n4908* might also promote survival of the male-specific CEM neurons, which normally die in hermaphrodites (in contrast to the point mutation we originally isolated, which does not protect these cells) (H. SCHWARTZ, personal communication). Perhaps one of the genes identified in the yeast one-hybrid screen regulates the death of these cells, or alternatively the 309 bp deletion might eliminate another element that specifically contributes to the regulation of those deaths.

EXPERIMENTAL PROCEDURES

Strains and Genetics

Strains were cultured as described by BRENNER (1974) and grown at 20° C on NGM agar using *E. coli* OP50 as a food source. The Bristol strain N2 was the wild-type strain. The mutations and balancer chromosomes used in the study are as follows and are previously described (RIDDLE *et al.* 1997) unless otherwise indicated:

LGI, *dpy-5(e61)*, *nIs133* [*pkd-2::gfp*; *lin-15AB(+)*] (JAGER *et al.* 2004)

LGII, *rol-6(e187)*, *Y43F11A.3(n4909Δ)* (this study), *rrf-3(pk1426)*

LGIII, *unc-32(e189)*

LGIV, *ced-3(n717)*, *ced-3(n2427)* (HENGARTNER and HORVITZ 1994), *unc-30(e191)*

LGV, *dpy-11(e224)*, *rol-4(sc8)*, *unc-42(e270)*, *unc-76(e911)*, *egl-1(n1084 n3082)* (CONRADT and HORVITZ 1998), *egl-1(n4045*, *n4629*, *n4908Δ)* (this study), *Y38C9A.1(n4799Δ)* (this study), *Y38C9A.1(n4917Δ)* (this study), *B0238.11(n4904Δ)* (this study)

LGX, *lon-2(e678)*, *nIs106* [*lin-11::gfp*; *lin-15AB(+)*] (REDDIEN *et al.* 2001).

The translocation *nT1* IV;V with the dominant marker *qIs51* [*myo-2::gfp*] (SIEGFRIED *et al.* 2004) was used as a balancer for *B0238.11(n4908Δ)*.

Identification and Mapping of *egl-1(n4045)*

We mutagenized L4 *ced-3(n2427); nIs106* hermaphrodites with ethyl methanesulfonate (EMS) as described by BRENNER (1974). After letting the animals recover, single P_0 animals were transferred to 9-cm plates. After 6-8 days F_2 animals on these plates were screened for animals with five surviving GFP-positive VC-like neurons. *n4045* was

mapped to linkage group V using the markers *dpy-5* I, *rol-6* II, *unc-32* III, *unc-30* IV, *dpy-11* V, and *lon-2* X (BRENNER 1974). Further mapping placed *n4045* approximately 13/20 of the distance from *rol-4* to *unc-76* on LGV, a position consistent with those of two genes known to cause survival of VC-like neurons in hermaphrodites, *egl-1* and *sel-10*. Further investigation demonstrated that *n4045* did not cause defects consistent with a mutation in *sel-10* (H. SCHWARTZ, personal communication).

egl-1 Non-complementation Screen

We mutagenized L4 *nIs133*; *nIs106* males with EMS as described by BRENNER (1974) and mated them with *nIs133*; *rol-4(sc8) egl-1(n1084n3082) unc-76(e911)*; *nIs106* hermaphrodites. After 3-4 days, F_1 non-Rol non-Unc cross progeny were screened to identify animals with surviving GFP-positive VC-like neurons.

Quantitation of Cell Death in the VC-like neurons and the Anterior Pharynx

VC-like neurons expressing GFP were visualized using a dissecting microscope equipped with fluorescence optics (M^2BIO, Kramer Scientific, Valley Cottage, NY). Animals late in the fourth larval stage were picked using a standard dissecting microscope and transferred to the fluorescence-equipped dissecting microscope for counting of their surviving VC-like neurons.

Nomarski differential-interference contrast microscopy was used to count the number of extra cells in the anterior region of the pharynx as previously described (HENGARTNER *et al.* 1992).

Determination of Mutant Allele Sequences

We used PCR-amplified regions of genomic DNA to determine mutant sequences. For *egl-1(n4045)*, we determined the sequence of approximately 13 kb surrounding the gene. For *egl-1(n4629)*, we determined the sequence of the coding region and the interval containing the *egl-1(n4045)* mutation. For *egl-1(n4908Δ)*, we determined the sequence of the interval containing the deletion. Sequences were determined using an ABI Prism 3100 Genetic Analyzer.

Generation of Yeast One-Hybrid Reporter Constructs and Strains

To generate a DNA bait construct containing eight copies of the 31 base-pair DNA element centered on the *n4045* mutation, four pairs of complementary oligonucleotides, each containing two copies of the element, were annealed and ligated into pUC19. M13 primers flanked by Gateway-compatible cloning sites were used to PCR-amplify the 8X DNA element, after which the resulting PCR amplicon was cloned via a Gateway BP reaction into the entry vector pDONR-P4-P1R, as described (DEPLANCKE *et al.* 2004). One sequence-verified entry clone was then used for LR cloning into the destination vectors and integrated into the genome of YM4271 yeast, as described (DEPLANCKE *et al.* 2004). Bait strains were verified by PCR of yeast genomic DNA using vector-specific primers, after which PCR amplicons were sequenced. Self-activation of promoter bait strains was tested as described (DEPLANCKE *et al.* 2004).

Yeast One-Hybrid Screens

Mating experiments using strains of opposite mating types, one containing the promoter bait and the other containing the AD-TFs (Activation Domain-Transcription Factors) (B. DEPLANCKE and A. J. M. WALHOUT, unpublished data) were performed as described (WALHOUT and VIDAL 2001).

Promoter bait strains were transformed with the AD-wrmcDNA (*C. elegans* cDNAs) and AD-TF libraries as described (DEPLANCKE *et al.* 2004), and plated onto Sc-His, -Ura, -Trp media containing 40mM 3AT. 9.4×10^6 and 3.8×10^5 colonies were screened per strain for the AD-wrmcDNA and AD-TF libraries, respectively, for the wild-type reporter. 2.5×10^6 and 3.4×10^5 colonies were screened per strain for the AD-wrmcDNA and AD-TF libraries, respectively, for the *n4045* mutant reporter. Potential positives were picked and retested as described (DEPLANCKE *et al.* 2004). To sequence ORFs encoding potential interactors, yeast colony PCR was performed as described (WALHOUT and VIDAL 2001). Gap-repair-based phenotypic retesting was performed as described (WALHOUT and VIDAL 2001).

RNA Interference of Candidates Isolated in the Yeast One-Hybrid Screen

Vector-specific primers flanked by T7 binding sites were used to PCR-amplify the one-hybrid isolates that retested. To generate double-stranded RNA, a MEGAscript® T7 Kit (Ambion, Austin, TX) was used according to the manufacturer's specifications. dsRNA for each isolate was injected into the gonad of *rrf-3(pk1426); nIs106* worms. The F_1 progeny were scored for extra GFP-positive VC-like neurons and other abnormalities.

Isolation of Deletion Alleles

Genomic DNA pools from the progeny of EMS-mutagenized animals were screened to identify deletions using PCR as previously described (CEOL and HORVITZ 2001). Deletion mutant animals were isolated from frozen stocks and backcrossed to the wild type. *egl-1(n4908Δ)* removes nucleotides 1819 to 2127 of plasmid VF23B12L. *Y38C9A.1(n4799Δ)* removes nucleotides 530 to 2807 of YAC Y38C9A. *Y38C9A.1(n4917Δ)* removes nucleotides 8199 to 11139 of YAC Y38C9A. *B0238.11(n4904Δ)* removes nucleotides 28722 to 30199 of cosmid B0238. *Y43F11A.3(n4909Δ)* removes nucleotides 5846 to 7310 of YAC Y43F11A.

FIGURE LEGENDS

Figure 1: *egl-1(n4045)* Promotes Survival of VC-like Neurons

(A) In the ventral cord, the twelve P cells P1-P12 each divide post embryonically to generate a Pn.a (n is 1-12) neuroblast and a Pn.p hypodermal cell. P3-8 give rise to the six Pn.aap cells that survive and differentiate into the VC motor neurons, whereas P1, 2, 9 - 12 give rise to Pn.aap cells that undergo programmed cell death shortly after they are generated, in the late L1/early L2 larval stage. The modified *lin-11::gfp* reporter *nIs106* reliably expresses in any of the eleven Pn.aap cells generated by P2 - P12 if they survive through the fourth larval stage.

(B) Confocal images of *nIs106* expression patterns. In wild-type animals containing the modified *lin-11::gfp* reporter *nIs106*, four of the six VC neurons generated by the P3-5, and P8 lineages, can be easily observed by fluorescence microscopy, whereas the fluorescence from VC neurons generated by the P6 and P7 lineages is obscured by GFP fluorescence in vulval tissue. In *egl-1(n4045)* animals, P2 - P12.aap cells usually survive and express *gfp*.

Figure 2: *n4045* and *n4629* Are Identical Mutations in the VCK Element of *egl-1*

A schematic representation of the *egl-1* locus, showing the locations and sequences of the *egl-1(n4045)*, *egl-1(n4629)*, and *egl-1(n4908Δ)* alleles and their evolutionary conservation. In the alignment of the conserved DNA element 3′ of *egl-1* in *C. elegans*, *C. briggsae*, and *C. remanei*, identical nucleotides are shaded. The DNA element in *C. elegans* has the opposite orientation of that found in *C. briggsae* and *C. remanei.*

Figure 3: Three Proteins Bind to the VCK Element of *egl-1* in A Yeast One-Hybrid Assay

(A) Schematic of the *HIS3* reporters used in the yeast one-hybrid assays.

(B) Isolates from the yeast one-hybrid screen were tested for their ability to allow *HIS3*- yeast strains to grow in the absence of His by assaying for their abilities to activate the *HIS3* reporters containing eight copies of either the wild-type (+) or mutant *(n4045)* VCK element. Cells were plated on Sc -His, -Ura media supplemented with 20 mM 3AT.

(C) Schematic representation of genes that encode the proteins isolated in the one-hybrid screen, showing the location of the deletion alleles (*n4799*Δ, *n4917*Δ, *n4904*Δ, and *n4909*Δ) isolated for each gene.

Figure 4: *Y38C9A.1* Functions to Promote the Deaths of VC-like Neurons

(A) Confocal image of *Y38C9A.1(n4799Δ); nIs106* animals, which display fluorescence in VC-like neurons that failed to die.

(B) Model for the death of VC-like neurons. In VC-like neurons that die in wild-type animals, Y38C9A.1 binds to the VCK element in *egl-1* defined by *n4045* and promotes transcription of *egl-1*. In either the absence of a functional VCK element or in the absence of *Y38C9A.1*, *egl-1* transcription is not promoted and the cells inappropriately survive.

Table 1. Mutations in the VCK Element in *egl-1* Promote Survival of VC-like Neurons

Genotype	No. of VC-like neurons ± SE (*n*)
Wild type	0 ± 0 (109)
ced-3(n2427)	2.4 ± 0.2 (50)
ced-3(n2427);egl-1(n4045)	5.0 ± 0.1 (102)
egl-1(n4045)	4.5 ± 0.1 (50)
egl-1(n4629)	4.4 ± 0.1 (50)
egl-1(n4908Δ)	5.0 ± 0 (50)
egl-1(null)^	5.0 ± 0 (25)
ced-3(n717)	5.0 ± 0.1 (25)

VC-like neurons counted as described in Experimental Procedures. *n*, number of animals. SE, standard error.

All strains were homozygous for *nIs106*, the integrated *lin-11::gfp* reporter. Genotypes are otherwise indicated.

^ *egl-1(n1084 n3082)*

Table 2. Mutations in the VCK Element in *egl-1* Do Not Promote Survival of Extra Cells in the Anterior Pharynx

Genotype	No. of extra cells in anterior pharynx ± SE (*n*)
Wild type	0 ± 0 (20)
egl-1(n4045)	0 ± 0 (10)
egl-1(n4908Δ)	0.1 ± 0.1 (10)
ced-3(n2427)	1.3 ± 0.3 (10)
ced-3(n2427); egl-1(n4045)	1.3 ± 0.3 (10)
ced-3(n2427); egl-1(n4908Δ)	1.2 ± 0.3 (10)
egl-1(null)^	11.1 ± 0.1 (113)
ced-3(n717)	12.0 ± 0.3 (20)

Number of extra cells counted as described in Experimental Procedures. *n*, number of animals. SE, standard error.

^ *egl-1(n1084 n3082);* these data were taken from CONRADT and HORVITZ (1998).

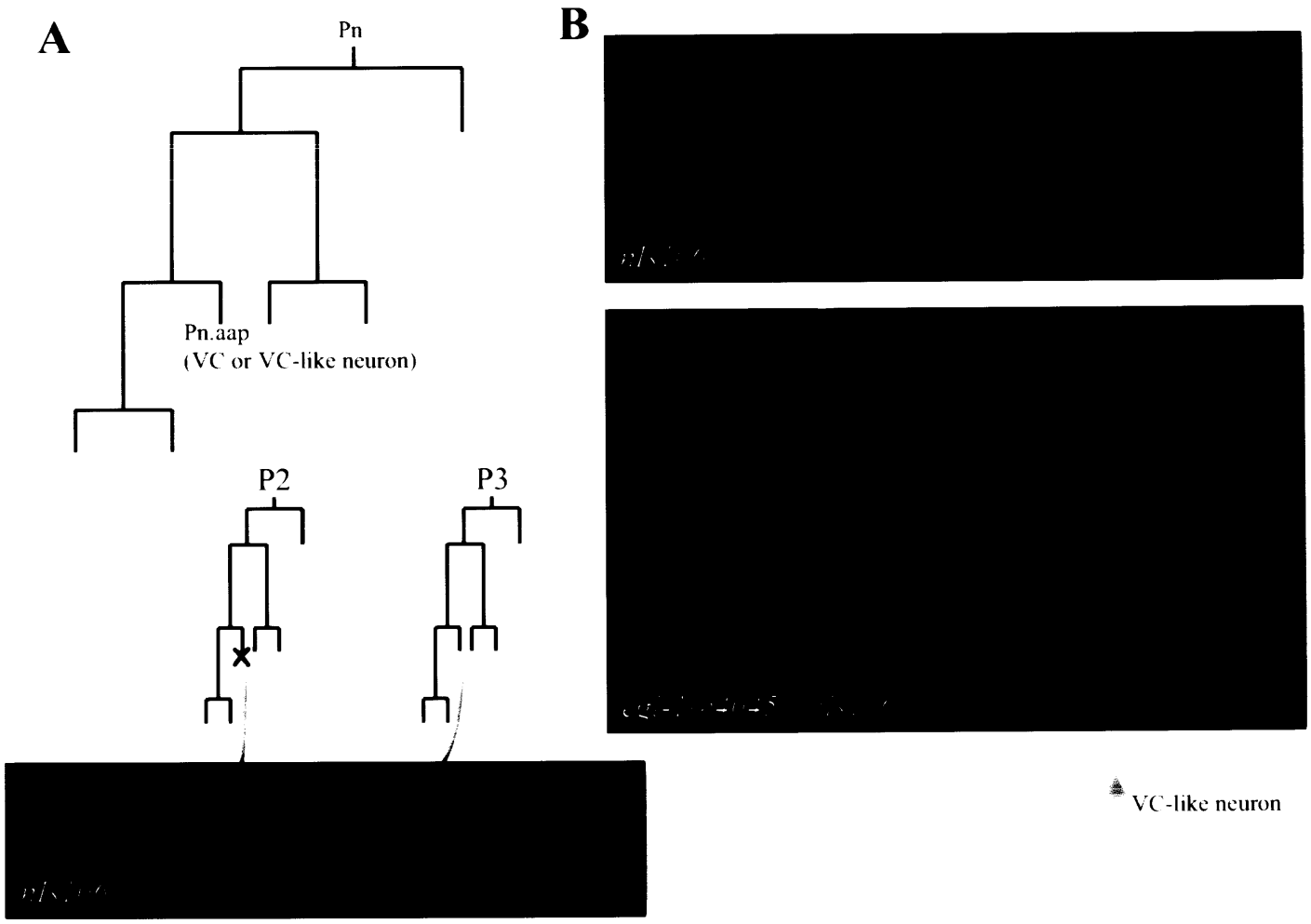

A
Pn
Pn.aap
(VC or VC-like neuron)
P2
P3
B
VC-like neuron
× location of dead VC-like neuron
location of GFP-positive VC neuron
location of GFP-positive vulva

Figure 1

VCK (VC-like Killing) Element

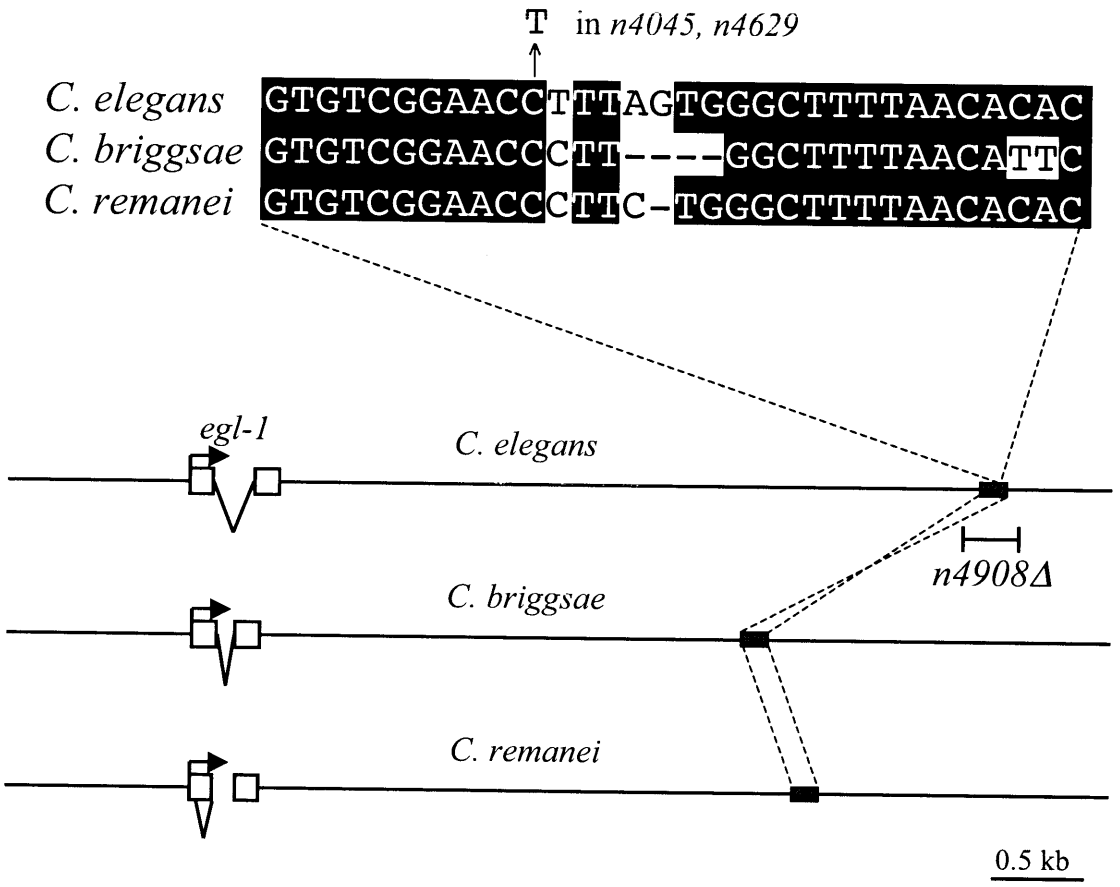


Figure 2

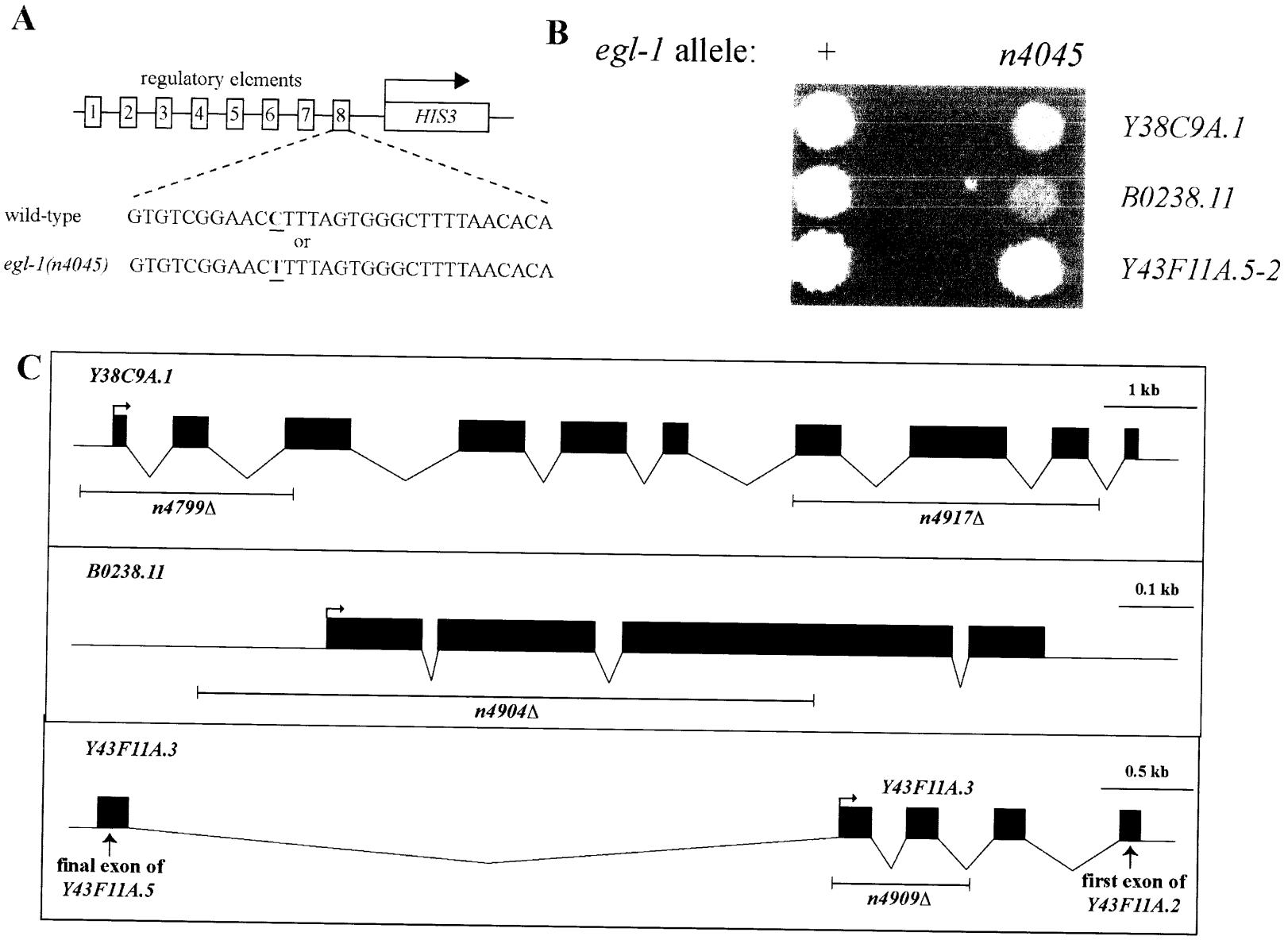
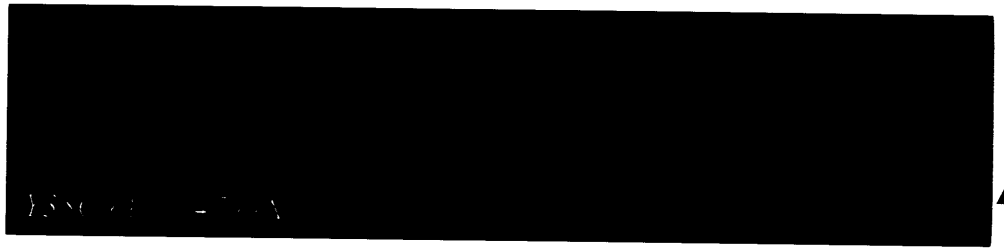

A
regulatory elements
1
2
3
4
5
6
7
8
HIS3
wild-type GTGTCGGAACCTTTAGTGGGCTTTTAACACA
or
egl-1(n4045) GTGTCGGAACTTTTAGTGGGCTTTTAACACA
B
egl-1 allele: +
n4045
Y38C9A.1
B0238.11
Y43F11A.5-2
C
Y38C9A.1
1 kb
n4799Δ
n4917Δ
B0238.11
0.1 kb
n4904Δ
Y43F11A.3
0.5 kb
Y43F11A.3
final exon of
Y43F11A.5
n4909Δ
first exon of
Y43F11A.2

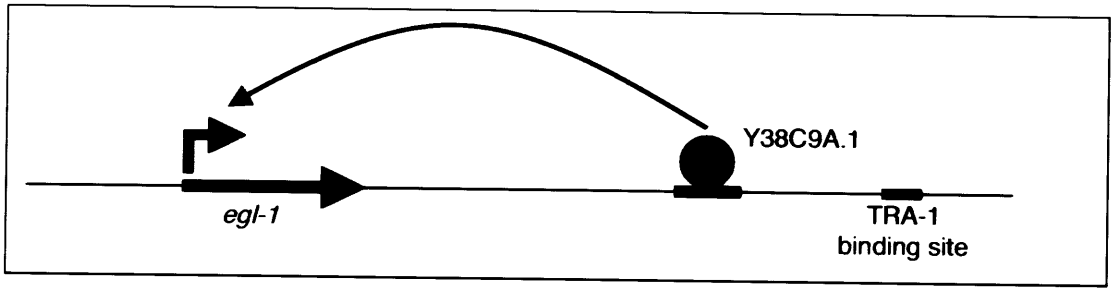
Figure 3

A



B

Genotype	*egl-1* transcription	VC-like neuron cell death
Wild type	HIGH	Yes



Genotype	*egl-1* transcription	VC-like neuron cell death
egl-1(n4045)	LOW	No
Y38C9A.1(Δ)	LOW	No

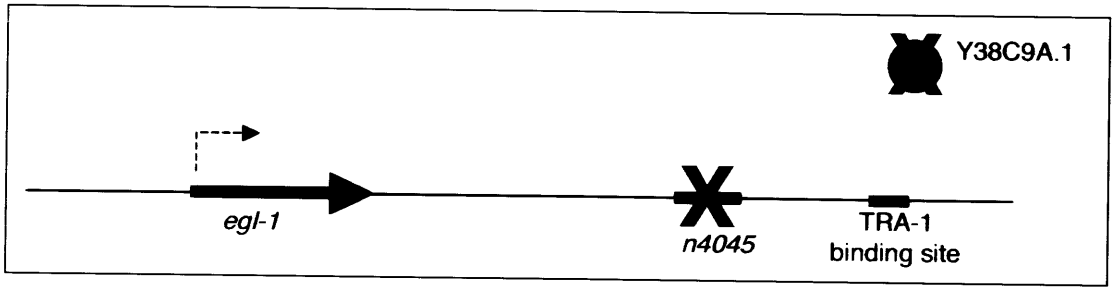


Figure 4

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Chapter Four

Mutations in Two Novel Genes, *lin-24* and *lin-33*, Cause Cytotoxic Cell Death in *C. elegans* that Requires Apoptotic Corpse Removal Genes

Brendan D. Galvin, Saechin Kim, and H. Robert Horvitz

Saechin Kim initiated preliminary characterization of *lin-24* and *lin-33* and cloned *lin-24*. Erika Hartwieg performed the electron microscopy.

SUMMARY

In *C. elegans*, two types of cell death have been studied extensively: programmed cell death and necrosis. In this work we characterize a novel type of cell death that occurs inappropriately in animals containing mutations in one of two novel genes, *lin-24* and *lin-33*. Gain-of-function mutations in the genes *lin-24* and *lin-33* can cause either the inappropriate deaths of the Pn.p cells or prevent these cells from adopting their proper fates. Since some of the Pn.p cells are vulval precursor cells, *lin-24* and *lin-33* gain-of-function mutations result in a vulvaless hermaphrodite. We showed that the corpses resulting from the Pn.p cells that die in *lin-24* and *lin-33* mutant animals are morphologically and ultrastructurally distinct from the corpses that characterize programmed cell deaths and cells that die by necrosis in *C. elegans*. We have molecularly identified both genes and found that *lin-24* encodes a protein containing a domain similar to one found in numerous bacterial toxins and that *lin-33* encodes a novel protein. We showed that the cytotoxicity caused by mutation of either gene requires the function of the other, demonstrating that these two genes work together in this process. The Pn.p cell cytotoxicity of *lin-24* and *lin-33* mutations also requires a subset of the genes necessary for the engulfment of cell corpses during programmed cell death. Thus, we conclude that the engulfment genes from the programmed cell death pathway also function in the process of cell killing initiated by *lin-24* and *lin-33* mutations, even though these deaths are distinct from programmed cell deaths and do not require the execution genes from this pathway.

INTRODUCTION

During the development of the *C. elegans* hermaphrodite, 131 of the 1030 somatic cells that are generated are eliminated by the process of programmed cell death or apoptosis (KIMBLE and HIRSH 1979; SULSTON and HORVITZ 1977; SULSTON *et al.* 1983). The identification of the genes that function in this pathway provided valuable insights into this conserved process. For example, molecular identification of the caspase gene *ced-3* defined the first biochemical mechanism of apoptosis (YUAN *et al.* 1993). Caspases are cysteine aspartate-specific proteases that act in apoptosis in all animals (DEGTEREV *et al.* 2003). Similarly, studies of *C. elegans ced-9* and human Bcl-2 revealed that the misregulation of apoptosis can cause human disease, in this case, cancer (HENGARTNER and HORVITZ 1994; VAUX *et al.* 1992). More than 20 genes have been identified in *C. elegans* that function in programmed cell death, and many have homologs that function in mammalian apoptosis (METZSTEIN *et al.* 1998).

Apoptosis is not the sole mechanism of cell death; cells can also die by necrosis and by autophagy. In autophagy, cells consume themselves from within, for example, under situations of nutritional deprivation (EDINGER and THOMPSON 2004). There are currently no examples of autophagic deaths in *C. elegans*. Necrosis is the cellular death process defined largely by ultrastructure, which includes the swelling of organelles and loss of plasma membrane integrity. Necrosis can be caused by injury or physiological insult and contributes significantly to human diseases, such as the immediate tissue damage that occurs after a heart attack due to lack of oxygen (ZONG and THOMPSON 2006). Some cell deaths that occur in *C. elegans* ultrastructurally resemble necrotic cell death. The best understood necrotic deaths in *C. elegans* are the deaths caused by gain-of-function mutations in the gene encoding the

mechanosensory sodium channel subunit MEC-4 (DRISCOLL and CHALFIE 1991). Mutations in several other genes, such as *deg-1* (encoding a related channel subunit) (CHALFIE and WOLINSKY 1990), *deg-3* (encoding an acetylcholine gated ion channel) (TREININ and CHALFIE 1995), and activated transgenic $G\alpha_s$ (BERGER *et al.* 1998; KORSWAGEN *et al.* 1997), probably act by a similar mechanism. The gain-of-function mutations in *mec-4* cause the hyperactivation of DEC/ENaC channels and cause the deaths of the six touch-receptor neurons in which this subunit is expressed (LAI *et al.* 1996). These deaths seem to require release of calcium from the endoplasmic reticulum (XU *et al.* 2001) and the activation of the calcium-activated calpain proteases and specific cathepsin aspartyl proteases (SYNTICHAKI *et al.* 2002). This death process is similar to that proposed for channel-mediated toxicity in mammals (DRISCOLL and GERSTBREIN 2003).

We have observed and characterized a third type of cell death in *C. elegans* that is distinct from necrotic cell death and programmed cell death, which we refer to as cytotoxicity. This novel type of cell death occurs in *C. elegans* containing mutations in either one of two genes, *lin-24* or *lin-33*. The genes *lin-24* and *lin-33* were identified in screens for mutants abnormal in vulval cell lineages (FERGUSON and HORVITZ 1985). These mutations cause a vulvaless phenotype. Additional alleles of each gene that cause the same phenotype were isolated in a screen for egg-laying defective mutants (TRENT *et al.* 1983) and in a screen for suppressors of the multivulva phenotype (S. G. CLARK & H. R. HORVITZ, unpublished results). In this work, we show that the vulvaless phenotype is due to a novel type of cell death that inappropriately occurs in the vulval precursor cells in *lin-24* and *lin-33* mutant animals.

RESULTS

Mutations in *lin-24* and *lin-33* Cause Both Inappropriate Cell Deaths and Aberrant Cell Fates of Pn.p cells

C. elegans strains with mutations in the genes *lin-24* and *lin-33* had been isolated previously as having a vulvaless phenotype or being unable to lay eggs (FERGUSON and HORVITZ 1985; TRENT *et al.* 1983). To determine the cause of the vulvaless phenotype, we directly observed the Pn.p cells in *lin-24* and *lin-33* mutants using Nomarski differential interference contrast microscopy. The Pn.p cells were examined because these cells normally give rise to the vulva, and the mutant animals are vulvaless. We confirmed that, in *lin-24* and *lin-33* mutants, these cells frequently look abnormal and either die inappropriately or survive but fail to adopt their proper cell fates, as previously reported (FERGUSON and HORVITZ 1985; FERGUSON *et al.* 1987). We observed that late in the first larval stage (L1) or early in the second larval stage (L2) in *lin-24* or *lin-33* mutant hermaphrodites, the nuclei of many of the Pn.p cells increase in refractility and form non-circular refractile bodies that can persist for minutes to hours (Figure 1). Once the refractility begins to decrease, one of three outcomes is observed: the cell dies, the cell survives but the nucleus is abnormally small, or the cell survives and the nucleus looks normal. The refractile bodies are distinct from the circular and more highly refractile corpses seen in programmed cell death (SULSTON and HORVITZ 1977). The refractile bodies are also distinct from necrotic corpses, which swell to several times their original diameter (CHALFIE and SULSTON 1981; CHALFIE and WOLINSKY 1990). We tracked 176 Pn.p cells from the mid-L1 larval stage to the early L2 larval stage in 16 *lin-24(n2050)* animals and observed that 31 died inappropriately (18%), two survived but had small nuclei, and 143 returned to looking normal. For *lin-33(n2003)*, 38 of the 99 cells

we tracked died inappropriately (38%), six had small nuclei, and the remaining 55 returned to looking normal.

The surviving Pn.p cells failed to adopt their correct cell fates in *lin-24* and *lin-33* mutants. Three of the 12 cells, P(5-7).p, normally undergo three rounds of division to generate the cells that form the vulva (SULSTON and HORVITZ 1977). Three neighboring cells P(3, 4, 8).p, although competent to make vulval cells, normally divide once to generate two descendants that fuse with the hypodermis (SULSTON and HORVITZ 1977; SULSTON and WHITE 1980). We examined the fates of P(3-8).p in *lin-24* and *lin-33* mutant animals and observed that these cells almost never divided. In *lin-24(n2050)* animals, of the 56 cells we tracked, all but two never divided. Instead these cells probably went on to fuse with the hypodermal syncytium, a fate normally assumed by P(1,2).p, P(9-12).p. Therefore, the abnormal death of 20-30% of the Pn.p cells, combined with the adoption of abnormal cell fates by the remaining cells, causes the vulvaless phenotype observed in *lin-24* and *lin-33* mutant animals.

The Abnormal Cells in *lin-24* and *lin-33* Mutant Animals Are Distinct from Cells that Undergo Necrotic or Programmed Cell Deaths

We examined the ultrastructure of the abnormal Pn.p cells in *lin-24* and *lin-33* mutants by electron microscopy. Hallmarks of programmed cell death, such as shrinking of cell volume and chromatin condensation (ROBERTSON and THOMSON 1982), were not observed. Similarly, the large vacuoles observed in *mec-4* and *deg-1*-induced necrotic deaths were not seen (HALL *et al.* 1997). Four ultrastructural characteristics are associated with the refractile Pn.p cells observed in *lin-24(n423)* animals, *lin-33(n1043)* animals, and

lin-33(n1043) lin-24(n432) doubly mutant animals (Figure 2): (1) disruption of mitochondrial ultrastructure, (2) electron dense puncta in the nuclei, (3) dilation of the nuclear membrane and its associated membranes, (4) electron-dense membranous whorls in the cytoplasm. These four characteristics distinguish *lin-24* and *lin-33*-induced cell deaths from both programmed cell deaths and the necrotic deaths associated with mutations in the genes *mec-4* and *deg-1*.

The *lin-24* and *lin-33* Alleles that Cause Cytotoxicity Are Not Loss-of-function Alleles

In several different screens performed previously, several alleles of *lin-24* and *lin-33* had been isolated. To rank them by penetrance, we scored the vulvaless phenotype by assaying the percent of the animals of each strain that could not lay eggs. The penetrance of the egg-laying defect of eight of the nine *lin-24* and *lin-33* alleles ranged from 75% to 100% (Table 1). Using this same assay, we also found that both *lin-24* and *lin-33* alleles can cause a semidominant vulvaless phenotype (Table 1). This result gave the first indication that these alleles were not loss-of-function mutations. We therefore sought to isolate mutations that reduce or eliminate *lin-24* and *lin-33* function to define the loss-of-function phenotype of these genes. Analysis with deficiencies suggested that *lin-24(n432)/lin-24(null)* and *lin-33(n1043)/lin-33(null)* were wild-type (data not shown). Thus, we predicted that we should be able to isolate *lin-24* and *lin-33* loss-of-function mutations as dominant suppressors of the vulvaless phenotype of *lin-24(n432)* or *lin-33(n1043)* in an F_1 reversion screen. We mutagenized strains that were homozygous for either *lin-24(n432)* or *lin-33(n1043)* and isolated non-Vul animals from the F_1 self-progeny. One isolate from each of these two screens was a good candidate to be an intragenic revertant allele based on its map position

(*lin-24(n432 n1503)* and *lin-33(n1043 n1502)*) (data not shown). Like the deficiencies, each of these mutations was able to dominantly suppress the vulvaless phenotype of *lin-24(n432)* or *lin-33(n1043)*, suggesting that they were loss-of-function alleles (data not shown). Animals homozygous for either of the intragenic revertant alleles appeared wild-type, suggesting that the null phenotype of these genes is not vulvaless and that the previously isolated alleles of *lin-24* and *lin-33* that cause the vulvaless phenotype are not loss-of-function alleles. This was confirmed once we molecularly identified the genes and isolated deletion alleles of the genes (see below). The deletion alleles did not cause a vulvaless phenotype, confirming that the alleles of *lin-24* and *lin-33* that cause cytotoxicity are not loss of function alleles.

Molecular Identification of *lin-24* and *lin-33*

lin-24 had been previously mapped between *unc-22* and *dpy-26* on linkage group IV (FERGUSON and HORVITZ 1985). We further mapped *lin-24* to the left of a polymorphism on cosmid C41D9 between *C. elegans* strains N2 and N62 (G. GARRIGA, personal communication) (Figure 3A). We cloned *lin-24* using transformation rescue by injecting wild-type cosmid DNA into *lin-24* mutant animals that displayed a recessive vulvaless phenotype. Although some alleles of *lin-24* are semidominant, we choose to use recessive alleles for these experiments to facilitate transformation rescue. Of the four cosmids we tested from within the defined interval between *unc-22* and the polymorphism, two overlapping cosmids (T20H7 and B0001) rescued the recessive vulvaless phenotype of *lin-24(n1821)* animals in germ-line transformation experiments. A 6.3 kb fragment within the overlapping region between T20H7 and B0001 containing a single predicted gene,

B0001.1, was sufficient to rescue the recessive vulvaless phenotype of *lin-24(n2050)* animals. We determined the sequence of the presumptive *lin-24* gene in each of the four different mutants and found that each contained a different missense mutation (Figure 3C). Six *lin-24* cDNAs were isolated from a mixed-stage cDNA library; the longest appeared to be full-length, as it contains an SL1 *trans*-spliced leader sequence. This cDNA established the gene structure of *lin-24* shown in Figure 3B. This gene is highly similar to an uncharacterized *C. elegans* gene, *C31H1.6*.

lin-33 was previously mapped between *dpy-13* and *unc-8* on linkage group IV (FERGUSON and HORVITZ 1985). We further mapped *lin-33* to the right of a polymorphism (at position 5412) on cosmid C10G6 between *C. elegans* strains N2 and CB4856 and to the left of *unc-44* (Figure 3D). We cloned *lin-33* by injecting DNA isolated from *lin-33* mutant animals (displaying the dominant vulvaless phenotype) into wild-type animals and determining the minimal fragment of DNA that caused animals to be vulvaless. Seven PCR products that had been generated from *lin-33(n1302)* genomic DNA (which together covered most of the relevant 156 kb interval were tested for the ability to phenocopy the vulvaless phenotype of *lin-33(n1302)* animals. A single 9 kb PCR product predicted to contain a single gene, *H32C10.2*, caused a vulvaless phenotype in wild-type animals. We determined the sequence of the *H32C10.2* locus from each of the five semidominant *lin-33* mutants and found that all five contain the identical missense mutation, changing glutamic acid 230 to lysine (Figure 3F). Two *lin-33* cDNAs have been isolated by Yuji Kohara and coworkers (KOHARA 1996); one, yk1122h12, appears to be full-length, as it contains a SL1 *trans*-spliced leader sequence. The gene structure of *lin-33* based on this cDNA is shown in Figure 3E.

LIN-24 Contains a Toxin Domain and LIN-33 is a Novel Protein

BLAST and related searches with the LIN-33 protein did not identify any significant domains or homologs in other organisms. Similar searches using the LIN-24 protein revealed that it contains a domain found in many bacterial toxins. This domain, spanning amino acids 62 to 176, is most similar to the *Bacillus sphaericus* mosquitocidal toxin Mtx3 and related toxins (Figure 4) (LIU *et al.* 1996). These toxins are thought to function by binding to eukaryotic cells and aggregating to form holes in the membrane, ultimately causing osmotic lysis by disrupting membrane permeability (GILBERT 2002). It is tempting to speculate that LIN-24 may be able to cause cytotoxicity by a mechanism similar to that of these bacterial toxins. Five of the seven isolated mutations in *lin-24* are located in the toxin-related domain (Figure 4), including three loss-of-function alleles that were isolated in reversion screens, suggesting that this domain has an important function in LIN-24.

The *lin-24(n432)* Mutation May Result in a Novel Gene Activity

We identified deletion alleles of *lin-24* and *lin-33* (*lin-24(n4294Δ)* and *lin-33(n4514Δ)*) by screening a library of mutagenized worms. Animals homozygous for either deletion allele appeared wild-type, demonstrating that the null phenotype of these genes is not vulvaless (Table 2). The *lin-24(n432)*/+ and *lin-33(n1043)*/+ strains have a more penetrant vulvaless phenotype than *lin-24(n432)*/*lin-24(Δ)* and *lin-33(n1043)*/*lin-33(Δ)*, suggesting that the presence of a wild-type copy of the respective gene can increase the toxicity of these alleles (Table 2). The relative penetrances of the vulvaless phenotype of the following *lin-24(n432)*-containing strains was *lin-24(n432)*/*Df* (0%) < *lin-24(n432)*/+/+ (34%) < *lin-24(n432)*/+ (55%) < *lin-24(n432)*/*lin-24(n432)* (95%). These results indicate

that an increase in the dosage of the *lin-24(+)* gene product can result in a decrease in the percentage of hermaphrodites that are vulvaless. These data suggest that the wild-type *lin-24* gene product may antagonize the vulvaless phenotype of the semidominant mutation. Therefore, the *lin-24(n432)* mutation probably does not result in an increase in the wild-type function of the gene product, but rather in the generation of an abnormal cytotoxic gene product. The complete suppression of the vulvaless phenotype of *lin-24(n432)* and *lin-33(n1043)* when in *trans* to their respective deletion alleles suggests that they need a minimum level of expression of either mutant or wild-type gene product to cause the vulvaless phenotype.

We also isolated a deletion allele of the gene that encodes a protein highly similar to LIN-24, *C31H1.6*. *C31H1.6(n4763Δ)* mutants also appear wild-type, both alone and in combination with *lin-24(n4294Δ)*. The wild-type phenotype of the doubly mutant strain, *lin-24(n4294Δ) C31H1.6(n4763Δ)*, suggests that these two genes do not function redundantly in a pathway that regulates an obvious process.

The *lin-24* and *lin-33*-induced Toxicities Each Require the Function of the Other Gene

In addition to isolating loss-of-function alleles of both *lin-24* and *lin-33* in their respective dominant suppressor screens, isolates of the other gene were also isolated in each screen. One loss-of-function allele of *lin-33*, *n1968*, was isolated in the *lin-24(n432)* screen and one loss-of-function allele of *lin-24*, *n2333*, was isolated in the *lin-33(n1043)* screen. These results suggested a genetic interaction between *lin-24* and *lin-33* that was further explored using the deletion alleles, which are molecular nulls. Strains of the genotypes *lin-33(n1043) lin-24(n4294Δ)/ lin-33(n1043) lin-24(n4294Δ)* or *lin-33(n4514Δ)*

lin-24(n1043)/lin-33(n4514Δ) lin-24(n1043) are wild-type (Table 2). Additionally, as suggested by their isolation in a F_1 screen, the loss-of-function alleles of *lin-24* and *lin-33* can suppress the vulvaless phenotype caused by mutations in the other gene when they are present only in one copy (data not shown). For example, an animal of the genotype *lin-33(n1043) lin-24(n4294Δ)/ lin-33(n1043) lin-24(+)* is not vulvaless, so a loss of one copy of *lin-24* is able to suppress the vulvaless phenotype of a *lin-33* mutant animal. These results show that the *lin-24* and *lin-33* requires the function of the other to induce cell death.

Genes that Function in the Engulfment Process of Programmed Cell Death Are Required for the *lin-24* and *lin-33*-Induced Cytotoxicity

To determine if genes known to be required for the programmed cell deaths in *C. elegans* are also required for the *lin-24* or *lin-33*-induced cytotoxicity, we tested whether mutations in such genes affect the vulvaless phenotype of *lin-24(n432)*/+ and *lin-33(1043)*/+ animals (Figure 5). We found that the penetrances of the Vul phenotype of *lin-24(n432)*/+ and *lin-33(n1043)*/+ animals were not affected by loss-of-function alleles of *ced-3* or *ced-4* or by a gain-of-function allele of *ced-9*. A loss-of-function allele of *egl-1* partially suppressed the Vul phenotype, although not to the same level as the modifiers discussed below. This result demonstrates that *lin-24* and *lin-33*-induced cell death does not require most of the killing genes in the pathway of programmed cell death.

The genes of the *ced-2*, *-5*, *-10*, and *-12* engulfment pathway were required for the *lin-24*- and *lin-33*-induced deaths (Figure 5). These four genes function together in one of two engulfment pathways involved in corpse removal. *ced-12* mutations almost completely eliminated the cytotoxicity of either *lin-24 or lin-33*. The *ced-1*, *ced-6*, and *ced-7* genes from

the second engulfment pathway are not required for these deaths. For example, the cytotoxicity is unchanged in *ced-7* mutant animals. The engulfment genes have been shown to influence the killing of cells that undergo programmed cell death (REDDIEN *et al.* 2001). Our findings indicate that some engulfment genes are also required for mediating the killing and cytotoxicity caused by mutations in *lin-24* and *lin-33*. Therefore, we believe that this cytotoxicity is due, in part, to injury mediated by the gene products of *ced-2*, *-5*, *-10*, and *-12*.

DISCUSSION

In this work we have identified two genes that, when mutated, can cause the inappropriate deaths of Pn.p cells, a subset of which give rise to the vulva. These genes appear to work together to mediate this cytotoxicity. The deaths caused by *lin-24* and *lin-33* mutations are cytologically distinct from the previously characterized deaths that have been observed in *C. elegans*, namely programmed cell death and necrotic death. *lin-24* and *lin-33* cytotoxicity does not require most of the execution genes of programmed cell death, as the cytotoxicity persists in animals harboring mutations in the genes the Bcl-2 family member *ced-9*, the Apaf-1 homolog *ced-4*, and the caspase encoding gene *ced-3*. *egl-1* may be partially required for the cytotoxicity, since *egl-1(lf)* (loss of function) can partially suppress the vulvaless phenotype of both *lin-24(n432)* and *lin-33(n1043)* animals. The partial suppression by *egl-1* is particularly interesting, as it suggests that the most upstream gene that functions in all somatic programmed cell deaths may also function independently from *ced-3*, *ced-4*, and *ced-9* in non-programmed cell deaths. The cytotoxicity requires the functions of the genes from one of the two partially redundant programmed cell death corpse engulfment pathways, mediated by *ced-2*, *-5*, *-10*, and *-12*.

ced-2, *-5*, *-10*, and *-12* function in parallel to the genes *ced-1*, *-6*, and *-7* for the rapid removal of corpses generated by programmed cell death. Whereas the function of the *ced-1*, *-6*, *-7* pathway is unclear, the *ced-2*, *-5*, *-10*, *-12* pathway is likely a signal transduction pathway, the output of which causes cytoskeletal rearrangement (MANGAHAS and ZHOU 2005; REDDIEN and HORVITZ 2004). CED-2 appears to be an SH2 (Src-homology 2) and SH3 (Src-homology 3) domain containing adapter protein (REDDIEN and HORVITZ 2000), CED-10 is a Rac1-like GTPase (REDDIEN and HORVITZ 2000), and CED-5 and CED-12

exchange Rac1 GDP for GTP (GUMIENNY *et al.* 2001; WU and HORVITZ 1998; WU *et al.* 2001; ZHOU *et al.* 2001). The mammalian counterparts of CED-2, CED-5, and CED-12 regulate Rac (AKAKURA *et al.* 2005; LU and RAVICHANDRAN 2006), a member of the Ras GTPase superfamily family that includes Rho, Rac, and Cdc42, each of which has a well-established role in cytoskeletal reorganization (WENNERBERG and DER 2004). Cytoskeletal reorganization is important for the process of polarized surface extension that is critical for cell migration, axonal outgrowth, and phagocytosis.

Whereas the *ced-1, -6, -7* pathway appears to be specific for programmed cell death, the *ced-2, -5, -10, -12* pathway has been shown to be involved in several processes in *C. elegans* such as: programmed cell death, distal tip cell migration, axonal outgrowth, and now *lin-24/lin-33*-mediated cytotoxicity (MANGAHAS and ZHOU 2005; REDDIEN and HORVITZ 2004). Our work suggests that the *ced-2, -5, -10, -12* pathway might respond to a signal presented on the cell surface of a cell that is sick or dying (Figure 6). It is possible that the signal presented by the cells affected by *lin-24/lin-33*-induced toxicity is the same as that recognized by the *ced-2, -5, -10, -12* pathway in programmed cell death. The *ced-1, -6, -7* pathway, in contrast, may recognize a different signal that is specific to corpses generated by programmed cell death. Although less likely, it is possible that *ced-2, -5, -10,* and *-12* function within the dying cells rather than engulfing cells (although this is certainly not the case in programmed cell death, where the engulfment genes are necessary in the neighboring cells that do the engulfing, but not in the dying cells). This possibility needs to be explored by determining the site of action of these genes in this *lin-24-* and *lin-33*-mediated cytotoxicity.

The observation that mutations in *ced-2*, *-5*, *-10*, and *-12* reduce *lin-24-* and *lin-33*-induced cytotoxicity parallels the observation that engulfment genes are involved not only in engulfing corpses generated by programmed cell death, but also in the execution of programmed cell death (REDDIEN *et al.* 2001). This conclusion was based on the observation that mutations in engulfment genes enhance the cell-death defect observed in a sensitized background containing a weak *ced-3* loss-of-function allele. There are also a few programmed cell deaths, such as death of one of the pair of cells B.alapaav and B.arapaav in the male tail, that appear to be entirely dependent on engulfment (HEDGECOCK *et al.* 1983). Additional studies of the *lin-24-* and *lin-33*-mediated cytotoxicity could provide valuable insight into how engulfment contributes to both this cytotoxicity and possibly programmed cell death. If the *ced-2*, *-5*, *-10*, *-12* pathway recognizes a signal presented by the sick Pn.p cells in *lin-24* and *lin-33* mutants, it may be possible to isolate mutations in this signal by screening for suppressors of the vulvaless phenotype caused by mutations in *lin-24* and *lin-33*.

In contrast to both programmed cell death and *lin-24-* and *lin-33*-mediated cytotoxicity, necrotic deaths do not involve the function of engulfment genes. Specifically, engulfment genes function in the engulfment of the necrotic corpses generated by mutations in *mec-4* and *deg-3*, but they affect only the timing of the disappearance of these corpses and not cell survival (CHUNG *et al.* 2000).

One other *C. elegans* cell death seems to require the function of the engulfment genes, which is the death of the Pn.p cells in animals containing a mutation in *pvl-5*, a gene not yet identified molecularly (JOSHI and EISENMANN 2004). When observed by Nomarski optics, the abnormal Pn.p cell nuclei in animals with the *pvl-5* mutation resemble those

observed in mutant *lin-24* and *lin-33* animals. These *pvl-5*-induced deaths are genetically distinct from *lin-24*- and *lin-33*-induced deaths, however, as the *pvl-5*-induced deaths require the caspase encoding gene *ced-3*. *pvl-5*-induced deaths do not require the activity of *ced-4*, which encodes the Apaf-1 homolog that activates CED-3, making these deaths also distinct from programmed cell deaths (JOSHI and EISENMANN 2004). *pvl-5*-induced deaths do occur in animals lacking any one engulfment gene (in contrast to the *lin-24* or *lin-33*-induced deaths, which require *ced-2*, *-5*, *-10*, and *-12*), but these deaths do not occur in *ced-1*; *ced-5* double mutants (JOSHI and EISENMANN 2004). Thus, at least one engulfment pathway must be functional in *pvl-5* mutant worms to allow killing. The authors suggest that *pvl-5* might function to protect the cells from inappropriate activation of *ced-3*-dependent programmed cell death by a pathway independent of its physiological activator *ced-4* (JOSHI and EISENMANN 2004). The mechanism of this protection is not known.

We are very interested in understanding how mutations in *lin-24* and *lin-33* induce cell death, and the toxin-like domain in LIN-24 suggests a possible mechanism. Related toxins are thought to form membrane complexes that alter eukaryotic membrane permeability and cause osmotic lysis (GILBERT 2002). Perhaps the *lin-24* and *lin-33* mutations inappropriately activate the toxin activity of the toxin-like domain of LIN-24, causing the deaths of the specific cells in which they are both expressed. The neomorphic activity of the allele *lin-24(n432)*, which causes the vulvaless phenotype, is consistent with our data.

Given the similarity of LIN-24 to bacterial toxins, what might be the wild-type function of the *lin-24* gene product in *C. elegans*? Neither loss of *lin-24* function alone, nor loss of *lin-24* function in combination with loss-of-function of its close homologue *C31H1.6*, results in any obvious abnormal phenotype. Members of the *Caenorhabditis* genus are one

of a small number of eukaryotic organisms that contain members of this family in their genomes. Almost all LIN-24 homologs are found in bacteria, which are the food source of *C. elegans*. In such bacteria, these toxins are produced and are capable of killing animals such as mosquitoes and cattle by forming oligomers that increase membrane permeability and cause osmotic lysis. Perhaps the wild-type LIN-24 normally functions to interact with bacterial toxins and inactivate them, possibly by a mechanism that requires LIN-33. Such an activity might allow *C. elegans* to be able to eat the bacteria that produce such toxins that are normally capable of killing.

FUTURE DIRECTIONS

The next step in this project is to determine the mechanism by which *lin-24* and *lin-33* mutations cause cytotoxicity. A genetic approach to this would be to perform a large-scale F_2 and F_3 suppression screen of the vulvaless phenotype caused by mutations in *lin-24* and *lin-33*. In addition to alleles of *lin-24* and *lin-33*, alleles of some engulfment genes should be isolated. Previously uncharacterized genes might also be identified, some of which may function in programmed cell death and some of which might function solely in the cytotoxicity of *lin-24* and *lin-33*. It would be very exciting to try to use this sort of screen to isolate the gene(s) encoding the signal presented by dying cells that are recognized by neighboring cells to trigger engulfment.

I would like to determine the expression patterns of *lin-24* and *lin-33*. Our hypothesis is that the expression patterns might inform us about why the Pn.p cells appear to be the only cells affected by these mutations. Perhaps they are the only cells that express both *lin-24* and *lin-33*. I have already made antibodies to LIN-24 and LIN-33 and transcriptional *gfp* reporter constructs for both genes, but analyses with these reagents have not been useful in determining the expression pattern of these genes. Also, I am interested in determining whether *ced-2*, *-5*, *-10*, and *-12* work non-cell autonomously in *lin-24*/*lin-33*-mediated cytotoxicity, as they do in programmed cell death. I would also like to determine whether *lin-24* and *lin-33* work cell autonomously or non-cell autonomously.

Establishing if there is a direct physical interaction between LIN-24 and LIN-33 would confirm part of our model for the activity of these two proteins. The possible interaction could be explored by either yeast two-hybrid analysis or co-immunoprecipitation experiments using our anti-LIN-24 antibody, which works on western blots.

I wish to test the hypothesis put forth in this chapter that the wild-type function of *lin-24* might be to defend worms against one of the toxins that their food source (bacteria) use to kill organisms. To test this initially, I would examine if loss of *lin-24* function and the function of the highly similar *C31H1.6* gene alters the ability of *C. elegans* to survive on *Pseudomonas aeruginosa*. We would examine *Pseudomonas aeruginosa* first, because survival assays have been established for this organism (TAN *et al.* 1999) and it possesses a cytotoxin that is somewhat similar to LIN-24 (HAYASHI *et al.* 1989).

EXPERIMENTAL PROCEDURES

Strains and General Techniques

Strains were cultured as described by BRENNER (1974) and grown at 20° C. The Bristol strain N2 was used as the wild-type strain, except in multifactor mapping experiments that used the polymorphic wild-type strains N62 and CB4856 (WICKS *et al.* 2001). The mutations used in the study are listed below, and a description, unless otherwise noted, can be found in RIDDLE (1997):

LGI: *ced-1(e1735)*, *ced-12(n3261)* (ZHOU *et al.* 2001)

LGIII: *ced-9(n1950)*, *ced-4(n1162)*, *ced-6(n1813)*, *ced-7(n1996)*

LGIV: *lin-24(n432, n1057)*, *lin-24(n1821, n2050)* (S. G. CLARK & H. R. HORVITZ, unpublished results), *lin-24(n2258, n2333, n4294)* (this study), *lin-24(n432 n1503)* (this study), *lin-33(n1043, n1044) lin-33(n1110)* (M. K. EDWARDS & H. R. HORVITZ, unpublished results), *lin-33(n1302)* (J. H. THOMAS & H. R. HORVITZ, unpublished results), *lin-24(n1968, n2003, n4514)* (this study), *lin-33(n1043 n1502)* (this study), *ced-3(n717)*, *ced-2(e1752)*, *ced-5(n1812)*, *ced-10(n1993)*, *unc-44(e362)*

LGV: *unc-76(e911)*, *egl-1(n1084 n3082)* (CONRADT and HORVITZ 1998)

Assay for Vulvaless Animals

Only worms propagated through two generations without starvation were scored. Five-centimeter plates were seeded 12 to 24 hours prior to assay with an overnight culture of the *E. coli* strain OP50. Single L4 animals were placed one per plate. Sixteen to 18 hours later, plates were examined for the presence of gravid adults. Plates with gravid adults were scored for the presence or absence of eggs. Plates without any eggs were scored as vulvaless.

To assay for suppression of *lin-24(n432)* and *lin-33(n1043)*-induced death by loss-of-function alleles of genes that function in programmed cell death, we modified the above assay. Homozygous males (*e.g.*, *ced-5(n1812) lin-24(n432)*) were generated by heat shock and mated with homozygous *unc-76(e911)* marked strains (*e.g.*, *ced-5(1812)*; *unc-76(e911)*), and three to five days later non-Unc L4 hermaphrodites were assayed as described above.

The assay for the dosage studies of *lin-24* not included in Table 2 was performed as previously described (FERGUSON and HORVITZ 1985).

Nomarski Observation of Pn.p Cells

Pn.p cells in *lin-24* or *lin-33* mutant animals were observed using Nomarski differential interference contrast microscopy at different times during development as previously described (SULSTON and HORVITZ 1977).

Electron Microscopy

Nomarski differential interference contrast microscopy was used to select a nematode with refractile bodies, and digital images were taken to note their positions. Mutant animals were recovered from the slide and fixed as previously described (BARGMANN *et al.* 1993). The fixed, embedded animals were sectioned as previously described (GUMIENNY *et al.* 1999) and photographed using a JEOL 12000CX electron microscope at 80 kv. After finding the location of the anus or the gonad in the EM photographs, nuclei were counted by examining EM photographs of adjacent sections and the cell that corresponded to the

refractile Pn.p cell identified in the original digital Nomarski image was identified and examined.

Dominant Suppression Screen for Revertant Alleles of *lin-24(n432)* and *lin-33(n1043)*

We mutagenized homozygous L4 or early adult hermaphrodites homozygous for either *lin-24(n432)* or *lin-33(n1043)* with ethyl methanesulfonate (EMS) as previously described (BRENNER 1974). After mutagenesis, three to four P_0 hermaphrodites were placed on each five-centimeter plate. F_1 self-progeny animals that were non-Vul were picked to individual plates. If greater than 10% of F_2 animals were non-Vul, individual non-Vul F_2 animals were picked to separate plates and assayed the next generation for suppression of the Vul phenotype.

Cloning of *lin-24* and *lin-33*

Using standard three-point and polymorphism mapping, we placed *lin-24(n1057)* and *lin-33(n1043)* in small map intervals. To clone the gene *lin-24* we performed transformation rescue experiments as previously described (MELLO *et al.* 1991) using pRF4 plasmid as a coinjection marker. To clone the gene *lin-33* we injected DNA from *lin-33* mutant animals into *unc-76(e911)* animals, using wild-type *unc-76* DNA as a co-injection marker, to identify DNA that caused the vulvaless phenotype. The DNA injected for the *lin-33* cloning were PCR products generated from *lin-33(n1302)* animals using the Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions.

Isolation of *lin-24* cDNAs

A 10.8 kb *SmaI-NcoI* fragment from cosmid T20H7 was used to screen more than 800,000 plaques of a λ ZAP cDNA library derived from mixed-stage poly(A)-positive RNA collection as previously described (BARSTEAD and WATERSTON 1989). Fourteen plaques were isolated, six of which hybridized to fragments of the 10.8 kb fragment required for rescue.

Determination of Mutant Allele Sequences

We used PCR-amplified regions of genomic DNA to determine mutant sequences. For all alleles of *lin-24* and *lin-33*, we determined the sequence of all exons and splice junctions. Sequences were determined using an ABI Prism 3100 Genetic Analyzer.

Isolation of Deletion Alleles

Genomic DNA pools from EMS-mutagenized animals were screened for deletions using PCR as previously described (CEOL and HORVITZ 2001). Deletion mutants were isolated from frozen stocks and backcrossed to the wild type six times. *lin-24(n4294)* removes nucleotides 2259 to 3253 of cosmid B0001. *lin-33(n4514)* removes nucleotides 28902 to 29937 of cosmid H32C10. *C31H1.6(n4763)* removes nucleotides 27211 to 28463 of cosmid C31H1.

FIGURE LEGENDS

Figure 1. The Pn.p Cells in *lin-24(n432)* and *lin-33(n1043)* Mutants Look Abnormal and Appear Different from Both Necrotic and Programmed Cell Death Corpses

(A) Normarski photomicrograph of representative abnormal Pn.p cells in a *lin-33(n1043)* L1 larva. The white arrows point to two Pn.p cells that have the refractile appearance often observed in *lin-24(n432)* and *lin-33(n1043)* animals.

(B) Nomarski photomicrograph of corpses generated by programmed cell death in a *ced-5(lf)* mutant.

(C) Nomarski photomicrograph of a necrotic corpse in a *mec-4(d)* mutant (photograph provided by Monica Driscoll).

Figure 2. Ultrastructural Characteristics of the Refractile Pn.p cells in *lin-24(n432)* and *lin-33(n1043)* Mutants

(A) Electron photomicrograph of the refractile body corresponding to P10.p in a *lin-33(n1043)* mutant. Four abnormal ultrastructural characteristics of these animals are indicated: (1) dilated mitochondrion with disrupted internal architecture, (2) electron dense puncta in the nucleus, (3) dilation of nuclear and associated membranes, and (4) dense membranous whorls. (5) One of several mitochondria in neighboring cells with normal architecture. Note that these four abnormal characteristics are not observed in the adjacent cells, which all have normal cellular architecture.

(B) Increased magnification of (A) showing the disrupted mitochondrion.

(C) Increased magnification of (A) showing a mitochondrion with normal architecture.

(D) Increased magnification of (A) showing the electron-dense nuclear punctations and membranous whorls.

Figure 3. Molecular Identification of *lin-24* and *lin-33*

(A) Genetic and physical maps of the *lin-24* region. The four cosmids shown were tested for their abilities to rescue the recessive Vul phenotype of *lin-24* mutants. The overlapping cosmids B0001 and T20H7 rescued the Vul phenotype. An *Nco*I-*Hind*III fragment containing the gene *B0001.1* also to rescued.

(B) The structure of the *lin-24* gene as deduced from genomic and cDNA sequences. The location of the deletion *lin-24(n4294)* is indicated by the horizontal line.

(C) The sequences of the *lin-24* mutations. The mutated bases are underlined.

(D) Genetic and physical maps of the *lin-33* region. PCR products generated from *lin-33(n1302)* genomic DNA spanning most of the shown interval were tested for the ability to phenocopy the dominant vulvaless phenotype of the mutant animals when injected into *lin-33(+)* animals. The 26 kb PCR product #6 was able to cause the vulvaless phenotype. The 9 kb PCR product containing only the gene *H32C10.2* was also able to cause the Vul phenotype.

(E) The structure of the *lin-33* gene as deduced from genomic and cDNA sequences. The location of the deletion *lin-33(n4514)* is indicated by the horizontal line.

(F) The sequences of the *lin-33* mutations. The mutated bases are underlined.

Figure 4. LIN-24 Contains a Domain Similar to that Found in Bacterial Toxins

Sequence alignment of LIN-24 and the 3.58-kilodalton-mosquitocidal toxin Mtx3 found in *Bacillus sphaericus*. Numbers at the right and left indicate amino acid positions. Identical residues are colored blue, and similar residues are colored red. Missense mutations found within the region of homology are indicated.

Figure 5. The Cytotoxicity of *lin-24* and *lin-33* Is Suppressed by Mutations in the Engulfment Genes *ced-2*, *ced-5*, *ced-10*, and *ced-12*

We analyzed *lin-33(n1043)*/+; *ced*/*ced* and *lin-24(n432)*/+; *ced*/*ced* mutants as described in Experimental Procedures. An average of 76 ± 29 (± standard deviation) animals were assayed for each genotype.

(A) The penetrance of the Vul phenotype of *lin-33(n1043)*/+ animals was modified by mutations in some of the genes required for programmed cell death. Genes on the X-axis are organized in the following order: cell killing genes in order of action (*egl-1*, *ced-9*, *ced-4*, *ced-3*); one partially redundant engulfment pathway (*ced-1*, *ced-6*, *ced-7*); the other partially redundant engulfment pathway (*ced-2*, *ced-5*, *ced-10*, *ced-12*).

(B) The penetrance of the Vul phenotype of *lin-24(n432)*/+ animals was modified by mutations in some of the genes required for programmed cell death.

Figure 6. Model for the Effects of Engulfment on *lin-24*/*lin-33*-induced Cytotoxicity

Mutations in *lin-24* and *lin-33* make the Pn.p cells sick, possibly by a mechanism related to the toxin domain of *lin-24*. A signal of this sickness is presented on the cell membrane and recognized by a neighboring cell. The neighboring cell then either removes or injures the sick cell in an process that requires the engulfment genes *ced-2*, *ced-5*, *ced-10*,

and *ced-12*, which likely act by reorganizing the actin cytoskeleton (adapted from REDDIEN and HORVITZ (2004)).

Table 1. *lin-24* and *lin-33* Allele Strengths

Genotype	% Vul (*n*)
Wild type	0 (89)
lin-24(n432)	92 (90)
lin-24(n432)/+	66 (109)
lin-24(n1821)	75 (89)
lin-24(n1821)/+	0 (72)
lin-24(n2050)	91 (87)
lin-24(n2050)/+	0 (71)
lin-24(n1057)	2 (90)
lin-24(n1057)/+	41 (74)
lin-33(n1043)	97 (90)
lin-33(n1043)/+	77 (121)
lin-33(n1044)	96 (90)
lin-33(n1110)	92 (88)
lin-33(n1302)	100 (90)
lin-33(n2003)	99 (90)
lin-33(n1043) lin-24(n432)	97 (89)

The penetrance of the Vul phenotype (%) was determined as described in Experimental Procedures. Heterozygous animals were generated by crossing homozygous mutant males with *unc-76(e911)* hermaphrodites, and non-Unc progeny were scored. *n*, number of animals.

Table 2. Gene Dosage and Gene Interaction Studies of *lin-24* and *lin-33* Alleles

Genotype	% Vul (*n*)
Wild type[c]	0 (89)
lin-24(n432)/lin-24(n432)[c]	92 (90)
lin-24(n4294Δ)/lin-24(n4294Δ)	0 (102)
lin-24(n432)/lin-24(n4294Δ)[a]	3 (89)
lin-24(n432)/+[c]	66 (109)
+/+/+[d]	0 (33)
lin-33(n1043)/lin-33(n1043)[c]	97 (90)
lin-33(n4514Δ)/lin-33(n4514Δ)	0 (72)
lin-33(n1043)/lin-33(n4514Δ)[b]	21 (77)
lin-33(n1043)/+[c]	77 (121)
+/+/+[e]	0 (53)
lin-33(n1043) lin-24(n432)[c]	97 (89)
lin-33(n1043) lin-24(n4294Δ)	0 (56)
lin-33(n4514Δ) lin-24(n432)	0 (60)

The number of Vul animals was counted as described in Experimental Procedures. *n*, number of animals.

[a] *lin-24(n432)* homzygous males were mated with *lin-24(n4294Δ)* hermaphrodites, which were *cis*-marked with *dpy-4(e1166)*, and non-Dpy cross progeny were scored.

[b] *lin-33(n1043)* homozygous males were mated with *lin-33(n4514Δ)* hermaphrodites, which were *cis*-marked with *unc-30(e191)*, and non-Unc cross progeny were scored.

[c] These data are from Table 1.

[d] This strain was of genotype *dpy-13(e184) unc-24(e138); mDp4;* only non-Dpy non-Unc animals were scored.

[e] This strain was of genotype *unc-30(e191) dpy-4(e1166); yDp1;* only non-Unc non-Dpy animals were scored.

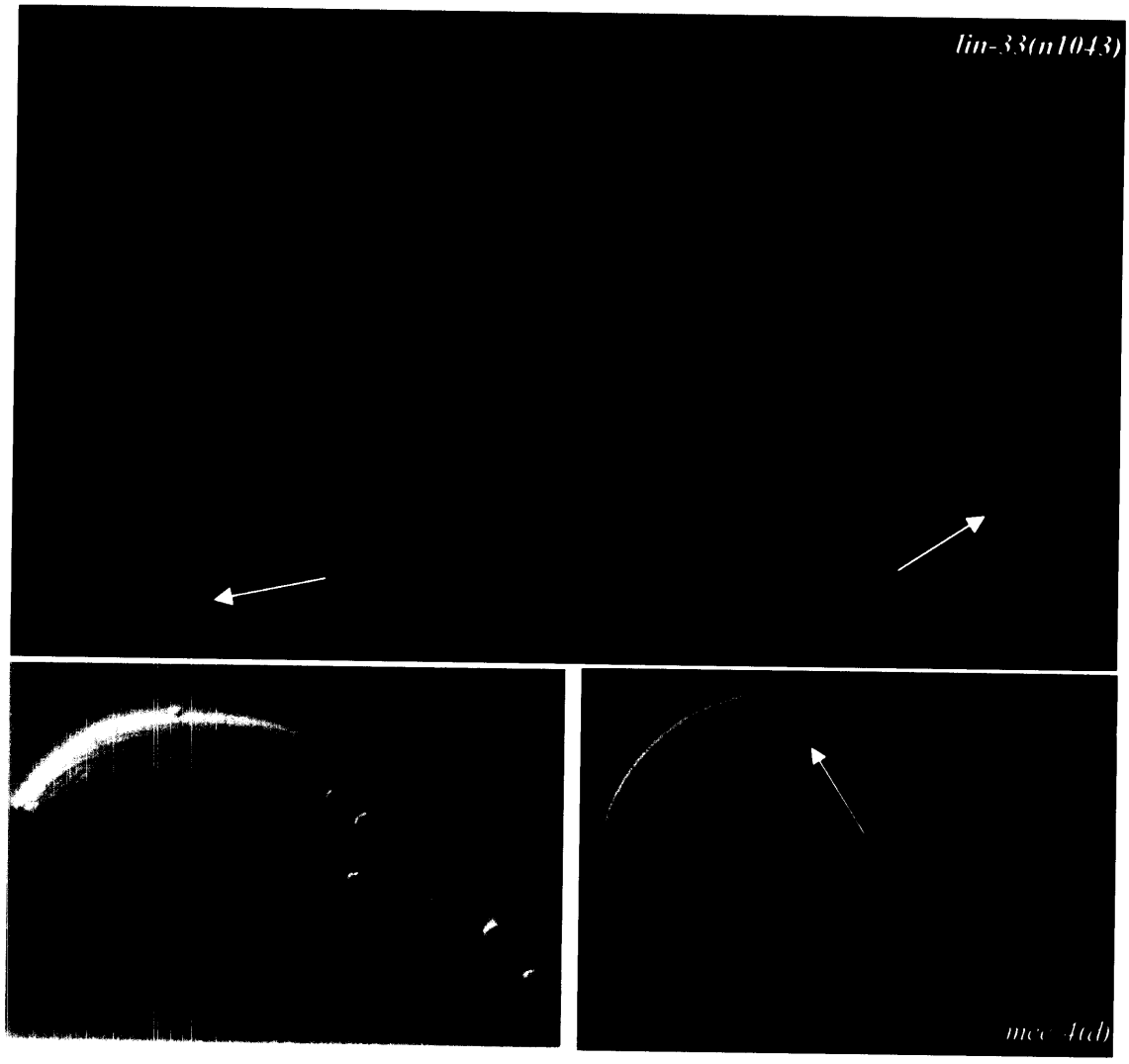
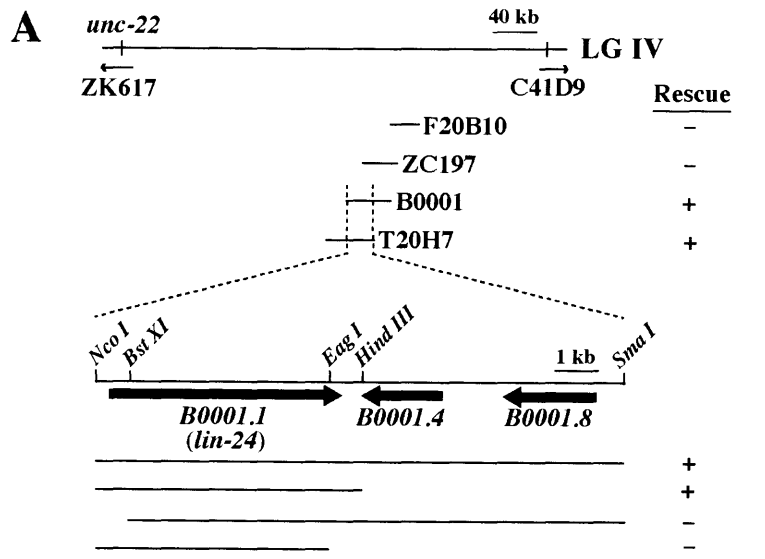
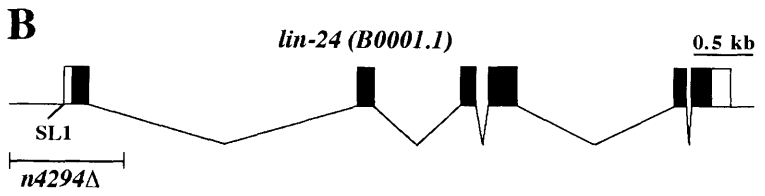
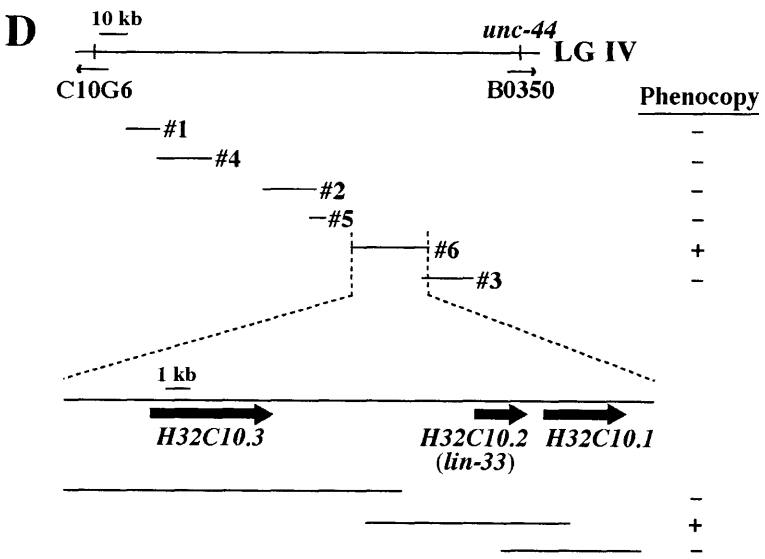


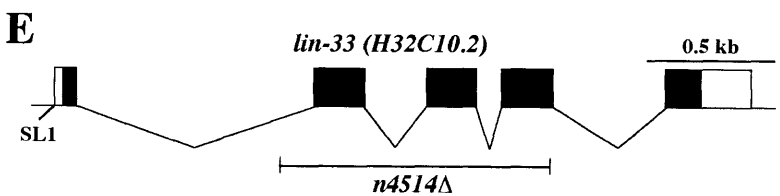
Figure 1

Figure 2









C

lin-24 mutations

Allele	Loss of function	Nucleotide change	Amino acid change
n1057	no	GAT to AAT	D20N
n1821	no	GAC to GGC	D31G
n2258	yes	TCC to TTC	S87F
n1503	yes	ACC to ATC	T94I
n2333	yes	GGA to GAA	G105E
n2050	no	ACG to ATG	T118M
n432	no	GCC to ACC	A127T
n4294	yes	deletion	-

F

lin-33 mutations

Allele	Loss of function	Nucleotide change	Amino acid change
n1502	yes	CCG to TCG	P192S
n1968	yes	TGG to TGA	W228opal
n1043	no	GAA to AAA	E230K
n1044	no	GAA to AAA	E230K
n1110	no	GAA to AAA	E230K
n1302	no	GAA to AAA	E230K
n2003	no	GAA to AAA	E230K
n4514	yes	deletion	-

Figure 3

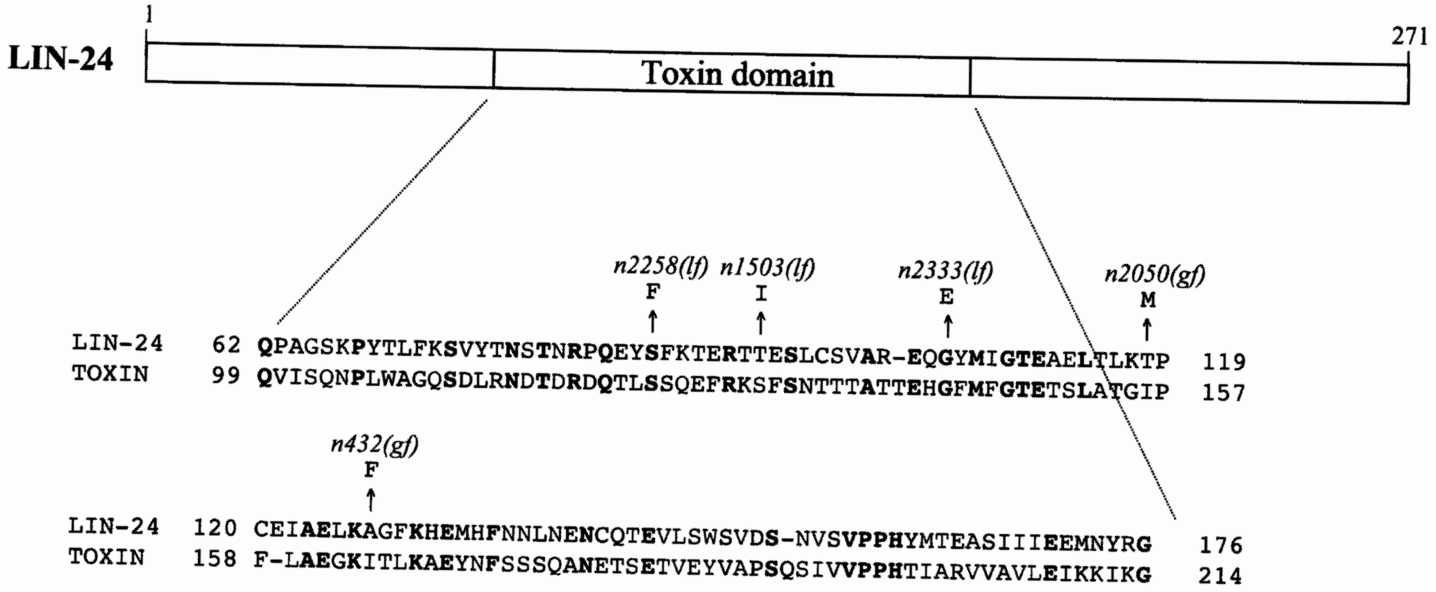

LIN-24
1
271
Toxin domain
n2258(lf)
F
n1503(lf)
I
n2333(lf)
E
n2050(gf)
M
LIN-24 62 QPAGSKPYTLFKSVYTNSTNRPQEYSFKTERTTESLCSVAR-EQGYMIGTEAELTLKTP 119
TOXIN 99 QVISQNPLWAGQSDLRNDTDRDQTLSSQEFRKSFSNTTTATTEHGFMFGTETSLATGIP 157
n432(gf)
F
LIN-24 120 CEIAELKAGFKHEMHFNNLNENCQTEVLSWSVDS-NVSVPPHYMTEASIIIEEMNYRG 176
TOXIN 158 F-LAEGKITLKAEYNFSSSQANETSETVEYVAPSQSIVVPPHTIARVVAVLEIKKIKG 214

Figure 4

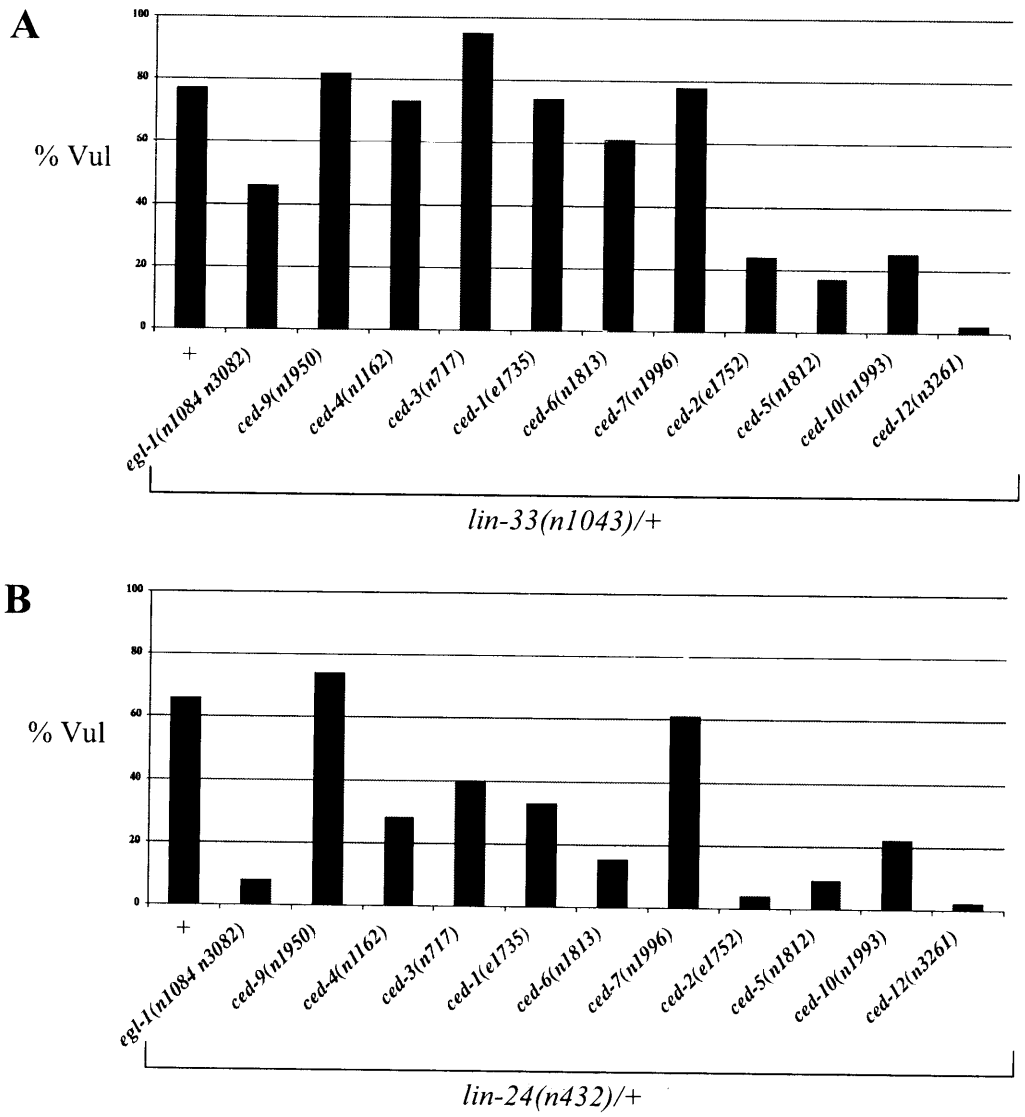

A
% Vul
100
80
60
40
20
0
+
egl-1(n1084 n3082)
ced-9(n1950)
ced-4(n1162)
ced-3(n717)
ced-1(e1735)
ced-6(n1813)
ced-7(n1996)
ced-2(e1752)
ced-5(n1812)
ced-10(n1993)
ced-12(n3261)
lin-33(n1043)/+
B
% Vul
100
80
60
40
20
0
+
egl-1(n1084 n3082)
ced-9(n1950)
ced-4(n1162)
ced-3(n717)
ced-1(e1735)
ced-6(n1813)
ced-7(n1996)
ced-2(e1752)
ced-5(n1812)
ced-10(n1993)
ced-12(n3261)
lin-24(n432)/+

Figure 5

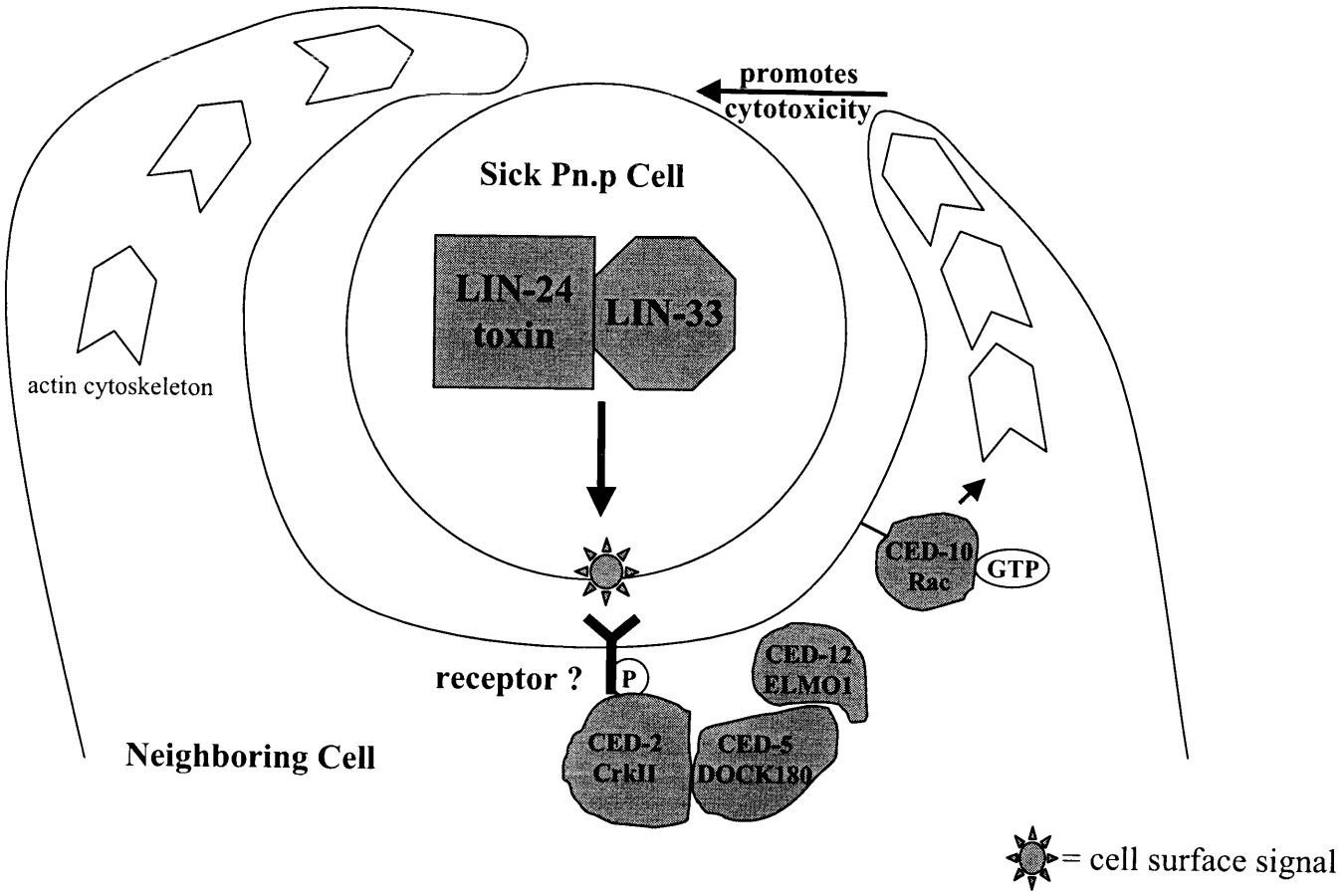

promotes
cytotoxicity
Sick Pn.p Cell
LIN-24
toxin
LIN-33
actin cytoskeleton
CED-10
Rac
GTP
receptor ?
P
CED-12
ELMO1
CED-2
CrkII
CED-5
DOCK180
Neighboring Cell
= cell surface signal

Figure 6

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