GENETIC ANALYSIS OF HOX TRANSCRIPTION FACTORS AND COFACTORS IN THE REGULATION OF PROGRAMMED CELL DEATH IN *C. ELEGANS*

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DEDICATION

I wish to dedicate this dissertation to my teachers, without whom I could never have become the scientist I am today. The first and most important teachers in my life were my parents, Jana and Don Knezek. I want to thank them for their constant love and support, and for teaching me about hard work, respect for others, and the pure joy of learning and discovery. I would also like to thank my classroom and music teachers for introducing me to science and for teaching me that seemingly impossible goals can be accomplished through dedication and determination. Furthermore, I want to thank my husband, Patrick Ryan Potts, for his love, support, and companionship. He is a true inspiration, and I continue to learn from him daily. Finally, I want to thank those who have taught me during my graduate studies, including my Thesis Committee and especially my mentor, Scott Cameron. Without their guidance and instruction this project would not have been possible.

GENETIC ANALYSIS OF HOX TRANSCRIPTION FACTORS AND COFACTORS IN THE REGULATION OF PROGRAMMED CELL DEATH IN *C. ELEGANS*

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It has been well established that blocking apoptosis can promote cancer. Throughout the animal kingdom, apoptosis is exquisitely regulated in cell-specific and context-specific ways to ensure proper development and tissue homeostasis. In many cases, transcriptional pathways carry out this regulation by mechanisms that are not completely understood. Studies of programmed cell death in the nematode *Caenorhabditis elegans* provided an essential foundation for understanding the more complex pathways of apoptosis in mammals. More recent work, including my thesis research, has focused on the mechanisms that decide whether individual cells of *C. elegans* survive or undergo programmed cell death, and has revealed a striking concordance of transcription factors that regulate cell death and that cause cancer in humans when altered by mutation. These findings suggest that mutations affecting these transcriptional pathways can provide a survival advantage to cancer cells, and thus may represent promising novel therapeutic targets.

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LIST OF DEFINITIONS

ALL – Acute lymphoblastic leukemia

AML – Acute myeloid leukemia

Apaf-1 – Apoptosis protease-activating factor 1

APC – Adenomatous polyposis coli

Ash1 – Absent, small or homeotic discs1

Bad – Bcl-XL/Bcl-2-associated death promoter

Bcl-2 – B cell lymphoma 2

Bcl-XL – Bcl-2 related gene, long splice variant

BH3 – Bcl-2 homology 3 domain

Bid – Bcl-2 interacting domain

Bim – Bcl-2 interacting mediator of cell death

Cabin1/Cain – Calcineurin inhibitor

Cdx – Caudal-related transcription factor

Ced – Cell death defective

Ceh – *C. elegans* homeodomain

Ces – Cell death specification

CFP – Cyan fluorescent protein

ChIP – Chromatin immunprecipitation

Dpy – Dumpy

Egl – Egg-laying defective

EMSA – Electrophoretic mobility shift assay

GFP – Green fluorescent protein

Gli – Zinc finger transcription factor homologous to cubitus interruptus

HDAC – Histone deacetylase

Hh – Hedgehog

HLF – Hepatic leukemia factor

HLH – Helix-loop-helix transcription factor

Hox – Homeotic selector

HSN – Hermaphrodite-specific neuron

IAP – Inhibitor of apoptosis protein

L1-L4 – Larval stage 1-4

LGI-IV, X – Linkage group (chromosome) I-IV, X

Lin – Cell lineage defective

Mab – Male abnormal

Meis – Myeloid ecotropic viral integration site

MLL – Mixed-lineage leukemia

Muv – Multivulvul

N2 – Wild-type *C. elegans*

NFAT – Nuclear factor of activated T-cells

Nhr – Nuclear hormone receptor

ORF – Open reading frame

Pax – Paired homeodomain transcription factor

Pbx – Pre-B-cell leukemia homeobox

PCD – Programmed cell death

PCR – Polymerase chain reaction

Pn.aap – Posterior daughter of the anterior daughter of the anterior daughter of the Pn blast cell

Puma – P53 upregulated modulator of apoptosis

RNAi – RNA interference

shRNA – Short-hairpin RNA

TALE – Three amino acid loop extension homeodomain

Unc – Uncoordinated movement

VC – Motor neurons in the ventral nerve cord of *C. elegans*

YFP – Yellow fluorescent protein

CHAPTER ONE: INTRODUCTION

TRANSCRIPTIONAL CONTROL OF APOPTOSIS IN DEVELOPMENT AND CANCER: THE WORM SPEAKS

The ability to avoid apoptosis can promote cancer initiation, progression, and resistance to treatment. Cancer cells can obtain this ability by usurping the cell-specific pathways that regulate apoptosis. These pathways may become essential for cancer cell survival and represent an Achilles Heel; therefore attacking them may offer a promising approach to new treatments. Consequently, the identification and mechanistic study of pathways that regulate apoptosis in specific cell types has the potential to offer novel insights into the biology and treatment of human cancers. In this chapter, I highlight five cell-specific apoptotic regulatory pathways discovered in the small roundworm *Caenorhabditis elegans* and explore the evidence suggesting that the human counterparts of these pathways promote cancer by preventing apoptosis.

Introduction

Apoptosis is a genetically programmed mechanism of cellular suicide that evolved to safely remove unneeded cells from an organism. This process is critical for normal development and tissue homeostasis in the adult, and is tightly controlled by cell-specific and context-specific regulatory mechanisms (Strasser, 2005). Apoptosis is executed by cysteine proteases called caspases, which are self-activated upon recruitment by adaptor proteins including Apaf-1 (Riedl and Salvesen, 2007). Apaf-1, in particular, requires the release of cytochrome *c* from the mitochondria to facilitate caspase activation (Wang,

2001). The Bcl-2 family of proteins, which includes both pro- and anti-apoptotic members, controls the release of mitochondrial cytochrome *c* (Chipuk and Green, 2008). A number of regulatory mechanisms exist that can positively or negatively control each step in the process of apoptosis.

Mutations in genes encoding core components of the apoptotic pathway and regulators of apoptosis can promote the development of cancer by giving a survival advantage to cells that would otherwise die. This was first described in follicular lymphoma caused by the t(14;18) chromosomal translocation, which results in overexpression of the anti-apoptotic protein Bcl-2 and enhanced survival of the cancer cells (Hockenbery et al., 1990; Vaux et al., 1988). More recently, the study of knockout mouse models has solidified the role of apoptotic genes as *bona fide* tumor suppressors: mice deficient for one pro-apoptotic member of the Bcl-2 family, *Bid*, develop a myeloproliferative disease that resembles chronic myelomonocytic leukemia (Zinkel et al., 2003), and mice deficient for a second member, *Bad* develop diffuse large B cell lymphoma (Ranger et al., 2003).

In addition to promoting oncogenesis, blocks in apoptosis can also render cancer cells resistant to treatment. For example, melanomas are notoriously resistant to chemotherapeutic agents due to several mechanisms that inhibit apoptosis (Soengas and Lowe, 2003). The most commonly mutated gene in human cancers, *TP53*, encodes a tumor suppressor that regulates apoptosis by activating transcription of BH3-only genes and directly promoting cytochrome *c* release from the mitochondria, and *TP53* mutations correlate with resistance to multiple chemotherapies (Royds and Iacopetta, 2006).

The mechanisms that determine the propensity of cells to undergo apoptosis can be hijacked by cancer cells to obtain a survival advantage. For example, post-mitotic cells such as neurons and cardiomyocytes, which exhibit minimal oncogenic potential and must survive for long periods of time, maintain extremely low levels of the pro-apoptotic protein Apaf-1, allowing endogenous IAPs to successfully block apoptosis (Potts et al., 2005; Wright et al., 2004). Cancer cells can imitate this survival-oriented state through mutations that either inactivate Apaf-1 or overexpress IAPs, both of which have been shown to occur in human melanomas where they offer a survival advantage to the cancer cells (Soengas et al., 2001; Vucic et al., 2000).

Cell Death and *C. elegans*

Genetic studies of programmed cell death in *C. elegans* have significantly advanced our understanding of the mechanisms underlying the regulation and execution of apoptosis. Many of the essential aspects of apoptosis in animals, including its nature as a genetically-encoded mechanism of cellular suicide and the central importance of caspases, were revealed by the identification and analysis of genes required for programmed cell death in *C. elegans* (Ellis and Horvitz, 1986). The realization that the *ced-9* gene, which is required for cell survival in *C. elegans* (Hengartner and Horvitz, 1994), is orthologous to *Bcl2* has been cited as the "aha" moment in the path of discovery (Horvitz, 2003). This discovery suggested that the mechanism of cell killing was evolutionarily conserved and deregulated in human diseases including cancer.

The core pathway of cell killing in *C. elegans* includes four genes, each with one or more mammalian homologs that serve similar functions (Figure 1-1). Mutations that inactivate the *egl-1*, *ced-4* or *ced-3* genes block essentially all programmed cell deaths in somatic cells (Conradt and Horvitz, 1998; Ellis and Horvitz, 1986), indicating that these genes are essential for programmed cell death. Gain-of-function mutations in *ced-9* prevent cell death, and loss-of-function mutations result in ectopic cell death and embryonic lethality, consistent with this gene serving as a critical switch in the cell death decision (Hengartner et al., 1992). *egl-1* encodes a BH3-only protein that is the most upstream component of the pathway for cell killing in somatic cells, and the EGL-1 protein binds directly to CED-9 to initiate programmed cell death (Conradt and Horvitz, 1998). The *ced-4* gene encodes an *Apaf-1* homolog that enhances the efficiency of killing by the caspase, CED-3 (Shaham and Horvitz, 1996; Yuan et al., 1993; Zou et al., 1997). Cell death is initiated in many cells by the binding of EGL-1 protein to a complex composed of CED-9 and two molecules of CED-4 on the surface of mitochondria (Chen et al., 2000; Yan et al., 2005; Yan et al., 2006). Upon release, the dimer of CED-4 forms a tetramer, which enhances autocatalytic activation of CED-3, resulting in death of the cell (Figure 1-1).

Development of *C. elegans* is essentially invariant, making it possible to identify and study the mechanisms that control cell fates, including programmed cell death and survival, with extraordinary precision (Sternberg and Horvitz, 1984). During normal development of a hermaphrodite, the cell lineages give rise to 1090 cells, of which 131 undergo programmed cell death (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977b;

Sulston et al., 1983b). Why evolution has favored the production and often immediate destruction of these "unnecessary" cells warrants speculation (Figure 1-2). Perhaps this is the simplest way to incrementally modify existing body plans and developmental programs.

The time and place of birth and death for each somatic cell in *C. elegans* is known and reproducible, making it possible to identify and study pathways that control programmed cell death with single cell resolution. Through various genetic screens, five pathways have been identified that control the programmed cell death or survival of specific somatic cells (Figure 1-3). In each case, the genes encode transcription factors whose human counterparts are oncogenes (Table 1-1). I summarize here the important features of each regulatory pathway and the evidence that these pathways are evolutionarily conserved and essential for survival of human cancer cells.

Pathway 1: *ces* genes and acute lymphoblastic leukemia offer a paradigm for identifying pathways required for cancer cell survival

Mutations affecting the *ces-1* and *ces-2* genes (*ces* = cell death specification) result in the survival of specific cells in the pharynx of *C. elegans* (Ellis and Horvitz, 1991). *ces-1* gain-of-function mutations result in the survival of four cells that normally die (two sisters of the NSM neurons and two sisters of the I2 neurons), and *ces-2* loss-of-function mutations result in the survival of two of these cells (the NSM sisters). Other deaths occur normally in these mutants. Genetic analyses indicate that *ces-2* acts as a negative

regulator of *ces-1* to control the death of the two NSM sister cells (Ellis and Horvitz, 1991) (Figure 1-3).

ces-2 encodes the *C. elegans* homolog of the PAR-family bZIP protein Hepatic Leukemia Factor (HLF) (Table 1-1) (Metzstein et al., 1996). *ces-1* encodes a zinc finger protein and Snail family member that directly represses *egl-1* transcription to prevent cell death in the NSM sister cells (Metzstein and Horvitz, 1999). The gain-of-function mutations in *ces-1* that ensure NSM cell survival affect a regulatory sequence in the promoter that is immediately adjacent to an evolutionarily-conserved binding site for the CES-2 protein, suggesting that these mutations prevent cell death by inappropriately maintaining *ces-1* expression (Metzstein and Horvitz, 1999).

Chromosomal translocations affecting the *Hlf* gene are identified in rare children with acute lymphoblastic leukemia (ALL), and unlike most children with ALL, the prognosis of children with these translocations is exceptionally poor (Inukai et al., 2007). The t(17;19) translocations couple the DNA-binding properties of HLF to the transcriptional activation function of E2A and bring the fusion protein under the control of the E2A promoter, which is expressed in lymphocytes (Hunger et al., 1991; Inaba et al., 1996; Inaba et al., 1992b). As a consequence, inhibition of E2A-HLF induces apoptosis of leukemic cells, indicating that the fusion protein prevents apoptosis (Inaba et al., 1996). (That E2A-HLF prevents apoptosis while the HLF homolog CES-2 activates apoptosis is due to altered activity of the fusion protein; fusion with E2A converts HLF from a

transcriptional repressor to a transcriptional activator of the relevant target gene.) The E2A-HLF fusion protein acts through *Slug*, a human *Snail* and *ces-1* homolog that encodes a transcriptional repressor (Hemavathy et al., 2000; Inukai et al., 1999). In hematopoietic stem cells, *Slug* competes with p53 to directly repress transcription of the *egl-1* homolog *Puma* and protect hematopoietic stem cells from radiation-induced apoptosis (Wu et al., 2005).

Therefore, human HLF and Slug control the *egl-1* homolog and BH3-only gene, *Puma,* in a manner directly analogous to their counterparts in *C. elegans*. Furthermore, mutations that alter the expression and function of HLF give cells in the lymphoid lineage an inappropriate survival advantage that results in development of acute lymphoblastic leukemia. Thus, the discovery and characterization of a genetic pathway that regulates programmed cell death of specific cells of *C. elegans* provided a model for how a human oncogene functioned, and this model was subsequently tested and confirmed. This indicates that genetic pathways that regulate programmed cell death or survival of specific cells of *C. elegans* can be evolutionarily conserved and altered by mutation to ensure survival of human cancer cells. Furthermore, inhibition of these pathways in the malignant cells can induce their death.

Pathway 2: *tra-1/Gli* is a global regulator of cell fates in *C. elegans* and active in diverse human cancers

The programmed cell death initiator *egl-1* was first discovered in screens for animals defective in egg-laying (Trent et al., 1983). These gain-of-function mutations were found

to disrupt binding of TRA-1, the terminal, global regulator of sex determination in *C. elegans*, to the regulatory region of *egl-1* (Conradt and Horvitz, 1999). In wild-type hermaprodites, TRA-1 represses *egl-1* in the HSN neurons and ensures their survival (Figure 1-3) (Conradt and Horvitz, 1999). In *tra-1* mutants the HSN neurons, which are essential for egg-laying, are partially transformed to a male fate, express *egl-1* and undergo programmed cell death. The deaths of these neurons result in the egg-laying defect that originally identified *egl-1*. Subsequent loss of function mutations in *egl-1* revealed its essential role in initiating programmed cell death in all somatic cells.

The discovery of *egl-1* as a direct target of the sex determination pathway posed a tremendous challenge to recognizing the mechanisms that specify cell death or survival during development. In the *ces-1* and *ces-2* mutants described in Pathway 1, the survival of a few precisely defined cells is the only obvious abnormality; the *ces* genes were therefore excellent candidates for cell-specific regulators of PCD. In striking contrast, *tra-1* regulates the fates of approximately one third of the cells of hermaphrodites. Indeed, genetic hermaphrodites are converted to males in *tra-1* mutants. The role of TRA-1 as a regulator of the cell death decision was therefore a surprise, in that death of the HSN neurons was thought to be a consequence of the male transformation by a transcription factor that acted as a master regulator high up in a regulatory cascade. The discovery of direct regulation of cell death in specific cells revealed that in this case it also acts as a micro-manager. These data pose a serious challenge to recognizing genes that directly regulate the cell death decision by individual cells.

The human homologs of TRA-1 are the Gli oncogenic zinc-finger transcription factors that respond to Hedgehog (Hh) signaling (Table 1-1) (Zarkower and Hodgkin, 1992). Abnormally increased Hh signaling is a very common feature of diverse cancers (Taipale and Beachy, 2001). People with Gorlin syndrome are heterozygous for germline mutations that inactivate *Patched*, a negative regulator of Hh signaling (Hahn et al., 1996; Johnson et al., 1996). These patients have developmental anomalies and are predisposed to multiple cancers including basal cell carcinoma, medulloblastoma, and soft tissue sarcomas (Hahn et al., 1996; Johnson et al., 1996; Kimonis et al., 1997). Activation of *Gli* is also seen in many other cancers including glioma, basal cell carcinoma, multiple myeloma, childhood sarcoma including rhabdomyosarcoma, and cancers of the prostate and gastrointestinal tract (Fukaya et al., 2006; Karhadkar et al., 2004; Mori et al., 2006; Ragazzini et al., 2004; Taipale et al., 2000; Watkins et al., 2003). Activation of Gli in cancers can occur through mutation of *Patched* or other negative regulators of the Hh pathway or through direct amplification or overexpression of the *Gli* genes (Evangelista et al., 2006; Hahn et al., 1996; Johnson et al., 1996; Kinzler et al., 1987).

The specific contributions of Hh signaling to the malignant character of individual tumors and tumor types can differ, including effects on pattern formation, stem cell character, proliferation, metastatic potential, and apoptosis. In a recent example of the latter, Hh signaling from stromal cells in the *Eμ-Myc* mouse model of B cell lymphoma activates *Gli* and maintains expression of *Bcl2* in the lymphoma cells, and inhibition of Hh signaling induces apoptosis (Dierks et al., 2007). This mechanism may be important in human non-Hodgkin's lymphoma and multiple myeloma as well (Dierks et al., 2007). In

primary keratinocytes, Gli proteins directly regulate transcription of *Bcl2* (Bigelow et al., 2004; Regl et al., 2004), suggesting that direct regulation of apoptosis is a conserved function of *Gli* family members that ensures survival of cancer cells. It is not yet clear how broadly this mechanism may be applied in other cancers in which Hh signaling is abnormally active.

Pathway 3: *Hox* genes as direct regulators of programmed cell death in *C. elegans* and in leukemia

A mutation in the *Hox* gene *lin-39* was the first allele isolated in genetic screens that led to the elucidation of the programmed cell death pathway of *C. elegans* (Clark et al., 1993; Ellis and Horvitz, 1986). Consistent with the established role of Hox genes in determining cell fates along the body axis, in *lin-39* mutants the six VC neurons in the midbody are transformed and adopt the fate of their more anterior and posterior homologs, which undergo programmed cell death (Clark et al., 1993). But what is the mechanism of this transformation? Hox proteins often act in concert with TALE family homeodomain cofactors to increase DNA binding affinity and selectivity. In *C. elegans* these include CEH-20 (homologous to mammalian Pbx), and UNC-62 (homologous to mammalian Meis) (Chan et al., 1994; Liu et al., 2006; Liu and Fire, 2000; Moens and Selleri, 2006; Streit et al., 2002; Van Auken et al., 2002; Yang et al., 2005). My thesis research suggests that a heterodimeric LIN-39/CEH-20 complex directly represses *egl-1* transcription in the VC neurons through binding multiple, redundant regulatory sites in *egl-1* (Chapter 4). UNC-62 is required for nuclear localization of CEH-20 and survival of the VC neurons, but does not appear to participate in DNA binding at the *egl-1* locus.

Expression of *lin-39* is promoted by the Trithorax group gene and *Ash1* homolog *lin-59*, which is additionally required for survival of the VC neurons (Figure 1-3).

The Hox gene *mab-5* promotes programmed cell death of two cells in the posterior ventral nerve cord, P11.aaap and P12.aaap. In P11.aaap, a complex that includes MAB-5 and CEH-20 binds to a single site in the *egl-1* gene to induce *egl-1* transcription and programmed cell death (Figure 1-3, Chapter 3). Remarkably, the death of P12.aaap is regulated indirectly, through an as yet unknown mechanism (Liu et al., 2006), indicating that the survival or death of even very closely related cells can be controlled through surprisingly distinct mechanisms. Distinct Hox protein complexes in *C. elegans* therefore activate or repress transcription of a cell death gene to ensure the programmed cell death or survival of specific cells. For *lin-39* and *mab-5,* this role is consistent with their specification of fates along the body axis, in this case by determining regional patterns of programmed cell death.

Abnormal activation of *Hox* genes is common in human leukemias, and their roles in normal and malignant hematopoiesis have been intensively studied. *Hox* function can be activated through several types of chromosomal translocations, including those that directly affect individual *Hox* genes, the *Pbx* family cofactors, or the *Trithorax* homolog *Mll*. *Mll* encodes a clinically important component of a chromatin-modifying complex that directly the regulatory regions of Hox genes to activate their expression (Ernst et al., 2002; Krivtsov and Armstrong, 2007; Milne et al., 2002; Rowley, 1999). Ten percent of adults with secondary leukemia and seventy percent of infants with ALL have

translocations affecting *Mll* (Biondi et al., 2000; Grimwade et al., 1998; Moorman et al., 2007), and two to five percent of children or adults with ALL have translocations affecting *Pbx1* (Moorman et al., 2007; Pui et al., 2004). Direct activation of *Hox* genes by translocations are less common. Leukemias with increased *Hox* gene activity are significantly more difficult to cure, and these patients are typically treated on more intensive regimens (Grimwade et al., 1998; Mancini et al., 2005).

Individual *Hox* genes have defined roles in normal hematopoiesis revealed by gene disruptions in mice, although these experiments probably underestimate their importance because of compensatory changes in *Hox* gene expression in the knockout animals (Bijl et al., 2006; Brun et al., 2004; Ko et al., 2007; Lawrence et al., 1997; Magnusson et al., 2007). Individual *Hox* genes also have defined roles in malignant hematopoiesis, and *HoxA9* in particular mediates some of the essential properties of the leukemic stem cell in *Mll*-dependent AML (Ayton and Cleary, 2003; Krivtsov et al., 2006; Kumar et al., 2004; Somervaille and Cleary, 2006; Wong et al., 2007). The *Pbx* and *Meis* cofactors are probably essential for leukemogenesis, as is the motif in the Hox proteins that mediates interaction with Pbx proteins (Schnabel et al., 2000; Wong et al., 2007), suggesting that complexes between these proteins regulate at least some genes critical for the leukemic phenotype.

But what are the critical target genes regulated by the Hox protein complexes that confer essential properties upon the leukemic cells? HoxA9 directly regulates the *Pim1* kinase, stimulating proliferation and inhibiting apoptosis, suggesting this is one important target

(Hu et al., 2007). The *C. elegans* data from my thesis research suggest that Hox proteins may also directly regulate transcription of apoptosis pathway genes to ensure survival of leukemia cells (Chapter 3, 4). Indeed, recent work from Dr. Scott Armstrong's laboratory has shown that in MLL-AF9-induced leukemias, HoxA9 directly represses transcription of the BH3-only gene *Bim* and prevents apoptosis (Chapter 4). Thus the Hox pathway is another example of a *C. elegans* transcriptional pathway involved in cell-specific regulation of programmed cell death that is mechanistically conserved in mammals and subverted by mutations that provide a survival advantage to cancer cells.

Pathway 4: *egl-38/Pax* regulates *ced-9/Bcl2*

C. elegans egl-38 and *pax-2* encode Paired-homeodomain transcription factors (Park et al., 2006). Unlike the *ces*, *tra-1* and *Hox* genes described above, which appear to regulate surival in a small number of cells, *egl-38* and *pax-2* ensure the survival of many cells in the developing animal, including somatic cells and developing germ cells. Moreover, *egl-38* and *pax-2* prevent programmed cell death through a distinct mechanism: direct stimulation of *ced-9* transcription (Figure 1-3) (Park et al., 2006).

In human cancers, *Pax* genes are rearranged and fused to *Forkhead* family genes by the t(2;13) and t(1;13) chromosomal translocations in childhood rhabdomyosarcoma, and to the immunoglobulin enhancer in some non-Hodgkin's lymphomas (Busslinger et al., 1996; Davis et al., 1995; Epstein et al., 1994; Fredericks et al., 1995; Shapiro et al., 1993). The fusion proteins inhibit apoptosis in rhabdomyosarcoma cells through an

undefined mechanism (Bernasconi et al., 1996). Pax genes are broadly expressed in a large collection of tumor cell lines including the NCI-60 cell lines, and inhibition of individual *Pax* gene expression in many of these cell lines induces apoptosis (Muratovska et al., 2003). How *Pax* expression prevents apoptosis in these tumor lines is unknown, although direct stimulation of *Bcl-XL* expression by Pax3 has been reported (Margue et al., 2000). This mechanism would be consistent with the well-defined role of *Pax* genes in *C. elegans*.

Pathway 5: *pal-1/Cdx* promotes leukemia and colon cancer through distinct pathways

Most cells of *C. elegans* that are destined to die undergo programmed cell death immediately after they are born. One exception is the tail spike cell, which dies several hours after birth (Sulston and Horvitz, 1977b; Sulston et al., 1983a). Death of the tail spike cell is only partially controlled by *egl-1* and *ced-9*; transcriptional activation of the caspase *ced-3* is also critically important for efficient programmed cell death of this cell (Maurer et al., 2007). Transcription of *ced-3* is promoted by the homeodomain protein PAL-1, which directly binds the *ced-3* promoter to activate expression (Figure 1-3) (Maurer et al., 2007). For this unusual cell, *C. elegans* may have adopted an unusual mechanism of killing.

The mammalian homologs of PAL-1 are the Cdx1, Cdx2 and Cdx4 proteins (Table 1-1). In multiple hematological cancers including AML, ALL, chronic myelogenous leukemia in blast crisis, myelodysplastic syndrome, and AML after therapy for another cancer, rare

patients have a t(12;13) translocation that activates expression of *Cdx2* in hematopoietic cells (Chase et al., 1999). Consistent with the roles of *pal-1* in *C. elegans* and *Caudal* in *Drosophila* as activators of *Hox* gene expression, increased *Cdx2* or *Cdx4* activity in leukemias can lead to increased *Hox* gene transcription in mouse models, although *Hox* overexpression may not be observed in all cases of human cancers (Bansal et al., 2006; Rawat et al., 2004).

In colon cancers, the *Cdx1* and *Cdx2* genes are silenced rather than activated (Guo et al., 2004). Heterozygosity for *Cdx2* enhances the development of intestinal polyps in adenomatous polyposis coli (APC) heterozygous mice, indicating that *Cdx2* acts as a tumor suppressor in this model. It does so at least in part by affecting chromosome stability (Aoki et al., 2003). These observations suggest that *Cdx* family genes can promote or prevent cancer through distinct mechanisms involving activation of a *Hox* gene program in hematopoietic cells and dysregulation of chromosome segregation in the intestine. Whether *Cdx* gene mutations affect caspase transcription and apoptosis in mammals, as predicted by the *C. elegans* data, has not yet been explored.

Apoptosis-regulating pathways as therapeutic targets

The five pathways described here comprise cell-specific transcriptional regulators of programmed cell death in *C. elegans* (Figure 1-3). In each pathway, the human homologs of the genes involved promote cancer (Table 1-1). For two pathways (the *ces/Hlf/Slug* and *Hox* pathways), convincing evidence shows that these pathways prevent

apoptosis of cancer cells by direct transcriptional repression of proapoptotic cell death genes. This suggests that cell-specific apoptotic regulatory pathways that operate during normal development to eliminate unnecessary cells or to ensure the survival of other cells are co-opted by cancer cells to gain a survival advantage. Attacking such pathways may be a promising approach to new treatments, as cancer cell survival may continuously depend on them for survival. Alternatively, successful targeting of these pathways could be insufficient to kill cancer cells outright, but may sensitize them to other cytotoxic treatments. Regardless, one major challenge for this potentially fruitful strategy is the physical nature of the targets involved. Although transcription factors are markedly over-represented among genes that cause cancer when mutated (Futreal et al., 2004), targeting them has proven difficult because protein-protein interactions are not easily disrupted by traditional drugs (Gadek and Nicholas, 2003). However, there is reason to be optimistic that novel strategies for drug design may be able to overcome this problem in the future (Wells and McClendon, 2007).

Alternatively, detailed mechanistic studies of these pathways may reveal critical contributions by more traditionally "druggable" proteins, such as kinases or chromatin-modifying enzymes. The importance of abnormal protein kinase activity in cancer, coupled with the stunning clinical successes of the kinase inhibitor imatinib, has driven efforts to identify more small molecule inhibitors of the protein kinases, some of which are effective clinically (Giles et al., 2007; Krause and Van Etten, 2005). Drugs that inhibit the enzymatic activities of chromatin modifying factors are in clinical trials for solid tumors and hematological malignancies (Duvic et al., 2007; Gojo et al., 2007;

Kuendgen et al., 2006; Ramalingam et al., 2007). Drugs that target nuclear hormone receptors, which are transcription factors whose activities are regulated allosterically by binding of small molecules, significantly improve outcome for women with estrogen receptor-positive breast cancer and patients with acute promyelocytic leukemia (Lin and Winer, 2008; Wang and Chen, 2008).

What's next?

Drawing the parallel between *C. elegans* genes that regulate cell death and their homologous human oncogenes immediately proposes the hypothesis that the oncogenes promote cancer, at least in part, by transcriptionally blocking apoptosis. Future studies will determine how broadly the parallels between *C. elegans* programmed cell death and human cancer can be drawn, and will strive to target these pathways therapeutically. Moreover, exploring the pathways that control apoptosis in other organisms where the biology and experimental tools are available will likely also be fruitful. Most importantly, the pathways described here barely scratch the surface of cell-specific regulation of programmed cell death. There are 1090 cells born in each *C. elegans* hermaphrodite, and each must decide whether to live or die. To date, we understand only a portion of the mechanisms that control this decision in only a few of these cells. Elucidating these mechanisms will provide insight into the mechanisms of oncogenesis and provide novel targets for cancer therapy.

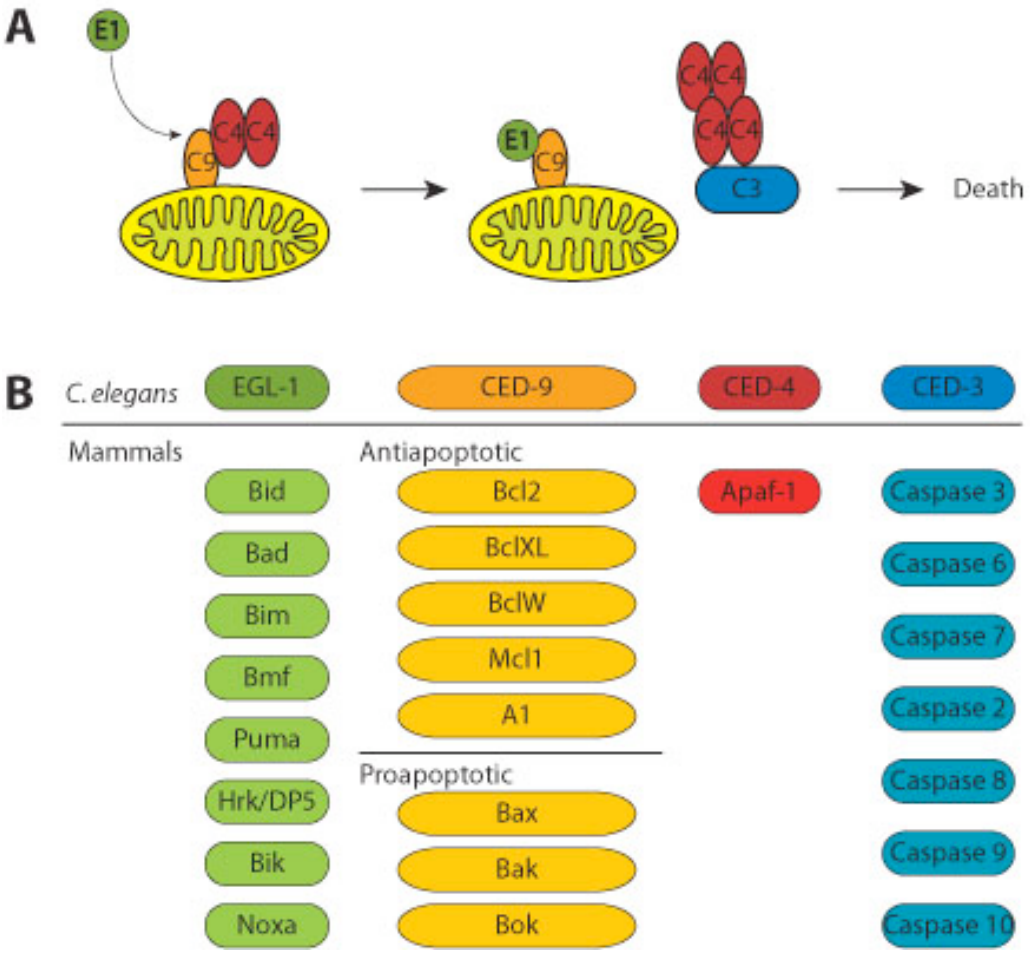


Figure 1-1. Programmed cell death is evolutionarily conserved.
A) The mechanism for programmed cell death in *C. elegans*. In living cells, CED-9 (C9) tethers CED-4 (C4) to the mitochondria and inhibits its activity. EGL-1 (E1) is produced in cells destined to die, where it binds to CED-9 and causes the release of CED-4. CED-4 then facilitates activation of the caspase CED-3 (C3), which cleaves many cellular substrates to induce death. B) Mammalian homologs of *C. elegans* programmed cell death proteins. EGL-1 is a pro-apoptotic BH3-only protein. Mammalian homologs are depicted below. Three additional mammalian proteins resemble BH3-only proteins but have not yet been functionally characterized: Bcl-rambo, Boo/Diva, and Bcl-b/Bcl2L10. CED-9 is an anti-apoptotic protein of the Bcl-2 family that contains multiple BH domains. Mammalian homologs include both anti- and pro-apoptotic members. CED-4 is homologous to the adaptor protein Apaf-1. CED-3 is a caspase.

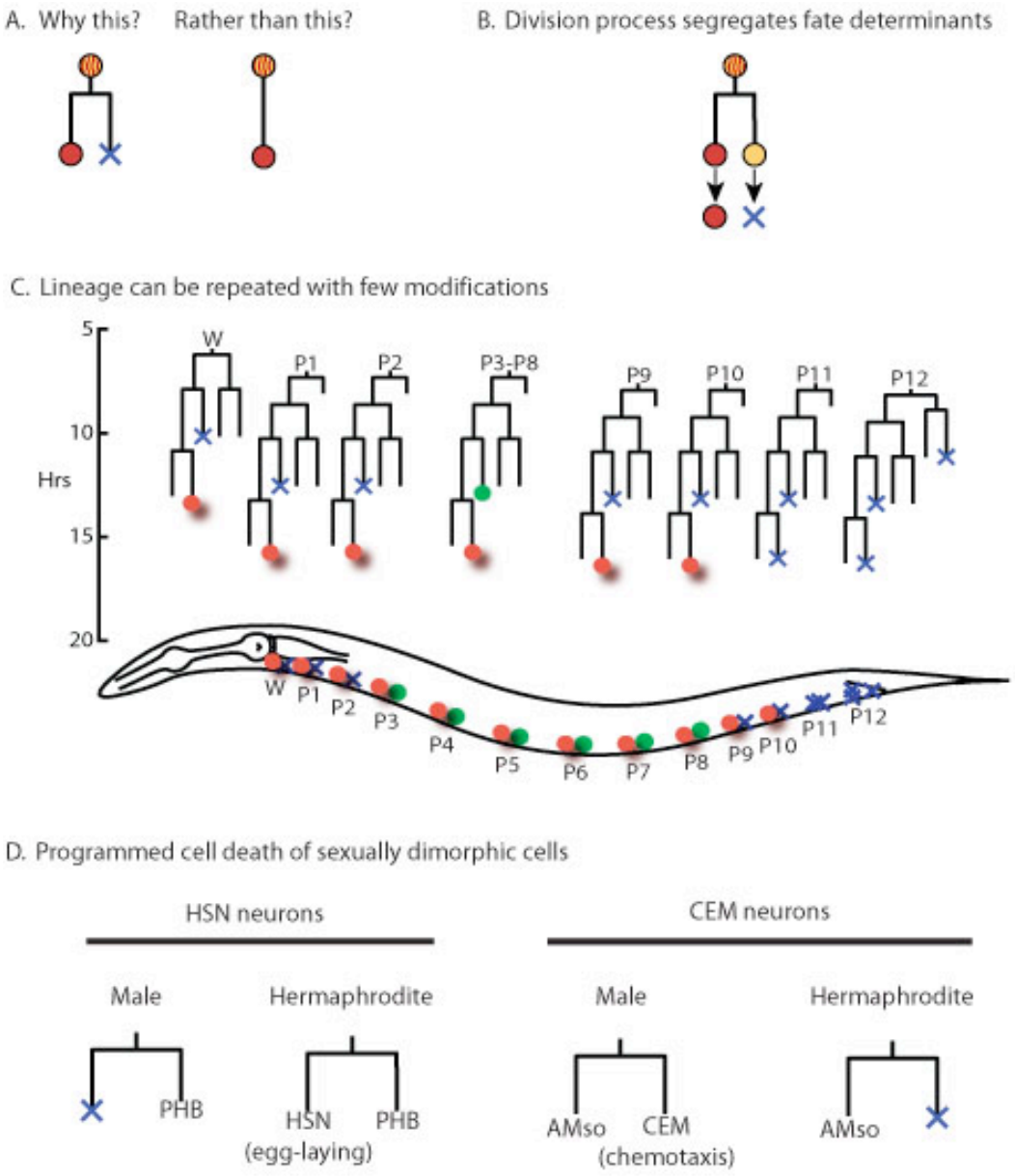


Figure 1-2. Cell deaths may play important roles in the cell lineages of *C. elegans* and mammals.
A) Most of the 131 cell deaths in *C. elegans* hermaphrodites occur immediately after cell division (left), producing one daughter cell of a particular fate (red circle) and another daughter cell that dies (blue X). Theoretically, the same outcome could have occurred without cell division or cell death, if the mother cell instead underwent a process of differentiation to acquire the correct cell fate (right). Why has evolution favored a process of coupled cell division and cell death, rather than the single process of

differentiation? B) One possibility is that critical determinants of cell fate cannot be appropriately segregated without division. For example, it might be mechanistically simpler to undergo asymmetric division and partition the desired determinants (red) into one daughter and the detritus (yellow) into a daughter that subsequently dies. C) Another possible explanation is that it is simpler to repeatedly use a basic cell lineage program and then to modify this program with programmed cell deaths rather than developing new cell lineage programs for specific cases. For example, the lineages of the 13 blast cells of the ventral nerve cord are strikingly similar to one another. The P3-P8 blast cells in the midbody region produce VC neurons (green), which innervate the vulva to aid in egg-laying. The vulva is located in the middle of the animal, so these neurons are presumably only needed in the midbody. The cells produced at the same point in the lineage in the anterior and posterior regions undergo programmed cell death. A similar evolutionary explanation exists for the VB neurons (red), which extend their axons posteriorly. Presumably, such neurons are not needed in the posterior so the VB equivalents undergo PCD. Thus, in the ventral nerve cord, it may be simpler to repeat the same lineage using cell death as a minor modification, rather than invent distinct lineages for each body region. D) A related process may allow the same lineage programs to be used by distinct sexes. Examples of sexually dimorphic cell deaths include the CEM, which is needed for chemotaxis in males, and the HSN, which is needed for egg-laying in hermaphrodites. Again, rather than invent completely novel lineages, evolution has opted to use programmed cell death to modify the existing lineage based on signaling inputs that convey the animal's sex. It seems highly likely that analogous programmed cell deaths occur in mammals, raising the possibility that cancer could result from the abnormal survival of cells destined to die in a developmentally programmed manner, rather than cells that have been damaged in some way and fail to engage the appropriate apoptotic response.

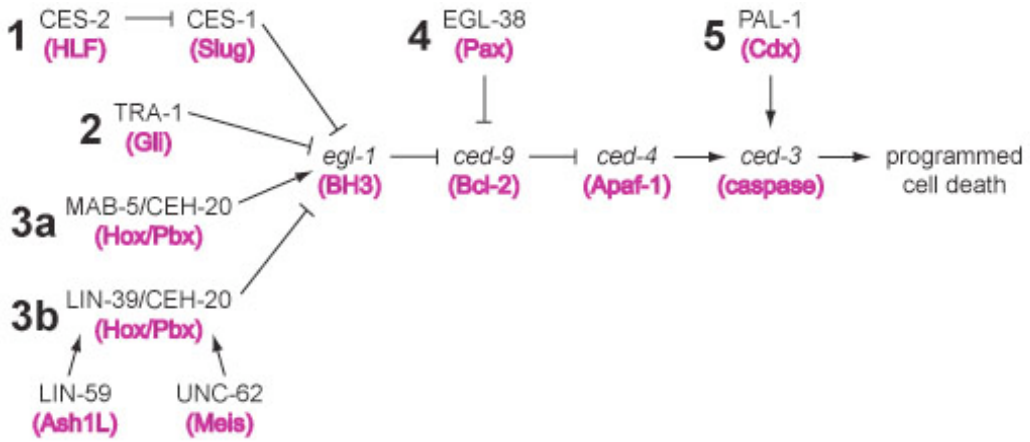


Figure 1-3. Five pathways that regulate cell death in specific cells in *C. elegans* contain transcription factors with human homologs that are oncogenes.
The interaction of five cell-specific regulatory pathways with the core programmed cell death genes are shown. The mammalian homolog (or homologous family) of each gene or protein is depicted in parentheses under the *C. elegans* name. Arrows indicate positive regulation, and ----| indicates negative regulation. The cells whose fates are determined by each pathway are: NSM sisters (1), HSN neurons (2), P11.aaap (3a), VC neurons (3b), germline and many somatic cells (4), and tail spike cell (5). These data suggest hypotheses about how oncogenic versions of the transcription factors my promote cancer; current evidence pertaining to each pathway is discussed in detail in the text.

Table 1-1. Genes controlling programmed cell death in *C. elegans* have mammalian homologs with roles in various cancers.

C. elegans genes	Mammalian homologs	Relevant cancers
ced-3	*caspase* family	not known
ced-4	*Apaf-1*	melanoma
ced-9	multidomain *Bcl-2* family	hematopoeitic malignancies
ceh-20	*Pbx* family	ALL
ces-1	*Snail* family (*Slug*)	acute lymphoblastic leukemia
ces-2	*hlf*	acute lymphoblastic leukemia
egl-1	BH3-only *Bcl-2* family	hematopoeitic malignancies
egl-38	*Pax* family	rhabdomyosarcoma, lymphoma
lin-39	*Hox* family	ALL, AML
lin-59	*Ash1L* (*trithorax* family)	ALL, AML
mab-5	*Hox* family	ALL, AML
pal-1	*cdx* family	colon cancer, leukemia
pax-2	*Pax* family	rhabdomyosarcoma, lymphoma
tra-1	*Gli* family	glioma, medulloblastoma, other
unc-62	*Meis* family	ALL, AML, other

C. elegans genes involved in execution or regulation of programmed cell death that are mentioned in Chapter 1 are shown, along with the homologous gene or gene family in mammals and a list of cancer types in which evidence exists that these genes play a role in cancer progression. For details and references, please see the text.

CHAPTER TWO: MUTAGENESIS-BASED SEARCH FOR NOVEL PCD MODIFIERS

INTRODUCTION

To discover novel cell-specific regulators of programmed cell death in *C. elegans* I focused on the ventral nerve cord, where the specific identity of cells can be determined by lineage analysis and thus PCD can be analyzed at single-cell resolution. The mechanisms that determine which cells survive and which undergo PCD in the ventral nerve cord are largely unknown, and identifying genes responsible for these decisions could lead to new insights into human cancer (Chapter 1). Mutagenesis screens in Dr. Robert Horvitz's lab produced two alleles with a defect in regulation of PCD that specifically affected the ventral nerve cord, and these alleles were sent to our lab for characterization. One turned out to be a new allele of *ced-4*, and thus not a novel PCD regulatory gene. The other is not a *ced-4* allele and thus could represent a novel gene, but its identity remains unknown at this time.

PCD in the Ventral Nerve Cord

The ventral nerve cord of *C. elegans* is made up of neurons and hypodermal cells, is located on the ventral side of the animal, and stretches along the entire body length (Sulston and Horvitz, 1977a). At hatching, 15 embryonic neurons in the ventral nerve cord are already differentiated and functioning. During the L1 larval stage, thirteen blast cells migrate into the ventral nerve cord and begin dividing in a stereotyped lineage that produces the post-embryonic neurons and hypodermal cells of the ventral nerve cord

(Figure 2-1A). By the end of the L2 larval stage these divisions are complete, but further neuronal development in the form of differentiation, as well as vulval development, continue through the young adult stage.

The pattern of division of each of the thirteen blast cells that make up the postembryonic ventral nerve cord is highly similar (Figure 2-1A). To accomplish specification along the anterior-posterior axis, a stereotyped pattern of cell death and other mechanisms eliminate cells not needed in a particular body region. One such cell type, the VC motor neuron, is needed in the midbody region to innervate the vulva and assist in egg-laying. One VC neuron is produced from each blast cell in the midbody region in the Pn.aap (posterior daughter of the anterior daughter of the anterior daughter of the Pn blast cell) position of the lineage. The blast cells in the anterior and posterior regions of the ventral nerve cord produce Pn.aap cells, but these cells undergo programmed cell death almost immediately after being born and therefore do not differentiate into VC neurons.

A Sensitized Background for PCD Modifiers

After VC differentiation occurs in the late L4 stage, the VC neurons express the marker $P_{lin\text{-}11}$*gfp* and can therefore be easily distinguished from the other neurons of the ventral nerve cord (Cameron et al., 2002; Reddien et al., 2007; Reddien et al., 2001). All six VC neurons express the marker, but only four can be reliably scored because the marker is also expressed brightly in the vulva where it obscures the signal of two VC neurons. Therefore, in the ventral nerve cord of wild-type animals expressing the $P_{lin\text{-}11}$*gfp* marker,

four neurons reliably express GFP (Figure 2-1A,C). When programmed cell death is blocked completely, five of the anterior and posterior Pn.aap cells that normally die instead survive, differentiate into VC neurons, and express the P_{lin-11}*gfp* marker (Figure 2-1B,C). Therefore a complete block of programmed cell death results in nine visible GFP-positive neurons in the ventral nerve cord (four VC neurons plus five "undead" Pn.aap cells).

To screen for mutations with weak effects on programmed cell death, Scott Cameron and Peter Reddien built a sensitized P_{lin-11}*gfp* reporter strain in which programmed cell death was incompletely blocked by the partial loss-of-function allele *ced-3(n2427)* (Reddien et al., 2007; Reddien et al., 2001). The individual animals of this strain show varying numbers of extra surviving Pn.aap cells (Figre 2-1C). Using this sensitized background, Peter Reddien performed a mutagenesis screen and discovered several alleles which enhance *ced-3(n2427)*'s weak effect on PCD (Reddien et al., 2007). He gave two of these alleles, *n3393* and *n3397*, to us to characterize (Figure 2-1D). The information that Peter Reddien provided with these alleles suggested that they complemented all known PCD regulators and did not affect PCD in the pharynx (Peter Reddien, personal communication). This information suggested that *n3393* and *n3397* might represent novel genes responsible for regulating PCD in the ventral nerve cord.

RESULTS AND METHODS

n3393 is a Novel Allele of *ced-4*

n3393 maps to LGIII

Worms were cultured at 20° C using standard techniques(Brenner, 1974a). To determine whether *n3393* was located on chromosome III, Matthew Jamison and I created the following mapping strain: *dpy-17(e164) unc-32(e189) III; ced-3(n2427) IV;* P_{lin-11}*gfp X.* We then crossed hermaphrodites from this strain to males of the genotype *ced-3(n2427) IV;* P_{lin-11}*gfp X; n3393* (obtained from Peter Reddien). Cross-progeny were easily identified as non-Dpy and non-Unc. These animals were allowed to self-fertilize, and from the F2 progeny all Dpy non-Unc and Unc non-Dpy recombinants were selected and isolated. The same phenotype was selected from the F3 generation to ensure homozygosity of the recombinant chromosome, and then F4 progeny were scored for P_{lin-11}*gfp* expression. All animals maintained *ced-3(n2427)* in the background, so those with a GFP pattern consistent with partial *ced-3* activation were presumed not to carry *n3393*, whereas those with enhanced PCD defects were presumed to carry *n3393*. Of 32 Unc non-Dpy recombinants, 30 showed enhanced PCD defects. In contrast, of 20 Dpy non-Unc recombinants, only one showed enhanced PCD defects. These data indicate that *n3393* is located on chromosome III, most likely to the left of *dpy-17* (Figure 2-2A).

n3393 fails to complement ced-4

The gene *ced-4*, which is required for PCD, is located on chromosome III just to the left of *dpy17* (Figure 1-2A). We attempted to rule out the possibility that *n3393* was an allele of *ced-4* using a standard complementation test. To perform the test, we crossed hermaphrodites of the genotype *ced-1(e1735); ced-4(n1162) unc-69(e587);* P_{lin-11}*gfp* with males of the genotype *ced-3(n2427);* P_{lin-11}*gfp; n3393*. The cross-progeny, which were

identified as non-Unc, showed complete survival of the Pn.aap cells (data not shown), indicating a complete defect in PCD. Thus *n3393* failed to complement *ced-4(n1162)*, suggesting that it was a new allele of *ced-4* rather than a novel PCD modifying gene.

n3393 exhibits a missense mutation in the ced-4 gene

To confirm that *n3393* was indeed a novel allele of *ced-4*, we sequenced the *ced-4* coding region in the *ced-3(n2427);* $P_{lin\text{-}11}$*gfp; n3393* animals. Indeed, we found a C->A missense mutation that would be predicted to convert the threonine at position 28 of the CED-4 protein to asparagine (Figure 2-2B). The remaining coding region of *ced-4* showed no mutations, and the C->A missense mutation was not present in the parent strain *ced-3(n2427)*; $P_{lin\text{-}11}$*gfp*. Because n3393 enhances the PCD defect of a weak allele of *ced-3*, maps to the left arm of chromosome III, fails to complement *ced-4(n1162)*, and displays a missense mutation in *ced-4*, we conclude that *n3393* is an allele of *ced-4*.

n3397 is Not an Allele of *ced-4*

n3397 complements ced-4

I initially wanted to map, identify, and characterize the *n3393* and *n3397* alleles Peter Reddien sent because they might be novel regulators of PCD in the ventral nerve cord. However, *n3393* turned out to be an allele of *ced-4*, which has already been extensively characterized and is not a cell-type specific regulator of cell death. To determine whether *n3397* was also an allele of *ced-4* or might represent a novel PCD modifying gene, I tested whether *n3397* complemented *ced-4(n1162)*. The cross-progeny from a cross

between *ced-1(e1735); ced-4(n1162) unc-69(e587);* $P_{lin\text{-}11}$*gfp* hermaphrodites and *ced-3(n2427);* $P_{lin\text{-}11}$*gfp; n3397* males did not show an enhanced PCD phenotype (Figure 2-3), indicating that *n3397* complements *ced-4(n1162)* and is therefore probably not an allele of *ced-4*.

The ced-4 coding region has no mutations in n3397

Further support that *n3397* is probably not an allele of *ced-4* comes from sequencing data. I sequenced the entire *ced-4* coding region in *ced-3(n2427);* $P_{lin\text{-}11}$*gfp; n3397* animals and did not find the C->A missense mutation found in *n3393* animals or any other mutation. Although this doesn't rule out the possibility that a mutation outside the coding region might affect *ced-4* function, the complementation test described above argues against that possibility. Thus, *n3397* appears to be a novel regulator of PCD in the ventral nerve cord, and further analysis should help to identify and characterize the affected gene.

DISCUSSION

The information obtained with the *n3393* and *n3397* alleles appears to be flawed, at least with regards to *n3393* complementing known PCD genes. Because of this, I recommend that any further characterization of *n3397* begin fresh with mapping. The results presented here indicate that *n3397* is not an allele of *ced-4*, but it is still possible that it is an allele of another known PCD gene such as *egl-1*, *ced-9*, or *ced-3*. However, if *n3397*

is a novel PCD modifying gene, then its identification and characterization could be extremely useful and interesting.

When I initially thawed the three strains obtained from Peter Reddien and scored their GFP patterns in the ventral nerve cord, the pattern of the parent strain *ced-3(n2427);* $P_{lin\text{-}11}$*gfp* was not consistent with what had been published (Reddien et al., 2007) (Figure 1-1C). Instead, almost all animals showed complete survival of the Pn.aap cells (data not shown). To recreate the original pattern, I selected animals with only one extra GFP-positive neuron to propogate the strain through several generations. After this selection pressure was applied, the strain exhibited the correct GFP pattern. Because this strain and *ced-3(n2427);* $P_{lin\text{-}11}$*gfp; n3393* did not perform as advertised, I think that it is possible that the identities of the strains may have been confused at some point between when the screen was performed and when I thawed and started to characterize them.

Alternatively, some natural mutations or polymorphisms may be affecting the GFP pattern in this sensitized strain. This explanation is supported by crosses I performed to determine whether *n3393* and *n3397* were recessive or dominant. Depending on which strains were used, these alleles appeared to be recessive in most cases but dominant in some. For example, cross-progeny obtained after crossing *unc-69(e587);* $P_{lin\text{-}11}$*gfp* hermaphrodites to *ced-3(n2427);* $P_{lin\text{-}11}$*gfp; n3397* males did not show enhanced PCD (Figure 1-4A), suggesting that *n3397* is recessive. This conclusion is also supported by the *ced-4* complementation data shown in Figure 1-3. However, cross-progeny obtained after crossing *unc-32(e189) dpy-17(e164); ced-3(n2427);* $P_{lin\text{-}11}$*gfp* hermaphrodites with

ced-3(n2427); $P_{lin\text{-}11}$*gfp; n3397* males did show enhanced PCD, suggesting that in this case *n3397* was not recessive (Figure 1-4B). These contradictory results are problematic and should be addressed if *n3397* is to be characterized in detail. They likely reflect the difficulties of genetics in a sensitized background.

In conclusion, Matthew Jamison and I determined that *n3393* is a new allele of *ced-4* rather than a novel PCD modifying gene. I showed that *n3397* is not a *ced-4* allele. The identity of the gene affected by *n3397* remains unknown.

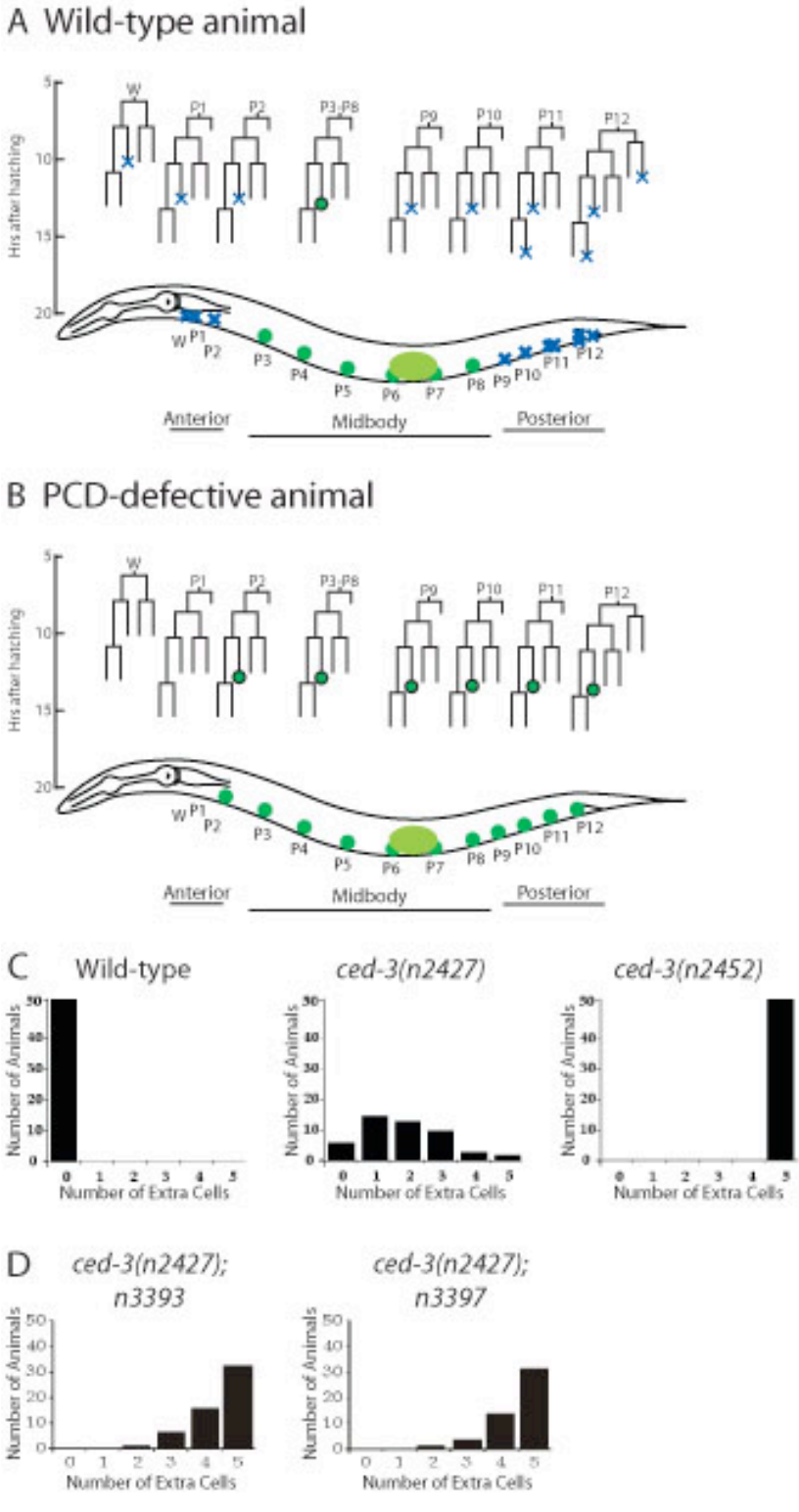

A Wild-type animal
Hrs after hatching
W
P1
P2
P3-P8
P9
P10
P11
P12
Anterior
Midbody
Posterior
B PCD-defective animal
C
Wild-type
ced-3(n2427)
ced-3(n2452)
Number of Animals
Number of Extra Cells
D
ced-3(n2427); n3393
ced-3(n2427); n3397

Figure 2-1. An enhancer screen for PCD modifiers in the ventral nerve cord produced two novel alleles.
A) The post-embryonic ventral nerve cord lineage of a wild-type animal. Cell divisions are indicated by horizontal lines; time is indicated by vertical lines. The green circles represent VC motor neurons, and the blue Xs represent cells that undergo programmed cell death. The position of these cells in the animal is schematized below. Vulval fluorescence of the $P_{lin\text{-}11}$*gfp* reporter is represented by a large, light green oval in the middle of the animal. B) When PCD is completely blocked, no cell deaths occur. Cells in the posterior and anterior that would otherwise die now differentiate into VC-like neurons that express the $P_{lin\text{-}11}$*gfp* reporter (indicated by green circles). C) The number of extra GFP-positive cells in the ventral nerve cord was scored in 50 animals each of the indicated genotype. *ced-3(n2427)* is a partial loss-of-function allele, whereas *ced-3(n2452)* is null and completely blocks PCD. D) The number of extra GFP-positive cells in the ventral nerve cord of two mutants obtained from a mutagenesis screen was scored (n=50 per genotype). Notice that the partial PCD defect of *ced-3(n2427)* is enhanced in these mutants.

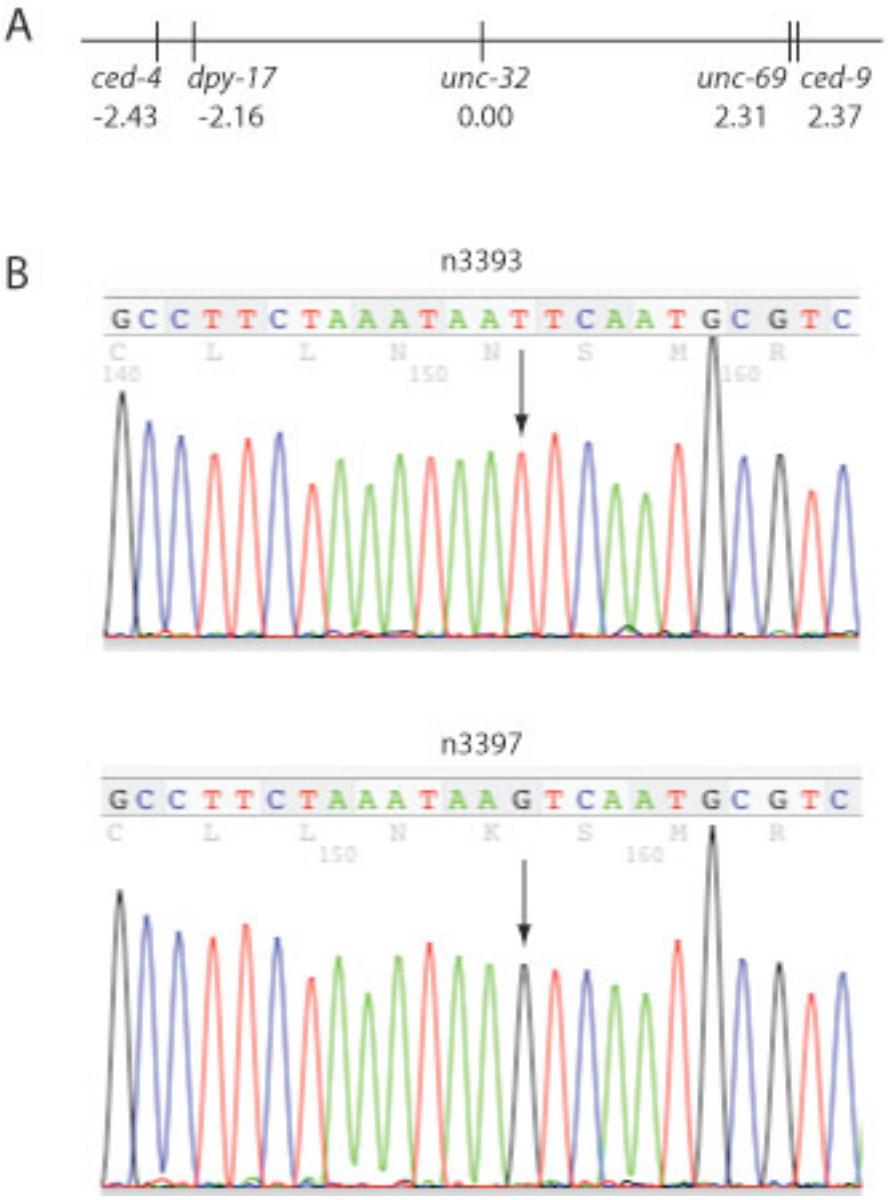


Figure 2-2. n3393 is an allele of ced-4.
A) Genetic map of the central region of LGIII. The location of each chromosome III gene mentioned in the text is shown. The number below the gene represents its distance from *unc-32* in genetic map units. B) Partial sequence of the *ced-4* gene from worms carrying n3393 or n3397 are shown. The sequence of the noncoding DNA is shown, with the G->T mutation (C->A on the coding strand) indicated by an arrow. This mutation is predicted to result in a T->N mutation at amino acid 28 of the CED-4 protein.

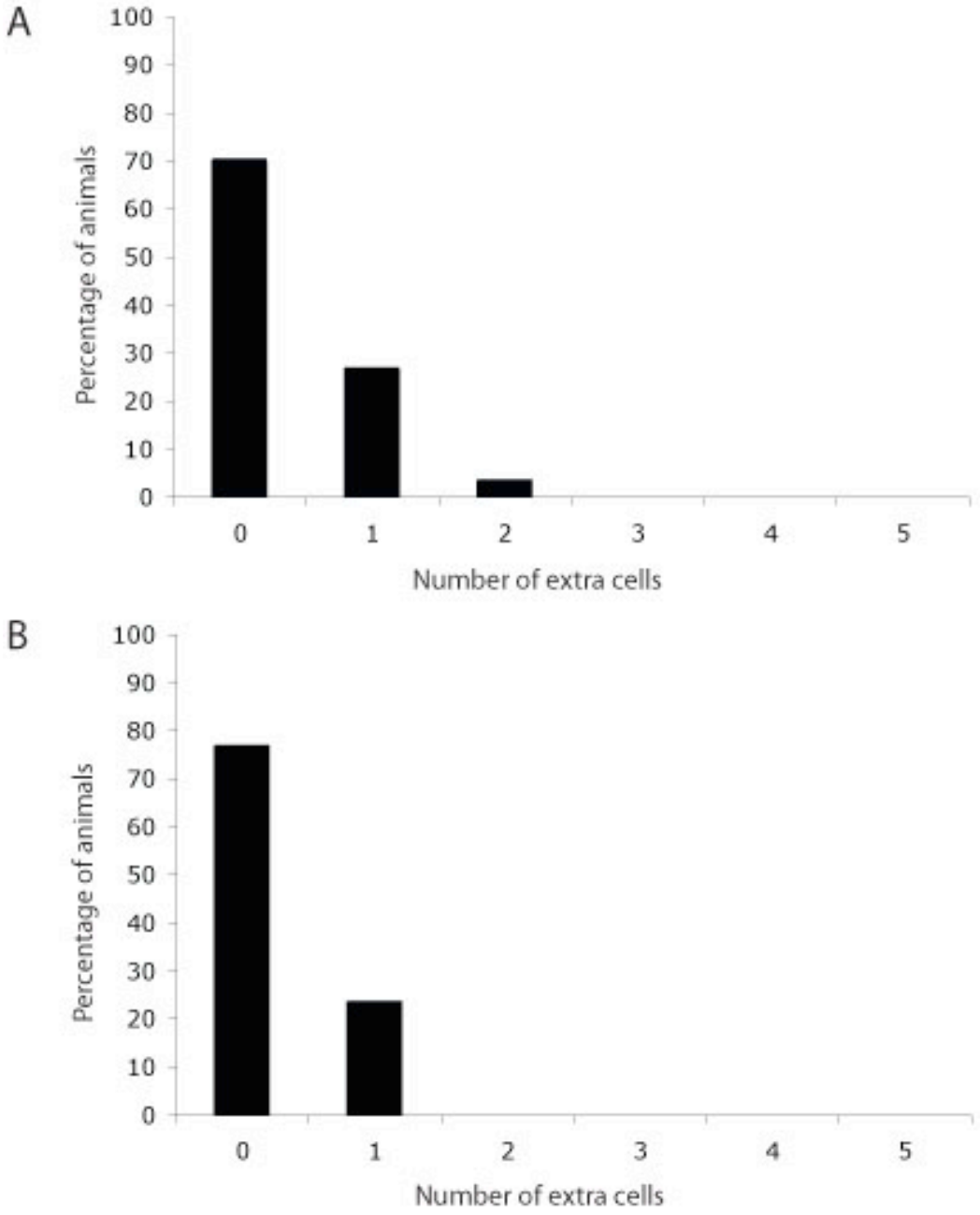


Figure 2-3. n3397 complements ced-4(n1162).
(A) Males of the genotype *ced-3(n2427);* $P_{lin\text{-}11}$*gfp; n3397* were crossed to hermaphrodites of the genotype *ced-1(e1735); unc-69(e587);* $P_{lin\text{-}11}$*gfp* and cross-progeny were scored for the number of extra GFP-positive nuclei in the ventral cord. This control

experiment demonstrates that *n3397* acts as a recessive mutation in the genetic background of this cross. (B) The same males as in A were crossed to hermaphrodites of the genotype *ced-1(e1735); ced-4(n1162) unc-69(e587);* $P_{lin\text{-}11}$*gfp* and cross-progeny were scored as in A. The lack of significant defects in PCD in these animals indicates that *n3397* complements *ced-4*.

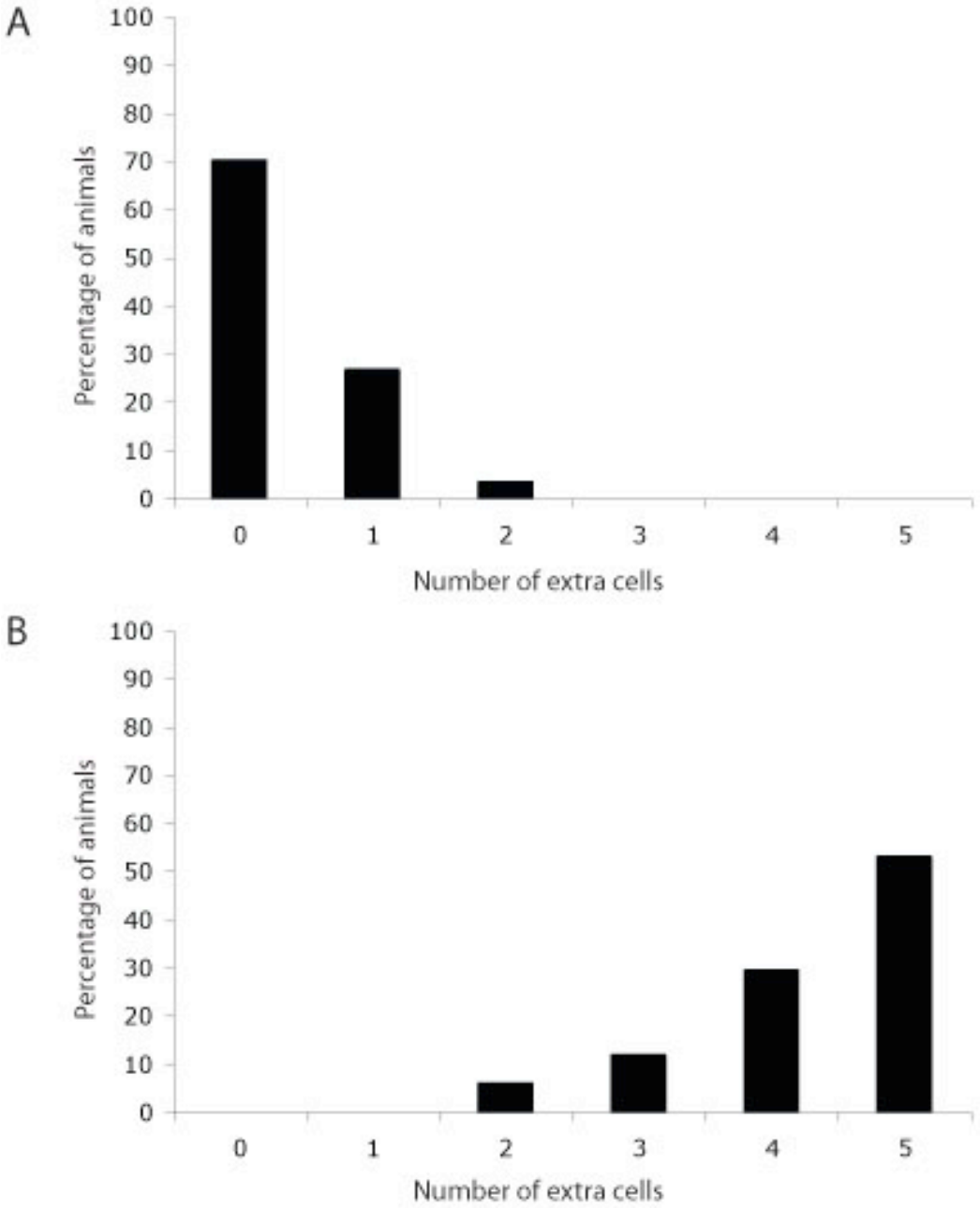


Figure 2-4. It is unclear whether n3397 is recessive or dominant.
ced-3(n2427); P*lin-11gfp; n3397* males were crossed to hermaphrodites of the genotype *ced-1(e1735); unc-69(e587);* P*lin-11gfp* (A) or *unc-32(e189) dpy-17(e164); ced-3(n2427);* P*lin-11gfp* (B) and cross-progeny were scored for number of extra GFP-positive nuclei in the ventral cord.

CHAPTER THREE: TRANSCRIPTIONAL REGULATION OF *EGL-1* AND PCD

DISSECTING THE EGL-1 GENE

Background

The most upstream gene in the PCD pathway, *egl-1*, encodes a pro-apoptotic protein with a single Bcl-2 Homology 3 (BH3) domain. This gene was first discovered in a screen for mutants with defects in egg-laying, which is why it is called *egl-1* (for egg-laying abnormal gene 1) (Trent et al., 1983). It was initially clear that *egl-1* mutants could not lay eggs because they were missing the HSN neurons, which are necessary for egg-laying. The gain-of-function *egl-1* mutations from this initial screen were later determined to block binding of the TRA-1 transcription factor to the regulatory region of *egl-1*, resulting in ectopic expression of *egl-1* in the HSN neurons of hermaphrodite animals, thus initiating PCD and killing these cells (Conradt and Horvitz, 1998; Conradt and Horvitz, 1999). In contrast, loss-of-function mutations in *egl-1* block PCD throughout the animal, demonstrating that *egl-1* is necessary for most (if not all) somatic PCD in *C. elegans*

TRA-1 normally acts to repress *egl-1* transcription in the hermaphrodite HSN neurons. A separate group of transcription factors controls *egl-1* expression in a different set of cells in the pharynx. The Snail family transcription factor CES-1 and the bHLH transcription factors HLH-2 and HLH-3 compete for a set of binding sites to repress (CES-1) or activate (HLH-2/3) *egl-1* transcription in the NSM sister cells (Ellis and Horvitz, 1991;

Metzstein et al., 1996; Thellmann et al., 2003). The HLF homolog CES-2 normally represses CES-1, allowing HLH-2/3 to activate *egl-1* transcription and kill the cells. Loss-of-function mutations in CES-2 or gain-of-function mutations in CES-1 tip the balance toward transcriptional repression of *egl-1*, and the NSM sisters survive in these mutant animals.

These TRA-1 and CES examples just described suggest the hypothesis that cell-specific transcriptional control of *egl-1* determines the pattern of PCD and cell survival in *C. elegans*. However, cell divisions produce 1090 cells in each hermaphrodite, 131 of which undergo PCD and 959 of which survive (Sulston and Horvitz, 1977a; Sulston et al., 1983b). Transcriptional control of *egl-1* has been demonstrated in only the HSN neurons and a few cells in the pharynx. Whether *egl-1* transcriptional status is the key factor determining death or survival of the more than 1000 remaining cells remains to be determined. If so, identifying the genes and mechanisms involved in transcriptional control of *egl-1* will be critical to understanding the regulation of PCD. To address these questions, I initiated an in-depth analysis of transcriptional regulation of *egl-1* and PCD in *C. elegans*.

Analyzing Evolutionary Conservation of *egl-1*

The minimal rescue fragment of *egl-1* that restores the wild-type pattern of PCD to *egl-1* mutants when introduced as a transgene is 7.66 Kb long, with most of the regulatory region lying 3' of the open reading frame (Conradt and Horvitz, 1999; Liu et al., 2006;

Thellmann et al., 2003) (Figure 3-1A). Like the majority of the *C. elegans* genome, this region is very A/T-rich. Because the *egl-1* regulatory region is so long, *in silico* searches for putative transcription factor binding sites in this region return hundreds of hits for a wide variety of transcription factors. In an attempt to narrow down the functional regions of the *egl-1* gene, I carried out an analysis of evolutionary conservation between the *egl-1* genes of *C. elegans* and two distantly related nematode species. This strategy was based on the assumption that if a particular region of DNA were critical for the proper expression pattern of *egl-1*, then evolutionary pressure would disfavor mutations in that region.

I obtained the sequence of *egl-1* from two nematode species distantly related to *C. elegans*: *Caenorhabditis briggsae* and *Caenorhabditis remanei*. These species are as distantly related as mice and humans, so DNA sequences not under selection pressure would be expected to have diverged radically. Indeed, most of the *egl-1* gene showed no detectable homology between the three species. However, by using the Cartwheel program from FamilyRelations (Brown et al., 2005), I was able to identify several regions of conservation between the *egl-1* genes of the three species (Figure 3-1A).

Interestingly, the highest degree of conservation was found in islands dispersed throughout the *egl-1* regulatory region, not in the protein-coding region of the gene. This pattern is very similar to what has been observed at the *rpr* locus in *Drosophila melanogaster* (Zhang et al., 2008). Reaper is an initiator of PCD in *D. melanogaster*, and although it is not directly homologous to EGL-1 and does not function via the same

mechanism, it does act as a transcriptionally-regulated initiator of cell death (White et al., 1996). The similar patterns of evolutionary conservation of these two genes may represent a relaxed restraint on protein sequence but a tight restraint on transcriptional regulation. This seems reasonable for a protein whose job is to kill the cell, because only those amino acids necessary for initiation of cell death (BH3 domain in EGL-1 and IAP inhibitor domain in Reaper) face selection pressure, whereas any mutation in the regulatory region that results in ectopic expression would cause excessive cell death and likely harm the organism's fitness.

Creation and Characterization of an *egl-1* transcriptional reporter

To ask whether the pattern of *egl-1* expression correlated with the PCD pattern in *C. elegans*, I built a transcriptional *egl-1* reporter and examined its expression pattern in various PCD-defective genetic backgrounds.

Cloning the $P_{egl\text{-}1}$histone:gfp *construct*

I used overlap PCR to replace the open reading frame (ORF) of *egl-1* with an AgeI restriction endonuclease recognition site within a plasmid containing the 7.66 minimal rescue fragment of *egl-1* in the Bluescript vector backbone. I then used PCR to amplify a fusion between the histone 2A gene R08C7.3 and *gfp* from the plasmid pNP5 (a gift from Jim Waddle) with AgeI sites on both ends. Finally, I digested the plasmid and PCR product with AgeI and performed a ligation to insert the *histone:gfp* fusion into the AgeI site of the plasmid. I confirmed the correct identity and orientation of the resulting

$P_{egl\text{-}1}$*histone:gfp* construct through diagnostic digests and sequencing. This plasmid, named pMP001, contains the full-length *egl-1* gene with the ORF replaced by a *histone:gfp* fusion (Figure 3-1B). It is therefore expected to drive H2A:GFP expression in a pattern identical to the endogenous transcriptional expression pattern of *egl-1*, at least in those cells where the PCD decision is controlled at the level of *egl-1* transcription.

Creating the $P_{egl\text{-}}$gfp *reporter strain*

I injected pMP001 at 15 ng/ul into *ced-1(e1735); ced-3(n717); lin-15(n765)* animals along with wild-type *lin-15* (50 ng/ul) as a coinjection marker. I included the *ced-3(n717)* mutation to block all PCD and allow observation of the transgene's expression pattern, because otherwise I expected any cells expressing $P_{egl\text{-}1}$*histone:gfp* to also express endogenous *egl-1* and die. I included the *ced-1(e1735)* mutation, which causes cells that die to persist as visible corpses rather than being engulfed by neighboring cells, to facilitate characterization of hits that might be obtained from future mutagenesis screens using this strain.

Creating useful transgenic lines by this method was inefficient for two reasons. First, most non-Muv (transgenic) F1 progeny from the injected animals failed to produce non-Muv progeny, and so did not represent true transgenic lines. Second, of the few lines that were produced, most exhibited GFP expression that was too dim to allow analysis of its expression pattern. I also used bombardment to produce transgenic lines, but all the lines produced by that method were too dim to analyze. Nevertheless, several lines with

relatively bright GFP expression in an identical pattern (described below) were obtained by injection, and the brightest of these was selected for integration.

To integrate the P_{egl-1}*histone:gfp* transgene, I irradiated L4 hermaphrodites carrying the transgene with 4000 rad of gamma irradiation. I then isolated 2000 non-Muv F2 progeny and kept only those animals that produced 100% non-Muv progeny. I chose a healthy line expressing GFP brightly in the same pattern as before integration, in which the integration mapped to the X chromosome, to characterize further. The P_{egl-1}*histone:gfp* transgene integrated on X was named *mxIs14*. I back-crossed this strain to wild-type N2 worms six times to remove any unwanted mutations introduced by irradiation.

egl-1 expression pattern resembles the wild-type PCD pattern

I next reintroduced the *ced-1(e1735)* and *ced-3(n717)* mutations into the *mxIs14* strain just described in order to examine the pattern of *egl-1* expression at single-cell resolution. PCD is blocked completely by the *ced-3* mutation, so all cells survive in this strain. GFP was first expressed in the comma stage embryos and continued to be expressed in distinct nuclei throughout development into the adult stage (Figure 3-2A). Expression grossly matched the pattern of PCD in wild-type animals. At the L3 larval stage, GFP expression was restricted to distinct nuclei in the head, tail, ventral nerve cord, and postderids as well as the HSN neurons (Figure 3-2B). Many neurons in the head and tail undergo programmed cell death in wild-type animals, so the presence of many GFP-positive cells in these regions was expected. Since identification of individual cells is so difficult in these regions, I did not examine them more closely. Neurons in the postderids also

undergo PCD in wild-type animals, so the presence of GFP-positive nuclei there was expected. However, the HSN neurons do not undergo PCD in wild-type hermaphrodites, so expression of the *egl-1* reporter observed in these two cells was unexpected. One possible explanation for this was that the presence of multiple copies of the *egl-1* regulatory region in the integrated reporter might provide too much competition for the transcription factor TRA-1, which is necessary in hermaphrodite HSNs to repress *egl-1* and prevent PCD. However, examination of the *mxIs14* PCD-competent worms revealed the presence and correct function of HSN neurons, arguing against this possibility. I think the likely explanation is that during rearrangement into the extrachromosomal array, some copies of $P_{egl\text{-}1}$*histone:gfp* were disrupted in such a way that the TRA-1 repressor binding site (which is located several kb away from the coding region, see Figure 3-1A) became disconnected from the coding region, thus allowing some transcription to occur despite the presence of TRA-1 protein in the nuclei. Regardless, the GFP expression in the HSNs and postderids was much dimmer than in other regions of the animal, so I did not examine it further.

The ventral nerve cord was amenable to evaluation of *egl-1* expression at the single-cell resolution because of its anatomy (nuclei arranged in a long, single row without significant overlap) and development (post-embryonic lineage that can be observed to determine each cell's identity). In addition, GFP expression was bright enough in this tissue to be examined carefully and photographed. During the L1 and L2 larval stages in wild-type hermaphrodites, the postembryonic ventral nerve cord lineages give rise to 78 cells, ten of which undergo PCD (Figure 2-1A). Three deaths occur in the anterior region

of the ventral cord, none in the midbody, and seven in the posterior. To determine how accurately the P_{egl-1}*histone:gfp* reporter reproduced this pattern with GFP expression, I counted the number of GFP-positive nuclei produced by each of the P2-P12 blast cells in 45 *ced-1(e1735); ced-3(n717); mxIs14* animals at the L3 larval stage (Figure 3-2B). I observed no GFP-positive nuclei in the midbody region (P3-P8), consistent with the survival of all cells produced by those lineages in wild-type animals. The anterior ventral nerve cord was not very consistent, perhaps because of embryonic deaths that occur in the same region, but in the posterior nerve cord the pattern of GFP expression consistently matched the PCD pattern of wild-type animals (Figure 3-2C). This indicates that at least in the midbody and posterior regions of the ventral nerve cord, *egl-1* transcription correlates exceptionally well with PCD (Figure 3-2).

The egl-1 *reporter responds appropriately to various genetic backgrounds*

There are four genes that have been identified as necessary to establish the correct pattern of PCD in the ventral nerve cord. In *pag-3* mutants, extra deaths occur in the anterior and posterior cord due to a lineage defect (Cameron et al., 2002). As expected, introduction of the *pag-3(n3098)* mutation into the *egl-1* reporter strain produced extra GFP-positive cells in the anterior and posterior ventral cord (data not shown). In *lin-39* mutants, the six VC neurons (P3-8.aap) in the midbody region of the ventral nerve cord undergo PCD rather than surviving as they do in wild-type animals (Clark et al., 1993). As expected, introduction of the *lin-39(n1760)* mutation into the *egl-1* reporter strain produced GFP-positive cells in the midbody region of the ventral nerve cord (Table 3-1). In *mab-5* mutants, two cells in the posterior region of the ventral nerve cord (P11.aaap and

P12.aaap) fail to undergo PCD (Kenyon, 1986). As expected, introduction of the *mab-5(n1384)* mutation into the *egl-1* reporter strain reduced the number of GFP positive cells produced by P11 and P12 by one each (Table 3-1). Finally, in *ceh-20* mutants, the VC neurons undergo PCD while P11.aaap and P12.aaap fail to die, which is a combination of the phenotypes seen in *lin-39* and *mab-5* mutants (Liu et al., 2006). As expected, introduction of the *ceh-20(ay9)* or *ceh-20(ay42)* mutation into the *egl-1* reporter strain increased the number of GFP positive cells in the midbody region and decreased the number of cells in the posterior region of the ventral nerve cord (Table 3-1). Expression of the *egl-1* reporter in the P11.aaap cell, specifically, required both *mab-5* and *ceh-20* function, as does PCD of P11.aaap (Figure 3-3). Taken together, these results show that *egl-1* transcription and PCD correlate perfectly in the midbody and posterior regions of the ventral nerve cord. This supports the hypothesis that *egl-1* transcription may be the major factor that determines whether a given cell lives or undergoes PCD within these tissues.

I also introduced the *him-5(e1490)* mutation into the *egl-1* reporter strain to examine the *egl-1* pattern in males. Normally *C. elegans* hermaphrodites produce only hermaphrodite progeny. However, certain mutations such as *him-5(e1490)* increase the frequency of male progeny from hermaphrodite self-fertilization. The *mxIs14* allele alone causes a low frequency of male progeny to be produced, but the introduction of the *him-5(e1490)* mutation increased this frequency dramatically, allowing observation of many male animals. The ventral nerve cord is one of many tissues in *C. elegans* that undergoes sexually dimorphic development, and as a result, the pattern of PCD in the male ventral

nerve cord is distinct from the pattern in hermaphrodites. As expected, the pattern of *egl-1* expression was also distinct in males. Unfortunately, the GFP signal overall in males was much dimmer than in hermaphrodites, perhaps because the reporter is integrated on the X chromosome. Because of this dim expression, the pattern in males could not be examined critically using mxIs14. I would expect that strains with the *egl-1* reporter integrated on other chromosomes might be brighter and thus more readily characterized, but I did not pursue this line of research.

The egl-1 transcriptional reporter is expressed in some cells that survive

Surprisingly, *mxIs14* worms showed GFP expression in several cells in the head and tail, despite the fact that PCD proceeded normally in these animals (Figure 3-4A). This result seems to contradict the hypothesis that *egl-1* transcription acts as the switch to determine whether somatic cells of *C. elegans* survive or initiate PCD. Alternatively, the reporter used here could lack some critical regulatory sequence (for example that lies outside the 7.66 kb fragment used, or in the small intron that was deleted when the ORF was replaced by the *histone:gfp* fusion). If the reporter inaccurately reports endogenous *egl-1* transcription, these GFP positive cells may not actually express endogenous *egl-1*. Another possibility worth considering is that endogenous *egl-1* may be transcribed in these cells, but post-transcriptional mechanisms may exist that prevent accumulation of active EGL-1 protein. A third possibility is that EGL-1 is expressed in these cells, but death does not occur because of some block in the PCD pathway downstream of EGL-1.

To further investigate these possibilities, I wished to determine whether any cells express EGL-1 protein but fail to undergo PCD in wild-type animals. In the absence of a good EGL-1 antibody, I designed, constructed (with the help of Matthew Jamison) and injected *egl-1* translational reporters that utilized the same 7.66 kb regulatory region described above to drive expression of *egl-1* cDNA fused to *gfp* to produce both C-terminal and N-terminal EGL-1:GFP fusion proteins. I reasoned that if GFP could be observed in some cells of PCD-competent worms expressing this transgene, this would provide strong evidence that some cells can survive even in the presence of EGL-1. I never observed GFP-positive cells in worms expressing these transgenes, regardless of whether the genetic background was competent or incompetent for PCD (data not shown). Because the transgene did not express at a detectable level even when PCD was blocked, no satisfactory conclusion could be drawn from these experiments. My guess as to why GFP was never observed is that the transgene was never expressed at levels high enough to detect GFP fluorescence. Perhaps overexpression of EGL-1 is toxic or detrimental to cells even when PCD is blocked downstream by mutations in the caspase *ced-3*.

In conclusion, $P_{egl\text{-}1}$*histone:gfp* is expressed in cells that survive, but whether functional EGL-1 protein is present in these cells remains unknown. Recent studies from the Chamberlin and Shaham laboratories suggest that transcriptional regulation of *ced-9* and *ced-3*, components of the PCD pathway that act downstream of *egl-1*, can regulate PCD in *C. elegans* (Maurer et al., 2007; Park et al., 2006). In light of this work, it seems likely that endogenous EGL-1 could be expressed in some cells that survive because they lack

CED-3 (or perhaps CED-4), indicating that transcriptional regulation of *egl-1* is not the only switch used to determine whether somatic cells live or die.

Dying cells express the egl-1 *transcriptional reporter*

To determine whether *egl*-1 was transcribed in cells that undergo PCD, I crossed the mxIs14 *egl-1* reporter into *ced-1(e1735)* mutants, in which many cells that undergo PCD persist as visible corpses because engulfment is defective. In this background, GFP expression was obvious in most corpses, indicating that *egl-1* is generally transcribed in dying cells, as expected (Figure 3-4B). Some healthy-looking nuclei also expressed GFP in the *ced-1(e1735); mxIs14* animals. This is probably due to the fact that *ced-1* is partially required for PCD downstream of *egl-1* (Reddien et al., 2001), in addition to the fact that we observed GFP expression in several cells even in otherwise wild-type worms carrying *mxIs14* (Figure 3-4A).

MAB-5 and CEH-20 directly activate *egl-1* transcription in P11.aaap

mab-5 encodes a Hox transcription factor, and *ceh-20* encodes a Pbx-class Hox cofactor. The fact that both are required for *egl-1* expression in the P11.aaap cell (Figure 3-3) raises the possibility that they may encode direct transcriptional activators of *egl-1*. Work from Kelly Liu, Tamara Strauss, Scott Cameron, and I demonstrated that this is indeed the case (Liu et al., 2006), and I will present my contributions to this work here.

Search for functional units in egl-1 *regulatory sequence*

Because the *egl-1* regulatory sequence is 7.66 kb long (Figure 3-1), I sought to find smaller functional units by examining a deletion series in the context of the P_{egl-1}*gfp* reporter. I designed six deletion constructs in which a series of ~1 kb fragments of the noncoding regions of *egl-1* were deleted, with entire conserved regions deleted in each construct (Figure 3-5). With help from Matthew Jamison, Jacquie Chen, Jenny Buseman, and Carmen Hernandez, I cloned these constructs by overlap PCR and injected each into *ced-1(e1735)*; *ced-3(n717)*; *lin-15(n765)* animals at 10 ng/µl with wild-type *lin-15* at 50 ng/µl as a co-injection marker. I then examined the GFP pattern of the resulting transgenic lines.

Two constructs, Deletion 3 and Deletion 4, appeared to be expressed in the same pattern as wild-type P_{egl-1}*histone:gfp* (Figure 3-5). This implies that the regions deleted in these constructs do not contain important functional elements that dictate the pattern of *egl-1* expression in the ventral nerve cord, or if they do, that they are redundant with elements in other regions of the regulatory sequence. However, the sequence deleted in Deletion 3 is known to contain an important, non-redundant element required for binding of CES-1 and HLH-2/3 in the NSM sister cells (Thellmann et al., 2003). Deletion 3 is probably not regulated correctly in the NSM sisters, but I didn't examine this. The only tissue I confidently analyzed with single-cell resolution was the ventral nerve cord.

Two additional constructs, Deletion 5 and Deletion 6, exhibited GFP expression that was extremely dim (Figure 3-5). It was not possible to determine the pattern of expression of

these constructs because the GFP was too faint. This implies that these deletions, which overlap, removed a functional element necessary for full expression levels of *egl-1* throughout the animal.

Deletion 2 showed normal levels and patterns of GFP expression throughout most of the animal, but was missing several GFP-positive nuclei in the ventral nerve cord (Figure 3-5). Specifically, only two P12 descendents expressed GFP, whereas three do with the wild-type reporter; only one P11 descendent expressed GFP, whereas two do with the wild-type reporter; and no other cells in the ventral nerve cord were GFP positive, whereas P2.aap, P9.aap, and P10.aap do express GFP with the wild-type reporter. This suggests that the region deleted in Deletion 2 contains an element that is required for *egl-1* expression in several cells throughout the ventral nerve cord. The simplest hypothesis as to the identity of these cells would be the Pn.aap cells, because lack of GFP expression in a single position of the P2-P12 lineages would give the same pattern as was observed with this construct. This hypothesis is supported by independent evidence from Bob Horvitz's lab, which will be described later in this chapter.

Deletion 1 also exhibited normal GFP expression throughout the animal, but with a specific defect in the ventral nerve cord (Figure 3-5). This construct showed only two GFP-positive nuclei from the P12 lineage and only one GFP-positive nucleus from the P11 lineage. This implies that some element in the region deleted in this construct is necessary for *egl-1* expression in one cell from the P11 lineage and one cell from the P12 lineage. Intriguingly, this is the same pattern observed for the wild-type *egl-1* reporter in

a *mab-5* mutant background (Table 3-1), suggesting that *mab-5* could bind an element in the region missing from Deletion 1 to directly activate *egl-1* transcription.

In addition to asking what smaller regions of *egl-1* are necessary for certain elements of its expression pattern by creating a series of deletion constructs, I also wished to ask what regions are sufficient on their own to convey certain elements of the expression pattern. To this end, I designed constructs in which each of the six regions deleted above was inserted upstream of a minimal promoter driving GFP, to test whether any were sufficient to drive GFP expression in particular cells. I also wished to place these regions upstream of the *pag-3* promoter, which is expressed in neurons of the ventral nerve cord (Cameron et al., 2002), to determine whether any regions were sufficient to repress expression in the VC neurons. These experiments have not yet been completed. Thus it is not currently known whether any regions of *egl-1* are sufficient, when isolated from the rest of the gene, to activate or repress expression in particular cells.

Analysis of Pbx-Hox consensus binding sites in egl-1

The Hox cofactor *ceh-20* is required for the correct pattern of *egl-1* expression and PCD in the ventral nerve cord. Specifically, *ceh-20* is required for *egl-1* repression and survival of the VC neurons, and for *egl-1* activation and death of P11.aaap and P12.aaap (Liu et al., 2006) (Table 3-1). *ceh-20* cooperates with the Hox gene *lin-39* in the VC neurons and with the Hox gene *mab-5* in P11.aaap and P12.aaap. To ask whether a complex composed of CEH-20 and a Hox transcription factor might bind *egl-1* directly to activate or repress transcription, Scott Cameron and I began by searching the *egl-1*

regulatory region for potential Pbx-Hox consensus binding sites. There are 116 sites that match the consensus TGATNNAT exactly or with one mismatch. Of these, only 12 are conserved between *C. elegans*, *C. briggsae*, and *C. remaneii*. I mutated these twelve sites in various combinations and examined the effects of these mutations on the P_{egl-1}*histone:gfp* expression pattern. To mutate each site, I used site-directed mutagenesis to convert the consensus Pbx-Hox site to an NcoI recognition site. Mutation of sites 2-12 had no effect on the expression pattern of the *egl-1* reporter (Table 3-2). However, mutation of site 1, whether alone or with other sites mutated, resulted in loss of *egl-1* expression in one cell from P11 and one from P12 (Table 3-2, Figure 3-6). This demonstrates that Pbx-Hox consensus site 1 in the regulatory region of *egl-1* is required for *egl-1* transcription in P11.aaap and one cell from the P12 lineage and suggests that MAB-5 and CEH-20 may kill these cells by binding directly to site 1 and activating *egl-1* transcription. In support of this hypothesis, electrophoretic mobility shift assays (EMSA) performed by Kelly Liu demonstrated that a complex composed of MAB-5 and CEH-20 could bind to wild-type but not mutant Pbx-Hox consensus site 1 *in vitro* (Liu et al., 2006).

CEH-20 and MAB-5 are expressed in P11.aaap

MAB-5 is expressed in many cells in the posterior body region of *C. elegans*. In the ventral nerve cord, MAB-5 is expressed in the P7-P12 lineages and is present in P11.aaap, but not in P12.aaap (Salser et al., 1993). Because MAB-5 is not expressed in P12.aaap, a complex consisting of MAB-5 and CEH-20 cannot directly activate *egl-1* transcription through Site 1 to kill that cell. Indeed, direct observation of the P11 and

P12 lineages performed by Scott Cameron showed that P12.aaap dies normally in animals where Site 1 has been mutated, whereas P11.aaap fails to die in these animals (Liu et al., 2006). Surprisingly, the missing death in the P12 lineage of Site 1 mutant animals comes from the failure of P12.pp to die. This death does not depend on *mab-5* or *ceh-20* genetically, but does depend on Pbx-Hox consensus site 1 in the *egl-1* regulatory region. Thus some unknown factor or factors act through Site 1 to activate *egl-1* transcription and initiate PCD in P12.pp. Furthermore, *mab-5* and *ceh-20* regulate *egl-1* transcription and cell death in P12.aaap, but they do so indirectly and not through binding Site 1 in this cell. Because MAB-5 is present in the P12 blast cell but not its descendents, MAB-5 and CEH-20 may act early in this lineage to specify the correct fate, with one consequence of this activity being the eventual death of P12.aaap.

In contrast, MAB-5 is present in P11.aaap, and the death of this cell requires Site 1 in *egl-1*, *mab-5*, and *ceh-20*. These results are consistent with the hypothesis that a complex composed of MAB-5 and CEH-20 directly activates *egl-1* transcription by binding Site 1 in P11.aaap. If this is the case, then CEH-20 should also be expressed in P11.aaap. To test this, I created a translational fusion of CEH-20 to CFP, determined whether it rescued *ceh-20* mutants, and examined its expression pattern. To construct the pMP0017 $P_{ceh\text{-}20}$*ceh-20:cfp* translation fusion plasmid, I amplified a 3.6 kb SacI PflMI fragment of the *ceh-20* genomic region, including ~1 kb of 5' sequence and 1 kb of 3' sequence, and cloned it into BlueScript. I then used site-directed mutagenesis to change the stop codon of *ceh-20* to an AscI restriction site and cloned the *cfp*-coding region from pPD134.96 into that site. pPD134.96 was a kind gift of Andy Fire, Stanford University, Palo Alto,

CA. I then injected pMP0017 at a concentration of 50 ng/µl into *ceh-20(ay42)* animals, where it rescued the egg-laying defect. This indicates that the *ceh-20* reporter contains the necessary regulatory regions to be expressed and function correctly. Finally, I injected pMP0017 (50 ng/µl) into *ced-3(n717); lin-15(n765)* mutants along with wild-type *lin-15* (50 ng/µl) as a coinjection marker, obtained transgenic lines, and observed the CFP expression pattern. CFP was expressed in many cells throughout the animal, including in P11.aaap (Figure 3-7A,B). This indicates that CEH-20, like MAB-5, is present in P11.aaap and could directly activate *egl-1* transcription through Site 1 to initiate PCD.

CEH-20 is probably not required for mab-5 *expression*

One alternative explanation for the data presented thus far is that MAB-5 binds site 1 and activates *egl-1* expression alone, or with the Pbx cofactor CEH-40. In this scenario, P11.aaap may fail to die in *ceh-20* mutants because *ceh-20* is required for expression of *mab-5*. This is not unreasonable, because Hox transcription factors and cofactors often participate in self-regulation or regulation of other Hox genes. This alternative hypothesis is not likely, however, because MAB-5 and CEH-20 only bind Site 1 efficiently as a heterodimer, and *ceh-40* mutations do not alter the pattern of PCD in the ventral nerve cord in wild-type animals or sensitized animals carrying a weak allele of *ceh-20* (Liu et al., 2006). Nevertheless, I examined whether *ceh-20* is required for *mab-5* expression using the $P_{mab\text{-}5}$*gfp* reporter *muIs16* (Cowing and Kenyon, 1996). Of the 30 otherwise wild-type *ced-3* mutants carrying the $P_{mab\text{-}5}$*gfp* reporter, 28 and 26 animals expressed *gfp* in P11.aaaa and P11.aaap, respectively (Figure 3-7 C,D). Of the 60

ceh-20(ay42) mutants carrying the P_{mab-5}*gfp* reporter, 51 and 50 mutants expressed *gfp* in P11.aaaa and P11.aaap, respectively (Figure 3-7 E,F). With the caveat that *ceh-20(ay42)* is a strong loss-of-function mutation but not null, and assuming that the P_{mab-5}*gfp* reporter accurately represents endogenous *mab-5* expression, these data suggest that *ceh-20* is not likely to be required for *mab-5* expression in P11.aaap. Taken together with the results described above and the other data presented in our paper (Liu et al., 2006), this demonstrates that MAB-5 and CEH-20 bind site 1 as a heterodimer and directly activate *egl-1* transcription to kill P11.aaap.

Conclusions

These studies expanded the number of cell-specific pathways known to regulate *egl-1* transcription and PCD to three. The MAB-5/CEH-20/Site1 pathway is even more specific than the TRA-1 and CES pathways, as it affects only a single cell. Furthermore, these results demonstrated an unexpected level of complexity in cell-specific regulation of PCD; two lineal equivalents regulate *egl-1* differently, which was never suspected in mammalian cells, and one *egl-1* site is bound by two different complexes in two different cells to activate transcription. The emerging picture seems to be that each cell's decision of whether to undergo PCD or survive is controlled by exquisitely specific transcriptional mechanisms that can involve regulation of *egl-1* or other genes of the core PCD pathway. If this is true, then many regulators of PCD remain to be discovered to explain all the deaths and survivals that occur in *C. elegans*.

More importantly, this finding demonstrates that Hox proteins and cofactors can directly regulate the transcription of BH3-only genes to determine whether cells survive or die. Although MAB-5 and CEH-20 directly activate *egl-1* transcription to kill a cell, this raises the possibility that LIN-39 and CEH-20 may directly repress *egl-1* in the VC neurons to promote cell survival. This is an exciting possibility, because if Hox-mediated repression of BH3-only genes and cell death also occurs in mammals, it could offer one explanation as to why Hox overexpression is oncogenic (see Chapters 1 and 4).

RNAI SCREEN FOR NOVEL REGULTORS OF EGL-1

Introduction

Creation of the P_{egl-1}*histone:gfp* reporter and analysis of the *egl-1* regulatory region provided novel information about how *egl-1* transcription and PCD are regulated. This strategy led to the discovery that MAB-5 and CEH-20 activate *egl-1* transcription directly through Site 1 to kill P11.aaap, demonstrating that Hox complexes can directly regulate transcription of BH3-only genes and influence the life vs. death decision in a cell. In addition, this research indicated that particular elements in the *egl-1* regulatory sequence control specific aspects of the *egl-1* expression pattern, although it did not lead to the identification of the factors that act through those elements to control *egl-1* transcription. For example, Site 1 is also required for death of P12.pp, but neither MAB-5 nor CEH-20 is involved in the death of that cell, and the factors that are involved remain unknown. In addition, an element deleted in the Deletion 2 construct appears to be required for *egl-1* expression in the Pn.aap cells, but the identity of the factor or factors involved in this

regulation also remains unknown. Furthermore, many aspects of the *egl-1* expression pattern remain completely mysterious. Certain factors could be required for *egl-1* repression or activation in specific cells even though previous analyses failed to uncover a particular region of *egl-1* necessary for that aspect of its regulation. For example, if a factor binds multiple, dispersed, redundant sites in the *egl-1* regulatory region to control expression in certain cells, that regulation would not have been uncovered by the deletion analysis described above. At this point, we can still only partially explain *egl-1* regulation in just a handful of the 1090 somatic cells in the worm.

To discover novel regulators of *egl-1* expression and PCD in *C. elegans*, I carried out an RNAi-based screen using the P_{egl-1}*histone:gfp* reporter. Although many screens have been performed in the past to look for regulators of PCD in the worm, my strategy was new in several aspects and therefore offered the opportunity to find novel PCD regulators. The most obvious difference between my strategy and those used in the past was that I could screen for alterations of the *egl-1* expression pattern in a PCD-defective background, rather than screening for alterations in corpse pattern or survival of particular cells in a PCD-competent background. This restricted my hits to genes that act upstream of *egl-1* and provided the opportunity to discover genes whose normal function is to prevent death of critical cells, which had previously been extremely difficult because loss of function of such genes would be lethal. Additionally, screening with the P_{egl-1}*histone:gfp* reporter allowed a greater combination of precision and scope than had previously been possible, because I could (in principle) examine with single-cell precision every cell in the worm. In reality, this was limited by my ability to distinguish

single cells in the various tissues and by the fact that the *egl-1* reporter did not mimic the wild-type pattern of PCD in all tissues. Because I was most familiar with the ventral nerve cord and because reporter accuracy was high in this tissue (Figure 3-2), it was the only tissue I confidently examined at single-cell resolution. Additional reasons for choosing the ventral nerve cord as a model tissue for studying regulation of PCD stem from the fact that it is composed of repeating lineages, which should increase sensitivity for partial loss-of-function or redundant genes as well as facilitate discovery of genes involved in regional specification and sex specificity. Finally, by using RNAi rather than mutagenesis to inactivate genes, I hoped to be able to identify essential genes more readily because RNAi partially and variably inactivates genes. This meant that some "escapers" exhibiting partial knock-down could be analyzed, even when RNAi of a particular gene was lethal overall.

Preparations for the screen

Creating and testing a RNAi-hypersensitive egl-1 *reporter strain*

One potential drawback to using RNAi to analyze the *egl-1* expression pattern is that RNAi does not work as well in neurons as in other cell types in *C. elegnans* (Simmer et al., 2002), and most cells that express *egl-1* are neurons. To overcome this problem, I introduced the *rrf-3(pk1426)* mutation into the P_{egl-1}*histone:gfp* reporter strain. This mutation enhances the effectiveness of RNAi in neurons (Simmer et al., 2002). Tracy Chow and Tugba Guven optimized the RNAi feeding protocol using this strain. I then used their optimized protocol to compare the RNAi efficiency in *ced-1(e1735);*

ced-3(n717); mxIs14 animals versus *rrf-3(pk1426)*; *ced-3(n717); mxIs14* animals. I fed L4-stage hermaphrodites from each strain RNAi to *lin-39* and *ceh-20* and scored GFP-positive nuclei in the midbody and posterior ventral nerve cord in 50 F1 progeny at the L3 stage (Table 3-3). The resulting GFP pattern after *lin-39(RNAi)* was equivalent between the two strains, but *ceh-20(RNAi)* worked more efficiently in the *rrf-3* strain, as evidenced by a greater number of GFP-positive cells in P3-P8 descendents and a smaller number of GFP-positive cells in P11 and P12 descendents (Table 3-3). Thus *rrf-3(pk1426)* enhances the efficiency of some RNAi clones but not others.

While performing the prior experiment, I noticed that *rrf-3* animals fed RNAi to *ceh-20* produced fewer surviving progeny than the control animals fed *ceh-20(RNAi)*. To quantitate this effect, I treated at least 10 L4-stage hermaphrodites from each strain with RNAi toward three genes that are known to be essential for viability based on characterization of null alleles. I then counted the number of viable progeny produced by each worm and plotted the average number of progeny per animal (Figure 3-8). I also included the strain *ced-1(e1735)* as a control. Lethality induced by RNAi was very similar between the reporter strain *ced-1(e1735); ced-3(n717); mxIs14* and the control strain *ced-1(e1735)*. However, lethality was markedly increased in *rrf-3(pk1426); ced-3(n717); mxIs14* compared to the other two strains. Because the RNAi-hypersensitive reporter strain showed greater sensitivity to some RNAi clones but increased lethality dramatically, I decided to carry out the screen using both the standard *egl-1* reporter strain and the hypersensitive *egl-1* reporter strain in parallel.

Choosing candidate genes

To examine the GFP pattern of the *egl-1* reporter animals, it is necessary to mount the animals in agar and sodium azide on a microscope slide and observe them using high-magnification fluorescent microscopy. Because of the time-consuming nature of this mounting procedure, I decided to use a candidate approach to screen a subset of hand-picked genes rather than performing a genome-wide RNAi screen. I chose three groups of genes to test based on informed conjecture that they might contain novel regulators of PCD: 1) essential transcription factors, 2) HDACs and corepressors, and 3) potential homologs of NFAT and CRZ1P. Appendix A lists all candidate genes chosen for the RNAi-based screen.

Essential transcription factors. As described above, one key advantage to using RNAi in a PCD-incompetent *egl-1* reporter strain to screen for regulators of PCD is that genes essential for viability or fertility should be much easier to identify by this method. Genes with sterile or lethal mutant phenotypes that regulate PCD could easily have been missed in previous screens but could potentially be detected in this screen. To take full advantage of this fact, I decided to screen genes whose published RNAi phenotypes included any degree of sterility, lethality, or growth arrest (Fraser et al., 2000; Kamath et al., 2003; Simmer et al., 2003). Since I was specifically looking for regulators of *egl-* transcription, I further restricted the candidates to transcription factors, which resulted in 61 candidate genes in this category (Appendix A).

HDACs and co-repressors. Because *egl-1* is expressed in the Pn.aap cells of the anterior and posterior regions of the ventral nerve cord, but not in the midbody Pn.aap cells that become VC neurons, I was particularly interested in mechanisms required for cell-specific repression of *egl-1*. Thus Scott Cameron and I compiled a list of histone deacetylase enzymes (HDACs) and worm homologs of other known co-repressors. This added 33 candidate genes to the list (Appendix A).

Potential homologs of NFAT and CRZ1P. The third category of candidates was born out of the discovery that the *egl-1* Deletion 2 construct is not expressed in the ventral nerve cord except in one descendent of P11 and one descendent of P12 (described above). This suggests that an element deleted in this construct is required for activation of *egl-1* transcription in the Pn.aap cells. This element was defined further by two *egl-1* alleles identified by Bob Horvitz's lab. Both alleles, *egl-1(n4045)* and *egl-1(n4629)*, exhibit a single C->T mutation in the conserved region of *egl-1* that is missing from Deletion 2. In addition, both alleles exhibit defects in Pn.aap cell death as quantitated using the $P_{lin\text{-}11}$*gfp* reporter described in Chapter 2. Consequently, these alleles identify a single nucleotide in an evolutionarily conserved region of the *egl-1* gene that is required for expression in the Pn.aap cells. In an attempt to find the transcription factor that binds this site to activate *egl-1* transcription in the Pn.aap cells, the Horvitz lab carried out yeast-one-hybrid experiments using multimerized wild-type or mutant sites as baits. Clones corresponding to two uncharacterized *C. elegans* genes interacted strongly with the wild-type, but not mutant constructs: B0238.11 and Y38C9A.1. RNAi and deletion alleles of B0238.11 produced larval arrest, with no escapers exhibiting defects in Pn.aap

death, suggesting that this gene is probably not involved in activating *egl-1* transcription. RNAi and deletion alleles of Y38C9A.1 exhibited defects in Pn.aap death and some masculanization. If the PCD defects observed with inactivation of Y38C9A.1 are not simply a result of masculanization, then this gene could potentially contribute to *egl-1* activation in the Pn.aap cells. However, Y38C9A.1 was completely uncharacterized and exhibited no obvious homology to any known genes, nor did it appear to contain any DNA-binding domains. To attempt to learn more about Y38C9A.1, I used iterative PSI-Blast to find distantly related homologous proteins. This search indicated clear homology between Y38C9A.1 and the calcineurin inhibitor CABIN1/CAIN. Based on this result, I suggested the possibility that Y38C9A.1 may have been a positive hit from the yeast one-hybrid screen not because it bound DNA directly, but because it regulated endogenous calcineurin and altered the transcriptional characteristics of the calcineurin target NFAT. According to this hypothesis, Y38C9A.1 does not bind *egl-1* to activate transcription, but instead is somehow important for the worm ortholog of NFAT to regulate *egl-1* transcription in the Pn.aap cells. In support of this somewhat fanciful hypothesis, the sequence immediately upstream of the mutated nucleotide in *egl-1(n4045)* and *egl-1(n4629)*, GGAAC, is similar to the NFAT consensus binding site, GGAAA. However, no known NFAT ortholog exists in *C. elegans* or in yeast. Yeast do have an unrelated transcription factor, CRZ1P, that responds to calcium signaling downstream of calcineurin in a manner similar to NFAT. The sequence surrounding the *egl-1(n4045)* and *egl-1(n4629)* mutation site also resembles a published CRZ1P binding site (Figure 3-9) (Stathopoulos and Cyert, 1997). To search for the transcriptional activator of *egl-1* in the Pn.aap cells that binds the region surrounding the *egl-1(n4045)*

and *egl-1(n4629)* mutation, I used Blast to find possible worm homologs of NFAT and CRZ1P to add to the list of candidate genes to screen. This added 37 genes to the list (Appendix A). An additional 30 genes were added to the list for other reasons, such as to function as positive and negative controls, because I was particularly interested to see if they were involved in PCD in the worm, or because other lab members had the RNAi clones out and I could test them with minimal effort. Thus the total number of candidate genes was 161 (Appendix A).

Sequencing RNAi clones

I obtained most of the feeding RNAi clones from a copy of the Ahringer library in Rueyling Lin's laboratory. If a gene was not represented in this library I generally did not screen that gene, with the exception of a few (like the HDACs) that I was extremely interested in and so constructed RNAi feeding clones myself. Before screening these RNAi clones, I partially sequenced each to confirm the identity of the clone. Several clones failed this sequencing step, and so were not screened. Appendix A lists all genes on the candidate list and indicates which were successfully screened. I obtained RNAi clones of the correct identity for 122 genes of the 161 candidates.

Conducting the screen and analyzing hits

The primary screen was performed by placing L4-stage hermaphrodites of the *egl-1* reporter and RNAi-hypersensitive *egl-1* reporter strains on HT115 bacteria expressing the RNAi clone of interest, then examining the GFP pattern of L3-stage progeny. The

candidate genes were given arbitrary numbers so that screening was performed blind. Positive (*lin-39* or *ceh-20)* and negative (*unc-22* or *unc-54)* RNAi clones were included in each batch, and the batch was discarded and re-screened if the controls did not look correct. Any clones appearing to alter the pattern of GFP expression at all were screened again in the same manner. If the phenotypes did not match between the first two rounds of screening, a third round was performed. RNAi clones that altered the *egl-1* expression pattern detectably in two rounds were considered potential hits and examined in a secondary screen. The clones did not have to show a phenotype in both the *egl-1* reporter strain and the RNAi-hypersensitive strain to move to secondary screening.

The secondary screen was performed using the same protocol as the primary screen. The only difference was that the observer in the secondary screen (Scott Cameron) was more experienced and held stricter criteria for what would be called a hit than the observer in the primary screen (me). Twenty-five clones were considered potential hits from the primary screen (Appendix A), and 14 of those were considered actual hits following the secondary screen (Table 3-4).

Of the 14 hits from this screen, nine exhibited generalized de-repression of the *egl-1* reporter in a very similar manner (Table 3-4). Many of the large hypodermal cells located laterally along the worm's body exhibited bright GFP expression throughout the animal's life. When examined, the eggs often exhibited bright GFP expression as well. GFP is not expressed at all in these locations from wild-type animals carrying the *egl-1* reporter. Because nine of 122 RNAi clones tested caused this phenotype, I suspected that

it might represent a non-specific de-repression of the integrated transgene rather than *bona fide* transcriptional regulation of *egl-1*. Nevertheless, repeated experiments demonstrated that the set of RNAi clones that produced this phenotype did so reproducibly, so it was not a random event. Two hits exhibiting this phenotype, *cir-1* and *skp-1*, are involved in the Su(H) signaling pathway. To determine whether this was meaningful or a coincidence, I created a feeding RNAi clone for *lag-1*, the worm ortholog of Su(H), and tested its phenotype in the *egl-1* reporter background. Inactivation of *lag-1* did not alter the normal expression pattern of the *egl-1* promoter, strengthening my suspicion that these nine hits might not represent true regulators of *egl-1* expression and PCD.

The remaining five hits from the screen each altered the *egl-1* expression pattern in a unique, reproducible, and specific way, suggesting that they were more likely to be *bona fide* regulators of *egl-1* transcription and PCD. RNAi against *unc-62* led to *egl-1* expression in the midbody region of the ventral nerve cord. This hit became my top priority because its mammalian homologs, the Meis proteins, contribute to leukemogenesis through unknown mechanisms. Chapter 4 addresses characterization of *unc-62*.

RNAi against the POU homeodomain transcription factor *ceh-18* resulted in a lack of GFP expression in P9.aap and P10.aap in the ventral nerve cord. The penetrance of this phenotype was less than ten percent. Nevertheless, I obtained a null allele of this gene, *ceh-18(mg57)*, from the CGC and crossed it into the *egl-1* reporter, $P_{lin\text{-}11}gfp$ reporter, and

ced-1(e1735) backgrounds to analyze its effect on PCD in the ventral nerve cord. I scored 50 animals of each strain and detected no difference between controls and *ceh-18(mg57)* animals in any background. Perhaps the phenotype observed with RNAi to *ceh-18* results from off-target effects. Because the null allele did not reproduce the RNAi phenotype, I did not investigate *ceh-18* further.

RNAi against the ftz-f1 homolog *nhr-25* caused a reduction in the number of GFP-positive nuclei in P9-P12 descendents in the ventral nerve cord. This gene may interact with *mab-5*, because ftz-f1 is a cofactor for the *mab-5* homolog ftz in *Drosophila*. Furthermore, *nhr-25* is known to be expressed in the P cells and their progeny. However, further analysis of this potential regulator of *egl-1* transcription and PCD was not feasible at this time because all known alleles are lethal prior to the L3 larval stage; survival to this stage is required to analyze the ventral nerve cord, which completes postembryonic development in L2 and needs time to express GFP from the *egl-1* reporter.

RNAi against the SCM/DTAFII85 homolog *lin-61* resulted in ectopic *egl-1* expression in non-neuronal cells along the body wall of the animal. The nuclei of these cells were smaller than those of hypodermal cells but larger than those of neurons, so I think that they were muscle cells. At first glance, this phenotype appears to resemble that of the nine hits described above that generally de-repress *egl-1* reporter transcription, but on closer examination the GFP-positive cells induced by *lin-61* RNAi are distinct. Because I was not certain about the identity of the affected cells, I did not further investigate *lin-61*

as a potential regulator of *egl-1* transcription and PCD. However, I think it would be interesting to do so in the future.

RNAi against the uncharacterized gene F53C3.11, which has no recognizable domains or homologs, resulted in extremely bright GFP expression in neurons in the postderids. Cells in this tissue do normally express GFP, but RNAi against F53C3.11 greatly augmented both the number of GFP-positive cells and the expression level. This phenotype suggests that F53C3.11 may repress *egl-1* in the postderids. I did not investigate this possibility further, but I think it might be interesting to do so in the future.

Conclusions

The RNAi-based screen for novel regulators of *egl-1* transcription and PCD was successful. The top hit, *unc-62*, will be described in the next chapter, but it is clearly an important and cell-specific regulator of PCD that had not been previously identified as such because it is essential for survival of the organism. In addition, it is required for repression of *egl-1* in the VC neurons, which was one category of gene we specifically sought to find. Several of the other hits exhibit potential as regulators of PCD as well, although they have not been well characterized yet. Thus, at least one novel regulator of PCD was identified by screening 122 candidate genes. Consequently, I think that conducting a genome-wide RNAi screen in a similar manner would likely be fruitful.

This screen failed to accomplish one of its specific goals, which was to identify the transcriptional activator that binds to the region of *egl-1* mutated in the *n4045* and *n4629* alleles to initiate PCD in the Pn.aap cells. There are several possible explanations for this failure. First, such a transcription factor may not exist, and the *n4045* and *n4629* alleles may prevent Pn.aap death through a different mechanism than the one hypothesized here. For example, perhaps the mutation forms an ectopic binding site for a potent transcriptional repressor that overrides the normal *egl-1* regulatory mechanisms in the Pn.aap cells to prevent death. In the context of wild-type *egl-1* regulatory sequence, RNAi of such a repressor would have no effect. This specific example is unlikely, however, because it does not explain how Deletion 2 phenocopies the mutant alleles. A second, and much more likely, possible explanation for our failure to detect the Pn.aap-specific activator of *egl-1* transcription is that it wasn't tested in this screen. Several of the potential NFAT and CRZ1P homologs were not tested because the correct RNAi clone was not available. Furthermore, the actual transcription factor involved may not have been selected as a candidate because it is more distantly related to NFAT or CRZ1P, or not related to these proteins at all. One potential class to test in the future would be the MEF-2 family, because these transcription factors are also regulated by calcineurin. Of course, it is also possible that the hypothesized connection to calcium signaling is incorrect, in which case any transcription factor could be the culprit. The third possible explanation for the failure of my screen to detect an activator of PCD in the Pn.aap cells is that RNAi could not inactivate its activity sufficiently to observe a phenotype. This could occur if the gene was particularly resistant to RNAi, redundant with other genes, or so essential to life that sufficient knockdown results in lethality

before the L3 larval stage. To find the identity of this *egl-1* activator, I would first conduct a genome-wide screen to see if any genes with the appropriate characteristics were found. If that was unsuccessful, I would then turn to a biochemical approach and screen candidates (or fractionated worm lysate) by EMSA using the region surrounding the *n4045* and *n4629* alleles as a probe. Although this approach has already been tried unsuccessfully by the Horvitz lab (Scott Cameron, personal communication), obtaining lysates from larval rather than embryonic animals might result in successful implementation in the future.

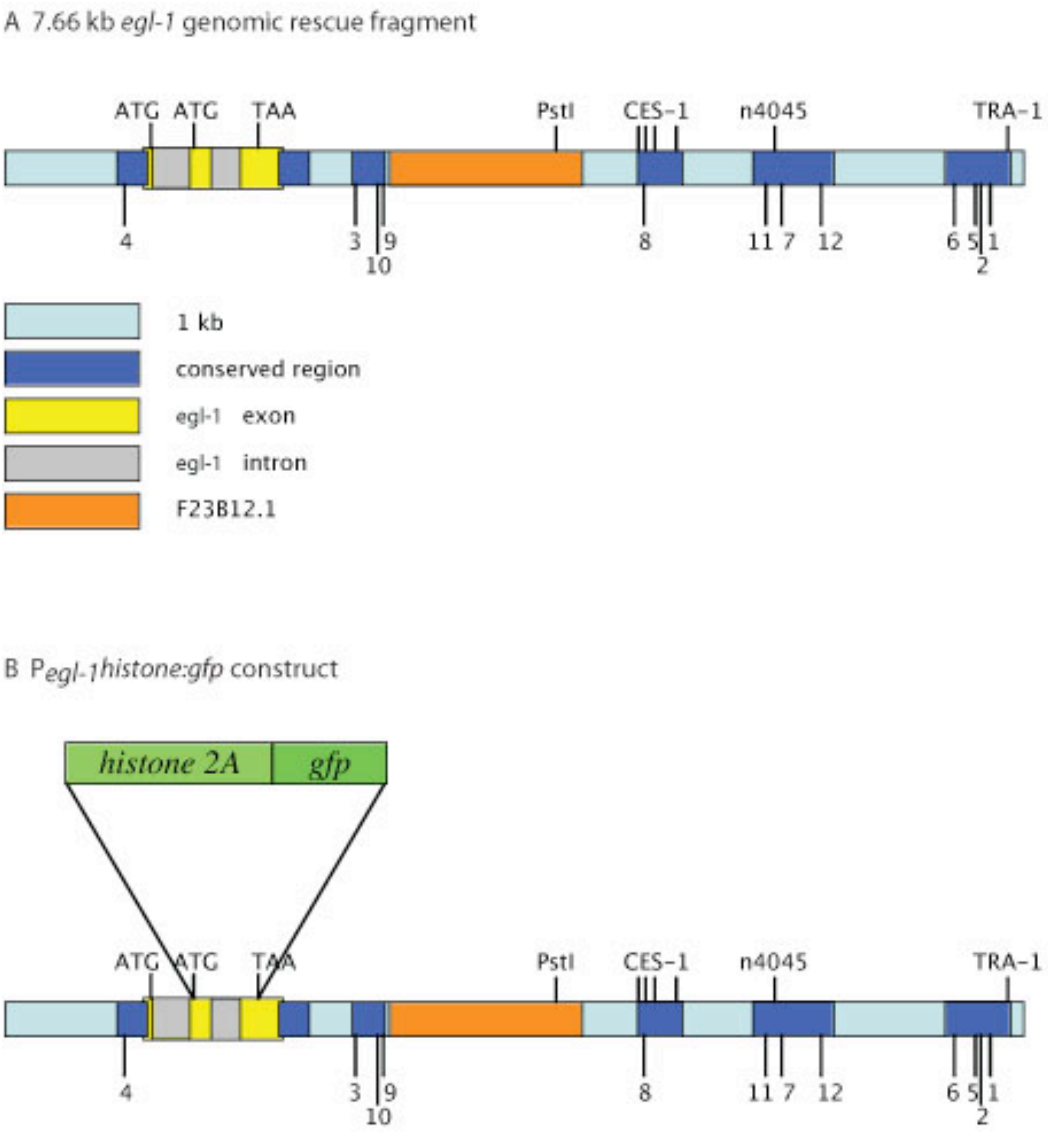
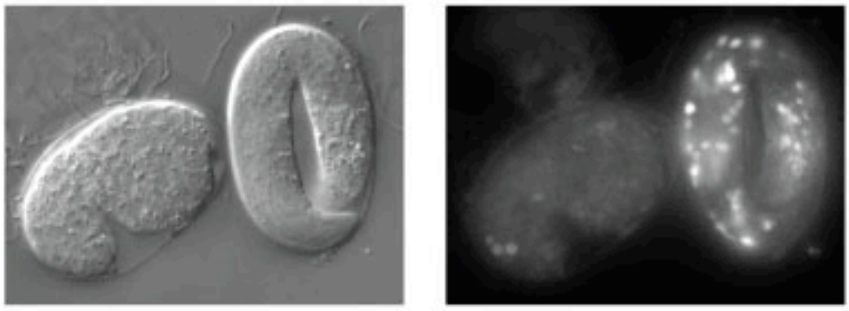


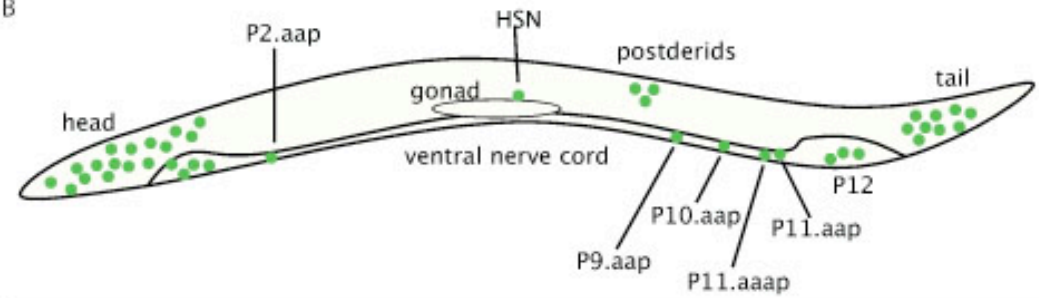
Figure 3-1. Diagram of the *egl-1* gene and a transcriptional *egl-1* reporter.
A) The 7.66 kb minimal rescue fragment of *egl-1* is depicted with features drawn to scale. The yellow rectangles represent exons and the gray rectangles represent introns. The stop codon (TAA) is indicated. Two potential start sites (ATG) are indicated. The first start is predicted by Wormbase, whereas the second is based on RACE data from Barbara Conradt. The orange rectangle represents another coding gene in the opposite orientation. The dark blue rectangles represent regions of evolutionary conservation. The numbers below the diagram represent conserved Pbx-Hox consensus sites. The binding site for CES-1 and TRA-1, as well as the location of the *egl-1(n4045)* mutation

are indicated above the digram. B) The open reading frame was replaced with a fusion between *histone2A* and *gfp* to produce the P_{egl-1}*histone:gfp* transcriptional *egl-1* reporter.

A



B



	P2.aap	P3-P8	P9.aap	P10.aap	P11.aaap	P11.aap	P12	HSN
# green cells expected	1	0	1	1	1	1	3	0
# worms correct (n=45)	19	45	44	44	44	44	42	2
% worms correct	**42.2**	**100**	**97.8**	**97.8**	**97.8**	**97.8**	**93.3**	**4.4**
average # of cells	1.71	0	1.02	1.02	0.98	0.98	2.98	0.96
range	1 - 3	0	1 - 2	1 - 2	0 - 1	0 - 1	2 - 4	0 - 1

C

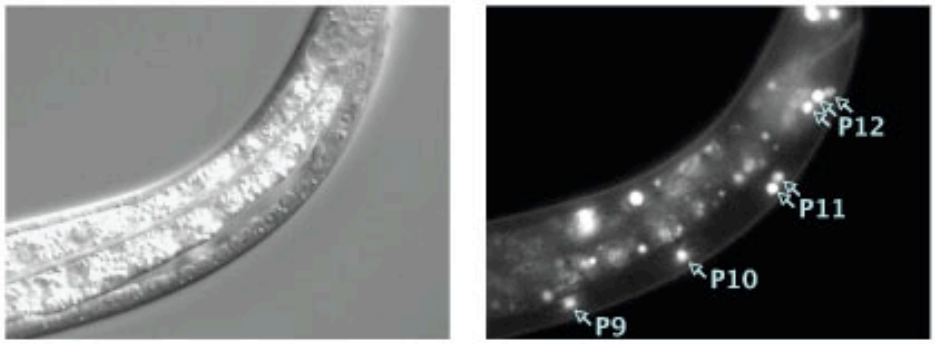


Figure 3-2. The expression pattern of the *egl-1* reporter generally mimics the pattern of PCD in the worm and matches this pattern precisely in the midbody and posterior regions of the ventral nerve cord.
A) DIC and fluorescent images of *ced-1(e1735); ced-3(n717); mxIs14* embryos are shown. The embryo on the left is at the comma stage, and a few GFP-positive cells are visible in the lower left corner of the right panel. The other embryo is much older and expresses GFP in many nuclei. B) The GFP expression pattern in 45 L3-stage larvae of the same genotype as A was quantitatively examined. The diagram shows the location of GFP-positive nuclei, and the table below provides quantitation. C) The posterior ventral nerve cord of one animal from B is shown. Note the pattern of three GFP-positive nuclei in the P12 descendents, two in P11 descendents, and one each in the descendents of P9 and P10.

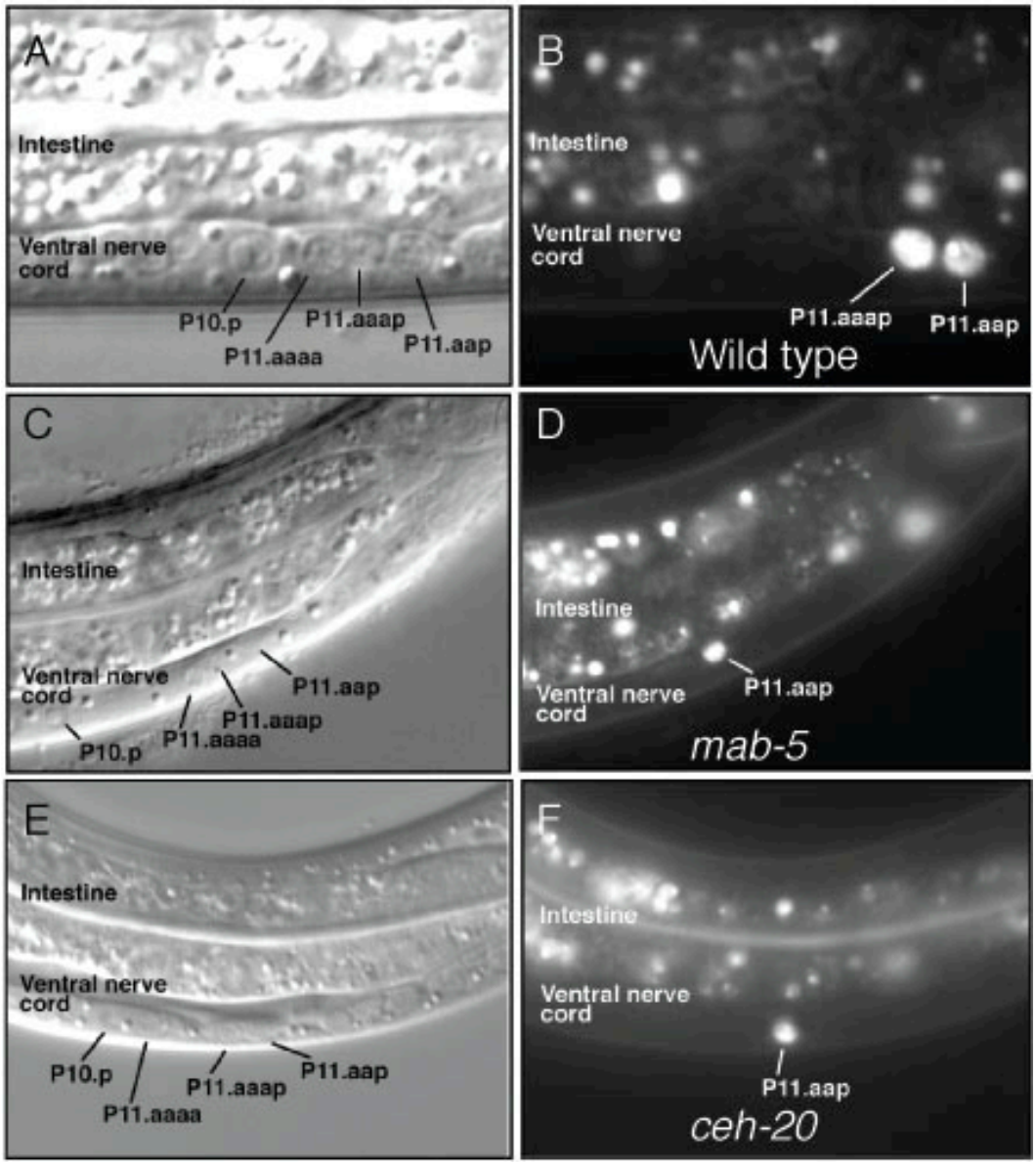


Figure 3-3. Transcription of *egl-1* correlates with programmed cell death in the P11 lineage.
Nomarski optics (A,C,E) and epifluorescnece (B,D,F) of L3-stage *ced-1(e1735); ced-3(n717); mxIs14* larvae. The descendents of P11.aaa are reproducibly located immediately posterior to the hypodermal cell P10.p. A) and B) In worms of the above genotype with no further mutations (30 out of 30 animals), *egl-1* is expressed in the two cells that undergo programmed cell death in the P11 lineage (P11.aap and P11.aaap). C) and D) In animals additionally carrying the *mab-5(n1384)* allele, the *egl-1* transcriptional reporter is not expressed (27 of 30 animals) in P11.aaap, which survives in

*mab-5*mutants. E) and F) P11.aaap also survives in *ceh-20* mutants, and *egl-1* is not expressed (58 out of 60 animals) in animals carrying the *ceh-20(ay42)* allele. Figure reproduced from (Liu et al., 2006).

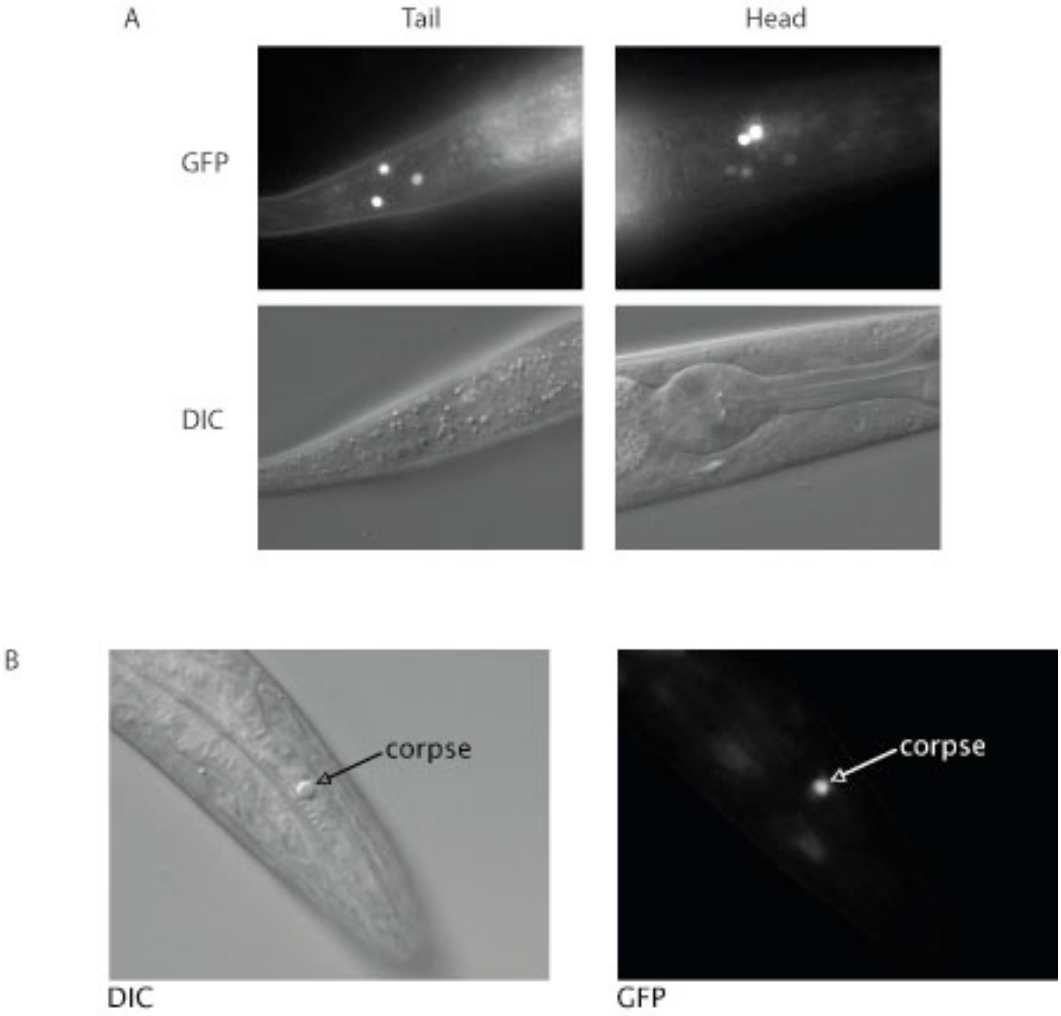
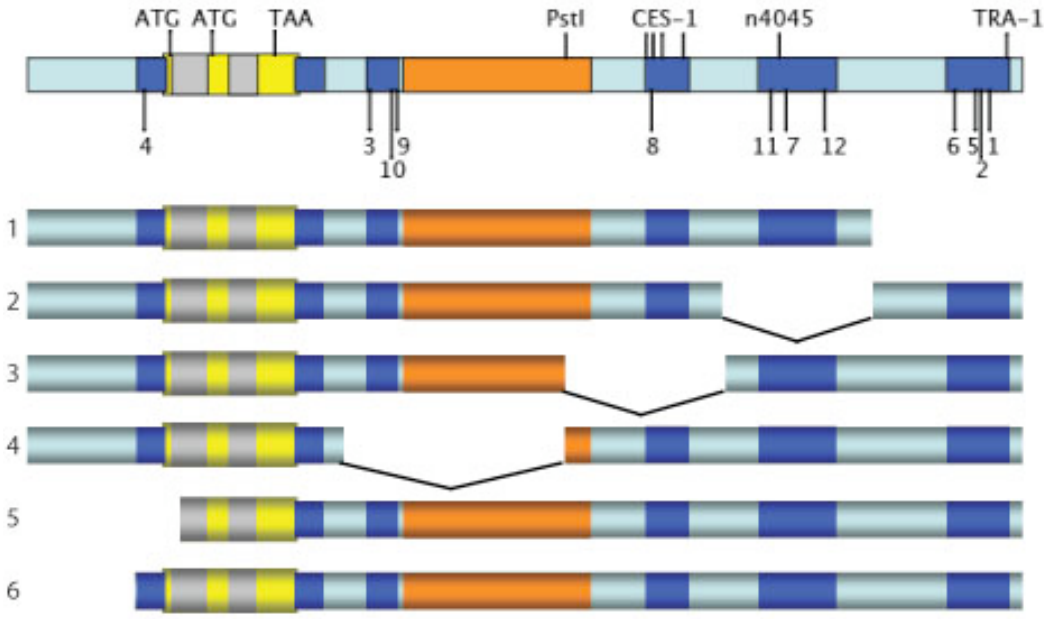


Figure 3-4. The *egl-1* reporter is expressed in some surviving cells and most dying cells.
A) *mxIs14* animals at the L3 stage were photographed using DIC and fluorescent optics. The *egl-1* reporter drives GFP expression in some cells in the tail and head. Because PCD is not blocked in this strain, this represents expression of the reporter in living cells. B) *ced-1(e1735); mxIs14* animals at the L1 stage were photographed using DIC and fluorecent optics. Most corpses were obviously GFP positive, indicating that the reporter is expressed in most dying cells. A single corpse expressing GFP is shown here.



Deletion	Construct	GFP Pattern
1	pMP048	Missing 1 cell each in P11 and P12
2	pMP071	2 cels in P12, 1 in P11, 0 in rest of cord
3	pMP049	Wild-type
4	pCH003	Wild-type
5	pCH001	GFP very faint
6	pCH002	GFP very faint

Figure 3-5. Analysis of an *egl-1* deletion series reveals small regions necessary for specific aspects of the expression pattern.
A series of six constructs delete ~1 kb segments of the *egl-1* regulatory region in the context of the P_{egl-1}*histone:gfp* reporter. Each construct was injected into *ced-1(e1735); ced-3(n717)* animals where its expression pattern was analyzed. The resulting patterns are described briefly here and in detail in the text.

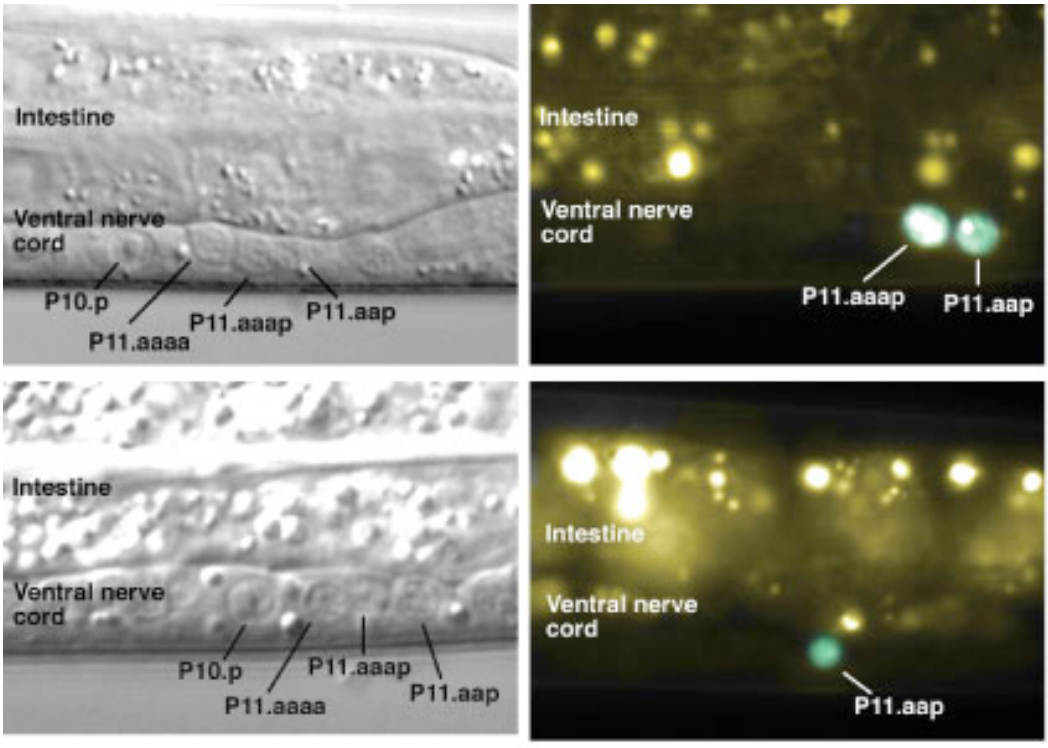


Figure 3-6. The Pbx-Hox consensus site, Site 1, is required for *egl-1* transcription in P11.aaap.
The top panels show *ced-1(e1735); ced-3(n717)* animals carrying the wild-type *egl-1* reporter. 30 of 30 animals expressed *gfp* in P11.aaap, and 29 of 30 expressed *gfp* in P11.aap. By contrast, the lower panels show *ced-1(e1735); ced-3(n717)* animals carrying the *egl-1* reporter with Site 1 mutated to an NcoI recognition site. Of these animals, only 11 of 90 expressed *gfp* in P11.aaap while 83 of 90 expressed *gfp* in P11.aap. P11.aap undergoes PCD in wild-type animals and *mab-5* and *ceh-20* mutants, whereas P11.aaap undergoes PCD in wild-type animals but not in *mab-5* and *ceh-20* mutants. Figure was modified from (Liu et al., 2006).

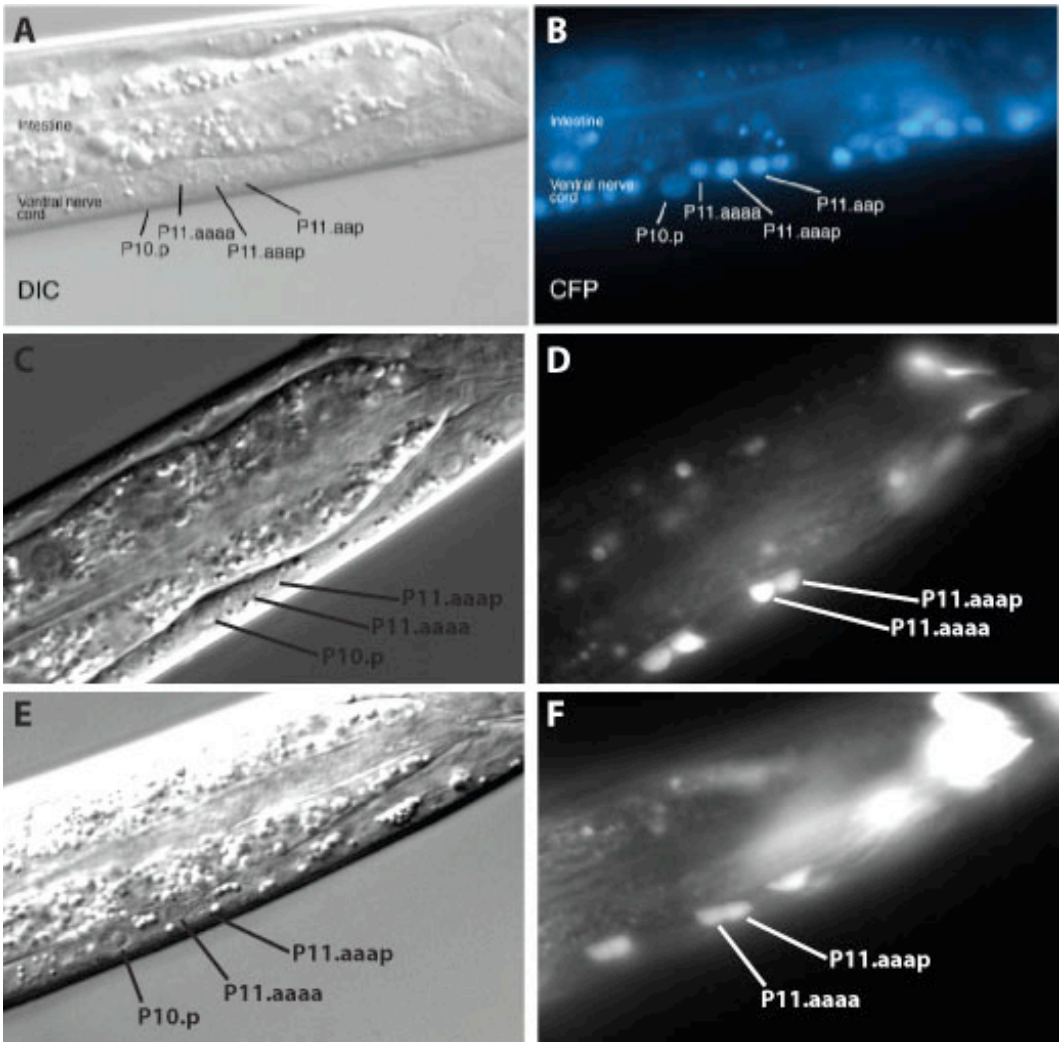


Figure 3-7. CEH-20 is expressed in P11.aap and may not be required for expression of a $P_{mab\text{-}5}$*gfp* reporter.

DIC (A,C,E) and epifluorescence (B,D,F) images. A) and B) The posterior ventral nerve cord of a late L2 stage animal carrying a rescuing $P_{ceh\text{-}20}$*ceh-20:cfp* fusion gene. C) and D) Of the 30 otherwise wild-type *ced-3* mutants carrying the $P_{mab\text{-}5}$*gfp* reporter, 28 and 26 animals expressed *gfp* in P11.aaaa and P11.aaap, respectively. E) and F) Of the 60 *ceh-20(ay42)* mutants carrying the $P_{mab\text{-}5}$*gfp* reporter, 51 and 50 mutants expressed *gfp* in P11.aaaa and P11.aaap, respectively. Figure is reproduced from (Liu et al., 2006).

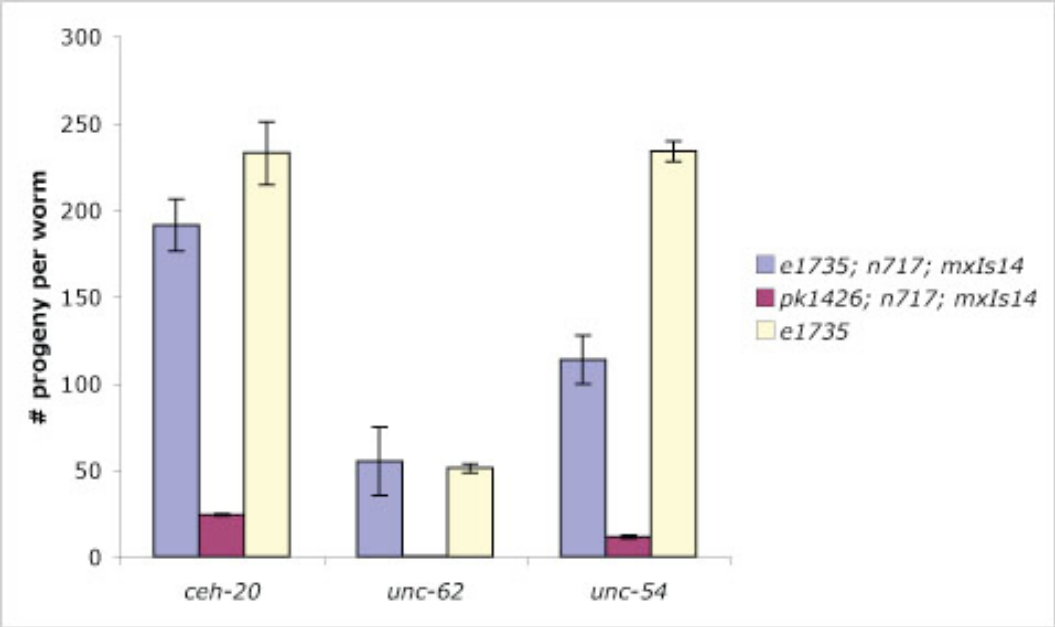


Figure 3-8. RNAi against essential genes causes more lethality in an *rrf-3(pk1426)* background.
At least ten L4-stage hermaphrodites from the indicated strains were fed RNAi to the indicated genes, and the number of progeny per worm was quantitated. The average number of progeny per worm is shown.

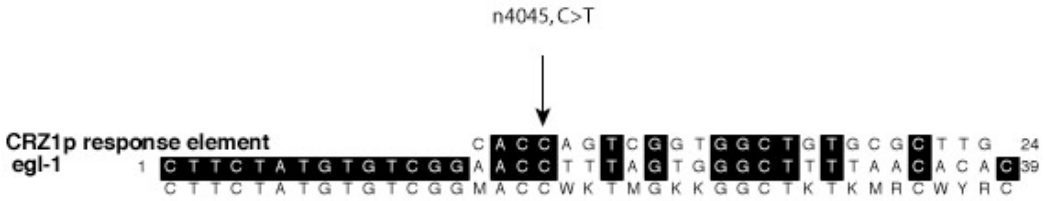


Figure 3-9. The region of *egl-1* surrounding the *n4045* mutation resembles a published CRZ1p binding site.
The sequence of a published CRZ1p response element (Stathopoulos and Cyert, 1997) is shown aligned to the region of *egl-1* surrounding the *n4045* mutation, which is indicated by an arrow. Nucleotides highlighted in black are identical and include the C that is mutated to T in *n4045*.

Genotype	W/P1	P2	P3-P8	P9	P10	P11	P12	n
Wild-type	3.7	1.3	0.0	1.0	1.0	2.0	2.7	30
lin-39(n1760)	3.2	1.0	6.0	1.0	1.0	2.0	2.8	50
mab-5(n1384)	3.1	1.3	0.0	1.1	1.0	1.0	1.4	30
ceh-20(ay9)	3.5	1.0	5.0	1.0	1.0	0.8	2.6	50
ceh-20(ay42)	1.8	1.0	5.7	1.0	0.9	0.9	1.6	50

Table 3-1. The *egl-1* reporter responds correctly to mutations that affect the pattern of PCD.
The indicated mutations were introduced into the *ced-1(e1735); ced-3(n717); mxIs14* background. The GFP pattern in the ventral nerve cord was quantitatively analyzed in 30-50 L3-stage hermaphrodites of each genotype. The average number of GFP-positive cells present in the descendents of each blast cell is shown. Standard error is not shown, but was between 0 and 0.2 for each data point. As expected, *lin-39* and *ceh-20* mutations resulted in GFP-positive cells in the descendents of P3-P8, whereas *mab-5* and *ceh-20* mutations resulted in a decrease in the number of GFP-positive nuclei in the descendents of P11 and P12.

Sites mutated/deleted	Pattern of *egl-1* expression
1	Missing 1 cell each in P11 and P12
1, 2, 3, 4	Missing 1 cell each in P11 and P12
1, 2, 5, 6	Missing 1 cell each in P11 and P12
7, 8	Wild-type
9	Wild-type
7, 8, 9	Wild-type
3, 4, 5, 7, 8, 9	Wild-type; general derepression
12	Wild-type
1 through 12	Missing 1 cell each in P11 and P12

Table 3-2. Pbx-Hox consensus binding Site 1 is required for *egl-1* expression in one cell in the P11 lineage and one cell in the P12 lineage.
Twelve conserved Pbx-Hox binding sites in the *egl-1* regulatory sequence were mutated within the context of the *egl-1* reporter, and expression patterns of the resulting constructs in the ventral nerve cord of *ced-1(e1735); ced-3(n717)* animals were analyzed. All constructs had the indicated sites mutated to NcoI restriction binding sites except the third construct, in which sites 1, 2, 5, and 6 were deleted along with the surrounding sequence. Most constructs exhibited a wild-type pattern of *egl-1* expression, except constructs in which Site 1 was mutated or deleted. Disruption of Site 1 resulted in one fewer GFP-positive nuclei each in the P11 and P12 lineages. The seventh construct, in which sites 3, 4, 5, 7, 8, and 9 were mutated, exhibited general derepression of the *egl-1* reporter in some transgenic lines such that low levels of GFP were visible throughout the ventral nerve cord. However, the cells that undergo PCD in wild-type worms always expressed significantly brighter GFP than the background levels expressed in surrounding cells.

Genotype	P3-P8	P9-P10	P11	P12
ced-1; ced-3; lin-39(RNAi)	4.8 ±0.2	2.0 ± 0.0	2.0 ± 0.0	2.9 ± 0.1
rrf-3; ced-3; lin-39(RNAi)	4.9 ± 0.1	1.9 ± 0.0	2.0 ± 0.0	2.4 ± 0.1
ced-1; ced-3; ceh-20(RNAi)	4.7 ± 0.2	2.1 ± 0.1	1.7 ± 0.1	2.2 ± 0.1
rrf-3; ced-3; ceh-20(RNAi)	5.2 ± 0.2	2.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.1

Table 3-3. The *rrf-3(pk1426)* mutation increases sensitivity of the *egl-1* reporter to RNAi-mediated phenotype.
Animals of the indicated genotype carrying the *mxIs14 egl-1* reporter were fed RNAi against *lin-39* or *ceh-20*. The number of GFP-positive nuclei in the lineages of the indicated blast cells in the ventral nerve cord was quantitated in 50 animals of each genotype. The average ± s.e.m. is shown. Inclusion of the *rrf-3(pk1426)* mutation did not affect the *lin-39(RNAi)* phenotype but did sensitize worms to the *ceh-20(RNAi)* phenotype as indicated by the increase in GFP-positive nuclei of P3-P8 lineage and the decrease in the P11 and P12 lineages.

Gene	Cells	Homolog/Description
unc-62	VC neurons	MEIS/hth hox cofactor
ceh-18	P9, P10	POU homeodomain TF
nhr-25	P9-12	ftz-f1 (drosophila), cofactor for ftz
lin-61	muscle?	SCM/DTAFII85
F53C3.11	postderids	unknown
cir-1	lat hyp	CBF1-interacting corepressor CIR
skp-1	lat hyp	mammalian SKIP (Ski interacting protein)
pdcd-2	lat hyp	Programmed Cell Death 2 / Rp8
lpd-2	lat hyp	C/EBP (maybe)
F55A3.3	lat hyp, egg	DRE4/dSPT16
ruvb-2	lat hyp	Reptin
tag-214	lat hyp, egg	DRING1
dis-3	lat hyp	yeast DIS3 exonuclease w/ homeodomain
F43G9.12	lat hyp	GC-rich seq. DNA-binding factor

Table 3-4. Fourteen genes from the RNAi screen are required for the correct pattern of *egl-1* reporter expression.
Each hit from the screen is presented along with the cells that depend on the gene for the correct pattern of *egl-1* reporter expression and a brief description of its homolog or family. For more detailed description of the phenotypes, see the text.

CHAPTER FOUR: HOX PROTEINS DIRECTLY INHIBIT APOPTOSIS BY REPRESSING BH3-ONLY GENES

Note about author contributions: The experiments described in this chapter were designed, performed, and analyzed by myself and Scott Cameron with two exceptions. All chromatin immunoprecipitation (ChIP) experiments were performed by David Wang in the Cameron laboratory. The results of these experiments are presented in Figure 4-6. All experiments in mammalian cells were designed, performed, and analyzed by Scott Armstrong, Matthew Stubbs, and Joerg Faber in the Armstrong laboratory. The results from these experiments are presented in Figure 4-10.

ABSTRACT

Mutations that aberrantly activate genes encoding Hox transcription factors, TALE-class Hox cofactors, and trithorax-group proteins promote leukemogenesis. Extensive work has established the importance of Hox transcriptional activity in promoting leukemia, but the target genes regulated by Hox proteins and critical for leukemogenesis remain largely unknown. Through genetic analyses in *C. elegans,* we identify a Hox-Pbx complex that represses the pro-apoptotic BH3-only gene *egl-1* to ensure cell survival. Homologs of the TALE-class homeodomain protein Meis and the trithorax-group protein Ash1 activate this Hox complex by promoting nuclear localization of the Pbx cofactor and transcription of the *Hox* gene, respectively. Ectopic expression of the *Hox* gene is sufficient to block death in some cells. Importantly, we show that Hox-mediated repression of BH3-only proteins is conserved in mammals and critical for the survival of human leukemia cells. This work identifies the BH3-only pro-apoptotic genes as direct targets of Hox-mediated

repression and suggests that aberrant activation of Hox networks may promote leukemia in part by inhibiting apoptosis.

INTRODUCTION

Hox genes encode transcription factors that bind DNA, often with Pbx and Meis cofactors, to activate or repress target genes (Pearson et al., 2005). In turn, Trithorax- and Polycomb-group complexes maintain the correct pattern of Hox expression by modifying chromatin to promote transcriptional activation and repression, respectively (Schuettengruber et al., 2007). Gain-of-function mutations affecting Trithorax group genes, Hox genes and Hox cofactors promote cancer. Most notably, translocations involving the *Trithorax* ortholog *MLL* are commonly found in particularly aggressive forms of acute lymphoblastic leukemia and acute myelogenous leukemia (Grimwade et al., 1998; Pui et al., 2004). Introduction of *MLL* fusion genes into mice is sufficient to cause leukemia (Chen et al., 2008; Corral et al., 1996; Lavau et al., 1997; So et al., 2003). MLL fusion proteins drive overexpression of Meis1 and a specific subset of Hox proteins in human leukemia, and these genes have a central role in promoting leukemogenesis (Argiropoulos et al., 2007; Armstrong et al., 2002; Ayton and Cleary, 2003; Kumar et al., 2004; Wong et al., 2007). Despite intensive study of the critical roles of Hox proteins, cofactors, and regulators in leukemogenesis, the biologically relevant target genes that contribute to the malignant phenotype remain largely unknown.

We propose here that transcriptional inhibition of apoptosis is a key mechanism through which Hox proteins promote leukemogenesis. The genetic basis and mechanism

of apoptosis, or programmed cell death, was first elucidated in the nematode *Caenorhabditis elegans* (Ellis and Horvitz, 1986), and analyses in this organism provided a foundation for understanding the similar but more complex pathways in mammals (Danial and Korsmeyer, 2004). A core pathway of four genes executes programmed cell death in *C. elegans* (Metzstein et al., 1998). The Bcl-2 homolog CED-9 promotes cell survival by inhibiting the Apaf-1 homolog CED-4 (Hengartner et al., 1992; Hengartner and Horvitz, 1994; Spector et al., 1997). The BH3-only protein EGL-1 antagonizes CED-9, allowing CED-4/Apaf-1 to activate the caspase CED-3, which kills the cell (Conradt and Horvitz, 1998; Shaham and Horvitz, 1996; Wu et al., 1997).

Appropriate regulation of apoptosis in a cell-specific manner is critical for normal development and prevention of cancer (Green and Evan, 2002), and mutations that block apoptosis lead to hematopoietic malignancies in mammals. BH3-only genes encode initiators of apoptosis that respond to stimuli in a cell-specific manner (Strasser, 2005). Mice with engineered mutations in specific BH3-only genes develop hematopoietic malignancies including myeloproliferative disorder, leukemia and lymphoma (Egle et al., 2004; Ranger et al., 2003; Zinkel et al., 2003). In humans, chromosomal translocations that cause overexpression of the anti-apoptotic protein Bcl-2 lead to follicular lymphoma (Hockenbery et al., 1990; Tsujimoto et al., 1984; Vaux et al., 1988), and translocations affecting the transcription factor HLF cause acute lymphoblastic leukemia by activating a repressor of the BH3-only gene *Puma* and promoting aberrant cell survival (Inaba et al., 1996; Inaba et al., 1992a; Inukai et al., 1999; Wu et al., 2005). Some cells therefore undergo apoptosis during normal hematopoiesis, and if their death is prevented malignancy can result.

In this study, we show that a Hox protein complex prevents programmed cell death of specific cells of *C. elegans* by repressing transcription of the cell death initiator *egl-1/BH3*. We also identify the Trithorax-group gene *lin-59/Ash1* and TALE gene *unc-62/Meis* as regulators of programmed cell death. Ectopic activation of this pathway can prevent the death of some cells that die during normal development. Furthermore, we show that Hox-dependent repression of BH3-only genes is conserved in mammals and promotes leukemia cell survival. These findings raise the possibility that direct transcriptional repression of pro-apoptotic BH3-only genes by mutationally-activated or ectopically expressed Hox proteins contributes to Hox-mediated oncogenesis.

RESULTS

unc-62/Meis represses egl-1/BH3 transcription

The BH3-only gene *egl-1* is required for programmed cell death of somatic cells of *C. elegans* and is transcriptionally regulated to control the survival or death of specific cells (Conradt and Horvitz, 1998; Conradt and Horvitz, 1999; Liu et al., 2006; Thellmann et al., 2003). To discover novel regulators of *egl-1/BH3* and programmed cell death, we developed a transcriptional reporter by integrating the P_{egl-1}*histone:gfp* construct into *ced-3/caspase* mutants (Liu et al., 2006). In this strain, which we refer to as P_{egl-1}*gfp*, the *ced-3/caspase* mutation prevents cell death, and a *histone:gfp* fusion gene is expressed under control of the *egl-1/BH3* regulatory region.

In P_{egl-1}*gfp* hermaphrodites, the pattern of GFP expression precisely matched the pattern of programmed cell deaths in the midbody and posterior regions of the ventral

nerve cord (shown schematically in Figure 4-1A). Specifically, in these regions all cells that undergo programmed cell death wild-type animals expressed GFP, and all cells that survive in wild-type animals did not express GFP (see Chapter 3). This suggests that the pattern of programmed cell death and survival in the ventral nerve cord is largely determined by controlling transcription of *egl-1/BH3*, and that the P_{egl-1}*gfp* reporter accurately portrays the wild-type pattern of *egl-1/BH3* expression in this tissue.

To identify genes that determine the *egl-1/BH3* expression pattern, we screened transcription factors with RNAi phenotypes of sterility or lethality (60 total) from the published genome-wide RNAi screen datasets (Kamath et al., 2003). Control *unc-22(RNAi)* did not alter the wild-type pattern of *egl-1/BH3* transcription, but *unc-62/Meis(RNAi)* resulted in ectopic expression of P_{egl-1}*gfp* in the midbody region of the ventral nerve cord (Figure 4-1B, Table 4-1A). The partial loss-of-function alleles *unc-62(e917)* and *unc-62(t2012)* also result in ectopic *egl-1/BH3* expression in the midbody (Table 4-1A). Mutants completely lacking *unc-62/Meis* gene function exhibit complete embryonic lethality and therefore could not be examined (Van Auken et al., 2002).

unc-62/Meis ensures survival of the VC motor neurons

To determine whether ectopic *egl-1/BH3* transcription resulted in the death of cells that normally survive, we used *ced-1(e1735)* mutants in which most cells that die persist as visible corpses. In a *ced-1* background, corpses are visible in the anterior and posterior regions of the ventral cord where cell death occurs, but never in the midbody region where all cells survive (Figure 4-2A) (Clark et al., 1993; Sulston, 1976). Control

unc-22(RNAi) does not alter this pattern, but *unc-62/Meis(*RNAi) results in ectopic corpses in the midbody region of the ventral nerve cord (Table 4-1B, Figure 4-2B). This phenotype is also observed in *unc-62(e917)* and *unc-62(t2012)* mutants (Figure 4-2 C,D). To determine which cells require *unc-62/Meis* for survival, we observed the lineages of the P5 and P6 blast cells in five *unc-62/Meis(RNAi)* animals and found that four of ten Pn.aap cells died (Figure 4-1 C-E). Pn.aap cells invariably survive and differentiate into VC motor neurons in wild-type animals (Sulston, 1976), so *unc-62/Meis* is required for survival of the VC neurons.

lin-39/Hox and ceh-20/Pbx promote VC survival through transcriptional repression of egl-1/BH3

Previous work has shown that survival of the VC neurons requires the Hox gene *lin-39* and the Hox cofactor *ceh-20/Pbx*, which act upstream of *egl-1/BH3* through unknown mechanisms (Clark et al., 1993; Liu et al., 2006). Because homologs of *unc-62/Meis* interact genetically and biochemically with Pbx and Hox homologs (Moens and Selleri, 2006), we considered the possibility that *lin-39/Hox*, *ceh-20/Pbx*, and *unc-62/Meis* may act together to repress *egl-1/BH3* and prevent death of the VC neurons. Indeed, reduction of *lin-39/Hox* or *ceh-20/Pbx* function by RNAi or mutation results in ectopic $P_{egl\text{-}1}$*gfp* expression in the VC neurons (Figure 4-1B; Table 4-1A), suggesting that like *unc-62/Meis*, *lin-39/Hox* and *ceh-20/Pbx* promote VC survival by repressing *egl-1/BH3* transcription.

lin-39/Hox acts cell-autonomously to prevent death of the VC neurons

If LIN-39/Hox, CEH-20/Pbx, and UNC-62/Meis directly bind *egl-1/BH3* to repress transcription in the VC neurons, then all three proteins should be expressed there and function in a cell-autonomous fashion. Indeed, LIN-39/Hox is present in neurons in the midbody region of the ventral nerve cord (Maloof and Kenyon, 1998; Wagmaister et al., 2006), and rescuing transgenic reporter constructs suggest that *ceh-20/Pbx* and *unc-62/Meis* are broadly expressed in all nuclei in the ventral nerve cord, including the VC neurons (Liu et al., 2006; Yang et al., 2005)(Figure 4-3). To determine whether LIN-39/Hox acts cell-autonomously in VC neurons, we introduced a P_{egl-1}*lin-39* transgene into *ced-1; lin-39* mutants and counted cell corpses in the ventral nerve cord (Figure 4-4A). Indeed, four independent transgenic lines showed similar levels of rescue in the midbody (Figure 4-4C, Figure 4-5). Because endogenous LIN-39/Hox is limited to the midbody region of the ventral nerve cord (Maloof and Kenyon, 1998; Wagmaister et al., 2006) and the *egl-1/BH3* promoter is expressed only in the VC neurons in this region in *lin-39/Hox* mutants (Figure 4-1B and Table 4-1A), these data suggest that *lin-39/Hox* acts cell-autonomously to ensure survival of the VC neurons.

Cooperative binding between LIN-39/Hox and CEH-20/Pbx is required to ensure VC survival

LIN-39/Hox contains a conserved hexapeptide motif N-terminal to the homeodomain (Clark et al., 1993), which in homologous proteins is required for binding to the Pbx cofactor but not for DNA binding or protein stability *in vitro* or *in vivo* (Chang et al., 1995; Knoepfler and Kamps, 1995; Neuteboom et al., 1995; Phelan and

Featherstone, 1997; Phelan et al., 1995). We created two LIN-39/Hox mutants in which the critical tryptophan of the hexapeptide was mutated, LIN-39/Hox(W>F) and LIN-39/Hox(WM>AA), expressed and purified these proteins from *E. coli* and tested their function in an electrophoretic mobility shift assay (EMSA). Wild-type LIN-39/Hox efficiently bound a consensus Hox-Pbx probe and cooperated with CEH-20/Pbx in a dose-dependent manner (Figure 4-4B, lanes 1-4). Both mutant proteins bound DNA as well as wild-type, but failed to cooperate with CEH-20/Pbx (Figure 4-4B, lanes 5-10).

We used the *lin-39/Hox in vivo* rescue assay to determine whether these mutant proteins were able to prevent death of the VC neurons. We found that although expression of wild-type *lin-39/Hox(+)* cDNA in the VC neurons rescued their death, *lin-39/Hox(W>F)* or *lin-39/Hox(WM>AA)* cDNA did not (Figure 4-4C, Figure 4-5). This suggests that cooperative binding between LIN-39/Hox and CEH-20/Pbx is required for *egl-1/BH3* repression and VC neuron survival.

LIN-39/Hox likely acts as a transcriptional repressor to prevent cell death

Hox proteins can function as transcriptional activators or repressors (Moens and Selleri, 2006). To determine whether *lin-39* acts as a repressor or activator to prevent cell death we fused *lin-39/Hox* coding sequences to the activation domain of VP16 or the repression domain of Engrailed (Jaynes and O'Farrell, 1991; Sze et al., 1997) and tested these fusion constructs using the rescue assay described above. *Engrailed:lin-39* promoted VC survival much more efficiently than *VP16:lin-39*, suggesting that LIN-39/Hox functions as a transcriptional repressor to prevent death of the VC neurons

in wild-type animals (Figure 4-4D, Figure 4-5). This model is consistent with direct repression of *egl-1/BH3* transcription by a LIN-39/HoxCEH-20/Pbx complex in the VC neurons.

Ectopic lin-39/Hox expression can prevent programmed cell death

The rescue assay used in Figure 4-4 drives *lin-39/Hox* expression in all cells that express *egl-1/BH3*, including cells outside the midbody region to which *lin-39/Hox* expression is normally restricted (Wagmaister et al., 2006; Wang et al., 1993). In the posterior region of the ventral nerve cord (P9-P12 descendants), seven cells undergo programmed cell death in wild-type animals, and approximately six corpses are observed in both *ced-1* and *ced-1; lin-39* mutants (Figure 4-4C) (Clark et al., 1993). Remarkably, expression of P_{egl-1}*lin-39* or P_{egl-1}*Engrailed:lin-39* suppressed programmed cell death in the posterior ventral cord (Figure 4-4D). This implies that ectopic expression of LIN-39/Hox in cells that normally die in wild-type animals can repress *egl-1/BH3* through cooperation with CEH-20/Pbx, blocking the intrinsic mechanism for programmed cell death that normally kills these cells.

LIN-39/Hox and CEH-20/Pbx bind the egl-1/BH3 locus at multiple sites in vivo

The results described thus far would be consistent with direct repression of *egl-1/BH3* transcription by a LIN-39/Hox-CEH-20/Pbx complex in the VC neurons. If this hypothesis were correct, we would expect to find one or more Hox/Pbx consensus sites in the promoter of *egl-1/BH3* that are required for repression in the VCs. The minimal fragment of the *egl-1/BH3* genomic locus required for rescue of the correct cell

death pattern in the ventral nerve cord of *egl-1/BH3* mutant animals is 7.66 Kb long, with the majority of the regulatory region lying 3' to the *egl-1/BH3* open reading frame (Figure 4-6A) (Conradt and Horvitz, 1999; Liu et al., 2006; Thellmann et al., 2003). A search for consensus Hox/Pbx binding sites in the promoter identifies 116 matches, of which 12 are conserved between *C. elegans, C. remaneii,* and *C. briggsae*, three distantly related nematode species. The locations of the 12 conserved sites are indicated by asterisks in Figure 4-6A. We disrupted all 12 conserved sites by site-directed mutagenesis in a single P_{egl-1}*gfp* construct. The resulting construct was correctly repressed in the VC neurons (see Chapter 3), suggesting that either LIN-39/CEH-20 represses *egl-1/BH3* indirectly through non-Hox consensus binding sequences, or it binds one or more of the remaining 104 sites.

To attempt to identify a single region of *egl-1/BH3* that is required for repression in the VCs, whether bound by a LIN-39/Hox-CEH-20/Pbx complex or by another hypothetical transcription factor, we made a series of six deletion mutants that systematically removed 1 kb segments of *egl-1/BH3* sequence, covering the entire noncoding region of *egl-1/BH3*. Each of these deletion mutants was correctly repressed in the VC neurons, suggesting that the factor or factors that repress *egl-1/BH3* in the VC neurons bind and act through multiple, dispersed and redundant sites (see Chapter 3).

We next asked whether LIN-39/Hox, CEH-20/Pbx, and UNC-62/Meis were enriched at the *egl-1/BH3* genomic locus *in vivo* using chromatin immunoprecipitation (ChIP). We performed immunoprecipitation of protein-cross-linked-DNA from the P_{lin-39}*lin-39:gfp* strain (Wagmaister et al., 2006) with anti-All Fluorescent Protein (anti-AFP) antibody or IgG control and used quantitative PCR to compare the amount of

egl-1/BH3 DNA bound to each. Primers designed toward widely separated regions of *egl-1/BH3* showed reproducible enrichment, indicating that LIN-39/Hox binds to *egl-1/BH3* at multiple, dispersed sites (Figure 4-6B). Primers designed toward a control genetic locus not known to be regulated by *Hox* genes did not show reproducible enrichment, suggesting that the immunoprecipitation specifically brought down DNA associated with the LIN-39:GFP fusion protein. Similar experiments using the same antibody were performed with the P_{ceh-20}*ceh-20:yfp* and P_{unc-62}*unc-62:cfp* rescuing translational fusions to examine CEH-20/Pbx and UNC-62/Meis association with the *egl-1/BH3* locus. We observed reproducible enrichment of CEH-20/Pbx, but not UNC-62/Meis, at several sites along the *egl-1/BH3* promoter (Figure 4-6 C,D). All ChIP experiments were performed by David Wang. These results, taken together with those described above, suggest that a complex composed of LIN-39/Hox and CEH-20/Pbx binds the *egl-1/BH3* regulatory region at multiple dispersed, redundant sites to directly repress *egl-1/BH3* transcription in the VC neurons.

unc-62/Meis is required for nuclear localization of CEH-20/Pbx

The fact that we did not observe enrichment of UNC-62/Meis at the *egl-1/*BH3 locus led us to ask whether this protein might be involved in *egl-1/BH3* repression in a less direct manner. In *Drosophila*, the *unc-62/Meis* homolog Homothorax is required for nuclear localization of the *ceh-20/Pbx* homolog Extradenticle (Stevens and Mann, 2007), and one report in *C. elegans* suggests the same may be true in this organism as well (Van Auken et al., 2002). We therefore examined the subcellular localization of CEH-20/Pbx

in animals carrying the $P_{ceh\text{-}20}$*ceh-20:yfp* reporter. In control *unc-22(RNAi)* animals, CEH-20/Pbx was localized exclusively in the nuclei of cells where it was expressed, which included the lateral hypodermal cells (Figure 4-7B) and all nuclei of the ventral nerve cord (Figure 4-7F). However, in *unc-62/Meis(RNAi)* animals, nuclear localization was lost in the lateral hypodermal cells (Figure 4-7D) and was absent or variable in many neuronal nuclei in the ventral nerve cord (Figure 4-7H). The discrepancy in severity of the defect in nuclear localization we observed between hypodermal cells and neurons may be due to reduced efficiency of RNAi in neuronal cells compared to non-neuronal cells in *C. elegans* (Simmer et al., 2002). These data suggest that *unc-62/Meis* contributes to *egl-1/BH3* repression and VC survival by promoting nuclear localization of CEH-20/Pbx.

The trithorax-group gene lin-59/Ash1 also ensures VC neuron survival

Hox transcription is controlled by many factors including the opposing activities of polycomb- and trithorax-group genes, which generally maintain the repressed and activated state of transcription, respectively (Schuettengruber et al., 2007). In a screen for genes that regulate programmed cell death in the ventral nerve cord (Cameron et al., 2002), we identified two alleles of the trithorax-group gene *lin-59*, which encodes a SET domain-containing protein homologous to the *Drosophila* Ash1 and human Ash1L histone methyltransferases (Chamberlin and Thomas, 2000). *lin-59(n3192)* and *lin-59(n3168)* encode premature stop codons that are predicted to truncate the protein just N-terminal and just C-terminal to the SET domain, respectively (Figure 4-8). Genetic

analyses suggest that both alleles result in partial loss of gene function (see Materials and Methods). In a *ced-1* background, *lin-59/Ash1* mutants and *lin-59/Ash1(RNAi)* animals exhibit cell corpses in the midbody region of the ventral nerve cord in a pattern like that of *lin-39/Hox,* ceh-20/Pbx*, and unc-62/Meis* mutants (Table 4-1B). Analysis of cell lineages of the P3-P8 blast cells in four *lin-59(n3168)* animals revealed programmed cell death of the VC neurons (Figure 4-9B). Thus survival of the VC neurons requires *lin-59/Ash1* in addition to *lin-39/Hox, ceh-20/Pbx,* and *unc-62/Meis.*

lin-59/Ash1 promotes transcription of lin-39/Hox

lin-59/Ash1 regulates expression of the posterior *Hox* genes *mab-5* and *egl-5* (Chamberlin and Thomas, 2000). We therefore investigated whether *lin-59/*Ash1 promotes VC neuron survival by maintaining transcription of lin-*39/Hox.* We found decreased *lin-39/Hox* mRNA in *lin-59(RNAi), lin-59(n3168)* and *lin-59(n3192)* mutants compared to wild-type animals (Figure 4-9 C,D). Levels of *ceh-20/Pbx* mRNA and two controls were normal (Figure 4-9E). Surprisingly, we observed a marked increase in *ceh-20/Pbx* mRNA levels in both *ceh-20(ay42)* and *unc-62(e917)* mutants compared to wild-type, suggesting that *ceh-20/Pbx* transcription may be controlled in part by a negative feedback mechanism that requires *unc-62/Meis* (Figure 4-9E). Taken together, these data suggest that *lin-59/Ash1* promotes cell survival by promoting transcription of *lin-39/Hox*, which encodes a Hox transcription factor that interacts with CEH-20/Pbx to repress the apoptosis-initiating gene *egl-1/BH3*, ensuring cell survival.

HoxA9 represses Bim transcription in human leukemia cell lines

Gain-of-function mutations affecting Hox genes are common in human leukemias, and act through unknown target genes to promote leukemogenesis. Our data suggest that direct repression by Hox protein complexes of cell death-initiating BH3 domain genes might contribute to leukemia cell survival. To test this idea we examined the human myeloid leukemia cell lines Molm14 and THP-1, which carry *MLL-Af9* fusions. Extensive work in human leukemia cells and mouse models has identified the *HoxA7* and *HoxA9* genes as being particularly important mediators of the transforming ability of *MLL-Af9* (Argiropoulos and Humphries, 2007). We therefore inhibited HoxA9 function with two independent short-hairpin constructs. When compared to cells treated with a control GFP shRNA, we observed a substantial induction of the BH3-only protein Bim and of the *Bim* mRNA (Figure 4-10 A,B), suggesting that HoxA9 represses transcription of *Bim* in human leukemia cells. All experiments in mammalian cells were performed by Matthew Stubbs and Joerg Faber in Scott Armstrong's laboratory.

Survival of Molm14 and THP-1 cells requires HoxA9

HoxA9-mediated repression of *Bim* could be necessary for survival in human leukemia cell lines, just as Hox-mediated repression of *egl-1* is necessary for survival of the VC neurons in *C. elegans*. To examine this possibility, we used trypan blue exclusion and Annexin V staining to quantitate cell viability in Molm14 and THP-1 cells infected with GFP or HoxA9 shRNA lentivirus. Indeed, depleting HoxA9 led to apoptotic death in both cell lines, indicating that HoxA9 is necessary for cell survival (Figure 4-10 A,B). To determine whether Bim was required for the cell death initiated by HoxA9 depletion,

we generated Molm14 lines expressing shRNA against Bim or Luciferase control and examined HoxA9-dependent cell survival (Figure 4-10C). Bim-depleted cells exhibited greatly enhanced survival compared to Luciferase-depleted controls upon HoxA9 inhibition, suggesting that *Bim* is a critical target of repression by HoxA9 that induces apoptosis when *HoxA9* function is compromised.

Primary bone marrow cells transduced with MLL-AF9 depend on HoxA9-mediated Bim repression for survival

Retroviral introduction of MLL fusion genes into mouse bone marrow cells followed by transfer into irradiated hosts induces leukemia and has been an important model used to study human leukemias. We used this system to ask whether bone marrow from Bim knockout animals transformed with Mll-Af9 was resistant to HoxA9 withdrawal. As we observed with the MLL fusion-positive cell lines, depletion of HoxA9 in MLL-Af9-transduced wild-type Bim+/+ cells induced Bim expression and cell death (Figure 4-10D). By contrast, HoxA9 depletion in MLL-AF9-transduced bone marrow from *Bim-/-* mice resulted in a decreased amount of cell death compared to *Bim*+/+ cells (Figure 4-10D). This suggests that primary mouse leukemia cells expressing MLL-AF9 are dependent on HoxA9 for *Bim* repression and cell survival.

Primary MLL-rearranged human infant leukemia blast cells also depend on HoxA9 for Bim repression and cell survival

To determine whether primary human leukemia cells depend on HoxA9 for *Bim* repression and cell survival, we obtained MLL-rearranged primary human infant

leukemia blast cells and examined the effects of HoxA9 depletion in these cells. As seen with human cell lines and primary bone marrow cells from mice, human primary leukemia cells also induced expression of *Bim* and cell death upon depletion of HoxA9 (Figure 4-10 E). This suggests that Hox-mediated repression of BH3-only genes is conserved from *C. elegans* to humans and that this mechanism promotes leukemia cell survival when Hox function is activated by mutation (Figure 4-11).

DISCUSSION

In this study, we identified and characterized a *C. elegans* Hox regulatory network that ensures survival of a specific set of six neurons through transcriptional repression of the BH3-only pro-apoptotic gene *egl-1* (Figure 4-11). We identified two new genes required for repression of *egl-1* transcription and programmed cell death of the VC motor neurons, *unc-62/Meis* and *lin-59/Ash1L*, which together with *lin-39/Hox* and *ceh-20/Pbx* make up this regulatory network.

Our data suggest that a complex composed of LIN-39/Hox and CEH-20/Pbx acts in the VC neurons through multiple dispersed and redundant sites to repress *egl-1/BH-3* transcription and prevent programmed cell death. UNC-62/Meis does not appear to be part of this DNA-bound complex, and is instead required for CEH-20/Pbx nuclear localization. LIN-59/Ash1 promotes LIN-39/Hox transcription. The functions of all four proteins in the network are essential for *egl-1/BH3* repression and VC survival.

We had previously described the mechanism whereby P11.aaap is induced to undergo programmed cell death by a complex made up of MAB-5/Hox and CEH-20/Pbx

(Liu et al., 2006). In the P11.aaap cell, binding of this complex to a single site in the *egl-1/BH3* regulatory region is sufficient to induce *egl-1/BH3* transcription and programmed cell death. CEH-20/Pbx is therefore required for *egl-1/BH3* activation and cell death in one cell-type and *egl-1/BH3* repression and cell survival in another. Repression of *egl-1/BH3* by the midbody Hox LIN-39 appears to be dominant over activation of *egl-1/BH3* by the more posterior Hox MAB-5, because misexpression of LIN-39 in the MAB-5 domain in the posterior ventral nerve cord prevents cell death. This is consistent with the theory that Hox-mediated repression dominates over Hox-mediated activation, even when this violates the posterior prevalence model for Hox dominance (Capovilla and Botas, 1998). The ability of LIN-39/Hox to suppress death in cells where it is not usually expressed provides a potential explanation for how mis-expression of Hox genes contributes to human leukemogenesis.

Of six pathways that directly regulate programmed cell death or survival of specific cells in *C. elegans*, all include transcription factors whose human counterparts are oncogenes (Conradt and Horvitz, 1999; Ellis and Horvitz, 1991; Liu et al., 2006; Maurer et al., 2007; Metzstein et al., 1996; Metzstein and Horvitz, 1999; Park et al., 2006). Thus transcriptional regulation of programmed cell death genes by oncogenic transcription factors may be a critical part of their ability to promote malignancy. The mechanism we describe here is strikingly similar to pathways important in human leukemias. Gain-of-function mutations affecting the SET domain gene *MLL,* specific *Hox* genes, and *Pbx1* are common in human leukemias (Pui et al., 2004; Schoch et al., 2003). *MLL* translocations induce abnormally high levels of *Meis1* and posterior *HoxA*

cluster gene expression (Armstrong et al., 2002), and *Meis1* determines critical aspects of the leukemic phenotype (Argiropoulos et al., 2007). The *Hox* genes are critical mediators of the leukemogenic phenotype (Armstrong et al., 2002; Ayton and Cleary, 2003; Ferrando et al., 2003; Kumar et al., 2004); however, the target genes regulated by the Hox proteins and important for the malignant phenotype are essentially unknown, with the exception of the *Pim1* protein kinase (Hu et al., 2007). Perhaps abnormally high levels of Hox activity in some hematopoietic progenitors can prevent their death by repressing BH3-only genes and thus promote leukemogenesis.

In support of the hypothesis that Hox-mediated repression of BH3-only genes contributes to human leukemogenesis, we presented evidence here that this mechanism appears to be conserved in mammals and necessary for survival of MLL-rearranged human leukemia cells (Figure 4-10). Data from human leukemia cell lines, primary mouse bone marrow, and primary human leukemia blast cells all suggest that MLL fusions drive HoxA9 expression, which represses the BH3-only gene *Bim* and promotes cell survival. Upon depletion of HoxA9, Bim levels increase dramatically and cell death ensues. This effect appears to be selective for Bim, as the levels of Noxa and Puma do not increase upon HoxA9 depletion (data not shown). The dependence of primary human leukemia cells on HoxA9 for survival has recently been further investigated in samples from patients with AML (Scott Armstrong, in press). Of 14 primary patient samples, knockdown of HoxA9 by RNAi resulted in apoptosis in seven cases. Interestingly, these seven lines were all positive for MLL rearrangements, whereas samples without MLL rearrangements were not dependent on HoxA9 for survival.

Further research is needed to determine how closely the mechanism in human leukemia cells mirrors that seen in *C. elegans*. For example, does HoxA9 bind the *Bim* promoter in a complex with a mammalian Pbx protein? If so, is this binding accomplished through multiple dispersed and redundant binding sites? Is the critical role of Meis1 in MLL-rearranged leukemias due to its ability to promote nuclear localization of Pbx proteins? Interestingly, Meis1 levels determine the rate of transformation in MLL fusion-induced leukemias, but require Pbx proteins to do so (Wong et al., 2007). The elucidation of the mechanistic details of Hox-mediated inhibition of apoptosis in *C. elegans* provides a framework from which to examine the mechanism for Hox-mediated inhibition of apoptosis in human leukemias.

More importantly, it will be essential to determine how widespread this mechanism is in human cancers and if it can be disrupted therapeutically. Hox overexpression can occur through mechanisms other than MLL rearrangement, and is not restricted to cancers of the hematopoietic system. Thus Hox-mediated inhibition of apoptosis through transcriptional repression of BH3-only genes could be important for survival of cancers other than MLL-rearranged leukemias. If this mechanism could be targeted therapeutically by down-regulating Hox, disrupting Hox-mediated repression of BH3-only genes, or activating or mimicking BH3-only proteins by other methods, cancer cell death would be the expected outcome. Consequently, the repression of BH3-only genes by Hox transcription factors presents a promising new therapeutic target for inducing apoptosis in cancer cells.

MATERIALS AND METHODS

Genetics and lineage analysis

All strains were derived from Bristol N2 wild-type and cultured as described (Brenner, 1974b). Strains were grown at 20° C unless noted otherwise. Alleles used were LGI: *lin-59(n3168); lin-59(n3192); ced-1(e1735).* LGII: *rrf-3(pk1426).* LGIII: *ceh-20(ay9); ceh-20(ay42); lin-39(n1760); unc-36(e251).* LGIV: *ced-3(n717).* LGV: *unc-62(e644); unc-62(e917); unc-62(t2012); dpy-11(e224); egl-1(n1084n3082).* LGX: *lin-15(n765); mxIs14,* an integrated P_{egl-1}*histone:gfp* transgene (Liu et al., 2006); *mxIs28,* an integrated P_{ceh-20}*ceh-20:yfp* transgene; *mxIs31*, an integrated P_{unc-62}*unc-62:cfp* transgene. We also used *deIs1*, an integrated YAC-based P_{lin-39}*lin-39:gfp* transgene (Wagmaister et al., 2006). Lineage analysis was performed as described previously (Sulston and Horvitz, 1977a).

lin-59 alleles

Two alleles of *lin-59* were identified in a screen of mutagenized *ced-1* mutants (Cameron et al, 2002). For characterization and mapping, we used the rectal patterning defect of *n3168* mutants to identify *n3168* homozygotes, and found that 28 of 36 *n3168* progeny of *ced-1 n3168/++* animals were Ced, suggesting linkage to LGI. From *n3168++/+dpy-5 unc-29* heterozygotes, 18 of 18 Unc-29 non-Dpy-5 recombinants carried *n3168*. Zero of 24 Unc-11 non-Dpy-5 recombinants from *n3168++/+unc-11 dpy-5* heterozygotes carried *n3168*. 12 of 12 Lin-17 non-Unc-11 recombinants from *+n3168+unc-29/lin-17+unc-11+* heterozygotes carried *n3168*. In crosses of *qDf3* and

qDf4 males into *n3168 unc-29* hermaphrodites, we failed to obtain identifiable non-Unc-29 cross progeny, suggesting that *qDf3* and *qDf4* delete *n3168*, that *n3168/Df* animals die, and that the *n3168* mutation results in a partial loss of *lin-59* function. In all cases, the ventral nerve cord cell corpse phenotype and rectal defect cosegregated. Sequencing of *lin-59* genomic DNA identified point mutations consistent with EMS-induced changes that result in an R844Opal mutation (in *n3168*) and a G616Amber mutation (in *n3192*).. *lin-59(n3168)* is a R844Opal mutation and *lin-59(n3192)* is a G616Amber mutation.

RNAi and imaging

The screen for regulators of *egl-1* expression was performed by RNAi feeding of *ced-1(e1735); ced-3(n717); mxIs14* or *rrf-3(pk1426); ced-3(n717); mxIs14* mutants, as described (Fraser et al., 2000). L3-stage progeny were observed using epifluorescence and photographed by subtracting the rhodamine filter (emission 610-675) image from the FITC band-pass filter image to reduce background.

Reporter constructs

Reporter constructs were made using standard cloning techniques, introduced into worms using microinjection, and integrated using gamma irradiation. $P_{ceh\text{-}20}$*ceh-20:yfp* was constructed identically to $P_{ceh\text{-}20}$*ceh-20:cfp* (Liu et al., 2006), except that *yfp* from pPD134.99 (a kind gift from Andy Fire, Stanford University, Palo Alto, CA) was cloned into the *Asc*I restriction site. This construct rescued the Egl phenotype

of *ceh-20(ay42)* mutants (data not shown). It was injected into *ced-1; ced-3; lin-15* mutants at 50 ng/µl along with a *lin-15* rescuing plasmid (50 ng/µl), integrated using gamma irradiation, and back-crossed to N2 nine times. P_{unc-62}*unc-62:cfp* was constructed from the cosmid T28F12 and the fosmid WRM061dC01 in several steps. First, a 20 kb *Pme*I-*Sac*II fragment from WRM061dC01 was inserted into pBSKSII+ digested with *Sac*II and *Sma*I (pMP040). Separately, a 12 kb *Sac*II fragment from T28F12 was ligated into the *Sac*II site of pBSKSII+ (pMP041). A 2 kb fragment of pMP041 containing the stop codon of *unc-62* was PCR-amplified and cloned into pBSKSII+ (pMP042), and the stop codon was replaced with an *Age*I restriction site using site-directed mutagenesis (pMP043). The coding region of *cfp* was PCR-amplified from pPD134.96 (a kind gift from Andy Fire) and inserted into the *Age*I site of pMP043 to produce pMP044. The *Nhe*I-*Pac*I fragment of pMP041 containing the *unc-62* termination codon was replaced with the 3 kb *Nhe*I-*Pac*I fragment of pMP044, essentially creating the *unc-62:cfp* fusion (pMP046). Finally, the 13 kb *Sac*II fragment from pMP046 was inserted into the *Sac*II site of pMP042 to produce the final P_{unc-62}*unc-62:cfp* plasmid, pMP062. This construct contains 32 kb of the *unc-62* genomic locus (including the coding region, 14 kb of upstream sequence, and 4 kb of downstream sequence) with a C-terminal *cfp* fusion, in the pBSKSII+ backbone. pMP062 was injected at 10 ng/µl into *lin-15(n765mat,ts)* mutants with a rescuing *lin-15* construct at 50 ng/µl, and integrated using gamma irradiation. The resulting animals were backcrossed to N2 six times. All DNA fragments subjected to PCR amplification were completely sequenced.

Protein purification and EMSA

Amino acids 141-229 of LIN-39 and 188-274 of CEH-20 were purified from *E. coli* using the IMPACT™ Protein Purification Kit (New England Biolabs, Ipswich, MA). EMSA binding reactions were performed at room temperature for 30 min. in 1x TBB (10 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1% NP-40, 5% glycerol), 500 ng poly dIdC, 200 ng LIN-39 where included, 400 or 800 ng CEH-20 where included, 20 fmol biotinylated Antp/Exd probe (Liu and Fire, 2000), and water to 25 µl. The reactions were run on an 8% polyacrylamide native gel in 0.5x TBE (45 mM Tris-borate, 1 mM EDTA) at 4° C and transferred to a nylon membrane that was developed using the LightShift® Chemiluminescent EMSA Kit (Pierce, Rockford, IL).

LIN-39 rescue assay

P_{egl-1}*lin-39* constructs were assembled by replacing the *histone:gfp* insert of pMP001 (P_{egl-1}*histone:gfp*) with *lin-39* cDNA. Site-directed mutagenesis was used to mutate W151 to P or WM to AA. The repression domain of Engrailed (corresponding to amino acids 1-298 of *D. melanogaster* protein) or the activation domain of VP16 (corresponding to amino acids 413-490 of HSV-1 protein) was PCR-amplified with primers containing *Age*I (5') and *Pac*I (3') sites, *lin-39* cDNA was amplified with primers containing *Pac*I (3') and *Age*I (3') sites, and a triple ligation was used to introduce these fragments into the *Age*I-digested pMP001. All constructs were fully sequenced. These were injected into *ced-1(e1735); lin-39(n1760)* animals at 50 ng/µl along with P_{myo-3}*gfp* (50 ng/µl) as a coinjection marker. The transgenic lines were

identified by GFP fluorescence, and a minimum of three independent lines per construct was scored for corpse pattern in the ventral nerve cord. We were unable to perform similar rescue experiments with *ceh-20/Pbx* and *unc-62/Meis* mutants because of the lethality associated with loss-of-function of either gene, as has been previously observed when attempting to construct transgenic animals with these mutants (Van Auken et al., 2002).

Chromatin Immunoprecipitation

Chromatin immunoprecipitations were performed using the anti-AFP antibody (Qbiogene, cat. no. AFP5002) and analyzed using real-time PCR. **Chromatin preparation.** Mixed stage P_{lin-39}*lin-39:gfp*, P_{ceh-20}*ceh-20:yfp* and P_{unc-62}*unc-62:cfp* animals were grown at 22.5°C, harvested and flash frozen with liquid nitrogen. 600 µL of frozen worms were subsequently ground into fine powder with a mortar and pestle. The powder was washed once with 4 mL of ice-cold M9 buffer. 4 mL of 1% formaldehyde in M9 was used for a 10 minute fixation at room temperature, and then the reaction was quenched for 5 minutes with 1.714 mL of 1 M glycine. After 10 minutes on ice, the extract was washed once with 4 mL of ice cold M9 buffer and twice with 4 mL of ice cold FA Buffer (50 mM HEPES/KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate). The extract was resuspended to 1.5 mL with ice cold FA Buffer plus 64 µL of 25x protease inhibitor cocktail (Roche, cat. no. 11873580001) and sheared to 100 bp-1000 bp size range with an Ultrasonic Processor sonicator. An output of 8 Watts was used for sonication (ten cycles of 30 s pulses followed by 2.5 min breaks in ice water). Cellular debris was removed by centrifugation

at 14,000 rpm for 30 minutes at 4°C, and the supernatant was frozen at –80°C.

Immunoprecipitation. 1 μg of anti-AFP antibody (Qbiogene, cat. no. AFP5002) or 1 μg of mouse IgG (Innovative research, cat. no. Ir-Ms-Gf) and 25 μL of protein A-sepharose beads (Invitrogen, cat. no. 10-1041) were incubated in 275 μL of FA Buffer for 2 hours rotating at 4°C. These beads were then washed with FA Buffer three times and blocked with herring sperm DNA (200 μg/mL) and BSA (0.1 mg/mL) in FA Buffer for 2 hours rotating at 4°C. The beads were washed with FA Buffer three times. The chromatin was diluted in six mL of FA Buffer containing 1x protease inhibitor cocktail and pre-cleared with 200 μL of 50% protein A-sepharose beads in FA Buffer rotating at 4°C for 2 hours. The pre-cleared chromatin was centrifuged at 2000 rpm at 4°C for 5 minutes. 10% of the supernatant was saved as input, and the remaining material was divided into two equal volumes. IgG-bound beads were added to one fraction of chromatin while anti-AFP-bound beads were added to the other fraction, and both were incubated overnight rotating at 4°C. **DNA Recovery.** The beads were centrifuged at 2000 rpm at 4°C for 5 minutes and then sequentially washed at 4°C with 1 mL of the following buffers: FA Buffer with 0.1% SDS (5 min, 2x), FA Buffer with 1M NaCl (5 min), FA Buffer with 500 mM NaCl (10 min), TEL Buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0) (10 min), and TE pH 8.0 (10 mM Tris pH 8.0, 1 mM EDTA) (5 min, 2x). Complexes were eluted from the beads with 100 μL Elution Buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% SDS) at 65°C for 10 minutes. After centrifugation at 2000 rpm at room temperature for 5 minutes, the supernatant was added to 80 μL TE pH 7.5 and 20 μL proteinase K (20 mg/mL).

Cross-linked polypeptides were digested at 42°C for 2 hours, and cross-links were reversed overnight at 65°C. 20 μL of 4 M LiCl was added and the DNA was purified by phenol/chloroform extraction. The DNA was dissolved in 35 μL of water and 2 μL was used for each quantitative PCR reaction.

RNA extraction and quantitative PCR

RNA was extracted from mixed-stage worms using TRIzol® Reagent (Invitrogen, Carlsbad, CA). RNA was treated with DNaseI (Invitrogen) and converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). An amount of cDNA corresponding to 50 ng of input RNA was used for each quantitative PCR reaction. Quantitative PCR was performed using the delta-delta Ct method with Power SYBR® Green PCR Master Mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions.

For quantitative RT-PCR from mammalian cells, total RNA was isolated using Trizol (Invitrogen), and cDNA was generated using the Retroscript kit (Ambion). Quantitative PCR was performed using SYBR GreenER (Invitrogen) on an ABI Prism 7700 (Applied Biosystems). Primer sequences are available upon request.

Cell culture and virus production

The Molm14 cell line was maintained in RPMI (Invitrogen) with 10% Fetal Calf Serum (Invitrogen) and supplemented with 100 units per ml penicillin/streptomycin (Invitrogen) and 2mM L-glutamine (Invitrogen). Bone marrow from Bim-/- mice was a

gift from Dr. Anthony Letai The murine cells were cultured in 'C10' media containing RPMI, 10% FCS, 100 units per ml penicillin/streptomycin, 2mM L-glutamine, 55 mM b-mercaptoethanol (Invitrogen), 0.1mM nonessential amino acids (Invitrogen), 1mM sodium pyruvate, (Invitrogen) and 10mM HEPES (Invitrogen), and supplemented with murine cytokines:10 ng/ml IL3, 10 ng/ml IL6, 10 ng/ml SCF (cytokines from Peprotech). Human patient sample cells were cultured in RPMI with 10% FCS, 10% Horse Serum (Invitrogen), 100 units per ml penicillin/streptomycin and 2mM L-glutamine, and supplemented with human cytokines: 2ng/ml IL3, 2ng/ml IL6, 5ng/ml SCF (cytokines from Peprotech).

pLKO-HoxA9-1F2 and pLKO-HoxA9-1F3, pLKO-GFP, pLKO-Luciferase, and pLKO-Bim were obtained from the RNAi Consortium (Broad Institute, MIT), and lentiviruses were produced as described previously (Krivtsov et al). Murine retroviruses were produced as described previously (Stubbs et al.). VSVG pseudotyped retroviruses from pMKO-HoxA9 shRNA or pMKO-neo vector control (Wang et al.) were produced as described above, with the addition of 5μg of vsvg expression plasmid to the transfections. The HoxA9 shRNA expressed from pLKO-1F3 and pMKO corresponds to the sequence 5'-GTGGTTCTCCTCCAGTTGATA-3' which is specific for and active against both murine and human HoxA9 RNA.

Cell survival and Annexin V analyses

For cell viability, cells were counted with a hemacytometer (VWR) using light microscopy and Trypan Blue (Invitrogen) exclusion. For apoptosis analyses, cells were harvested, washed with PBS (Invitrogen) and resuspended in annexin V buffer (140mM

NaCl (Fisher), 10mM HEPES (Invitrogen), 2.5mM CaCl2 (Fisher)) with annexin V-Cy3 reagent (Biovision) and analyzed on a FACSCalibur (Becton Dickenson).

Western blots

Cells were harvested 72 hours post infection and equivalent cell numbers were lysed in RIPA buffer (150mM NaCl, 1% IPEGAL (Sigma), 0.5% deoxycholate (Sigma), 0.1% SDS (Invitrogen), 50mM Tris pH 8.0 (Sigma))with Complete protease inhibitor cocktail (Roche), denatured and run on a 10% Acrylamide Bis-Tris gel (Invitrogen) and transferred to nitrocellulose (Whatman) for Western analyses. Rabbit anti-Bim (Calbiochem), rabbit anti-HoxA9 (Millipore-Upstate), mouse anti-pan Actin (Chemicon) were used according to manufacturer specifications.

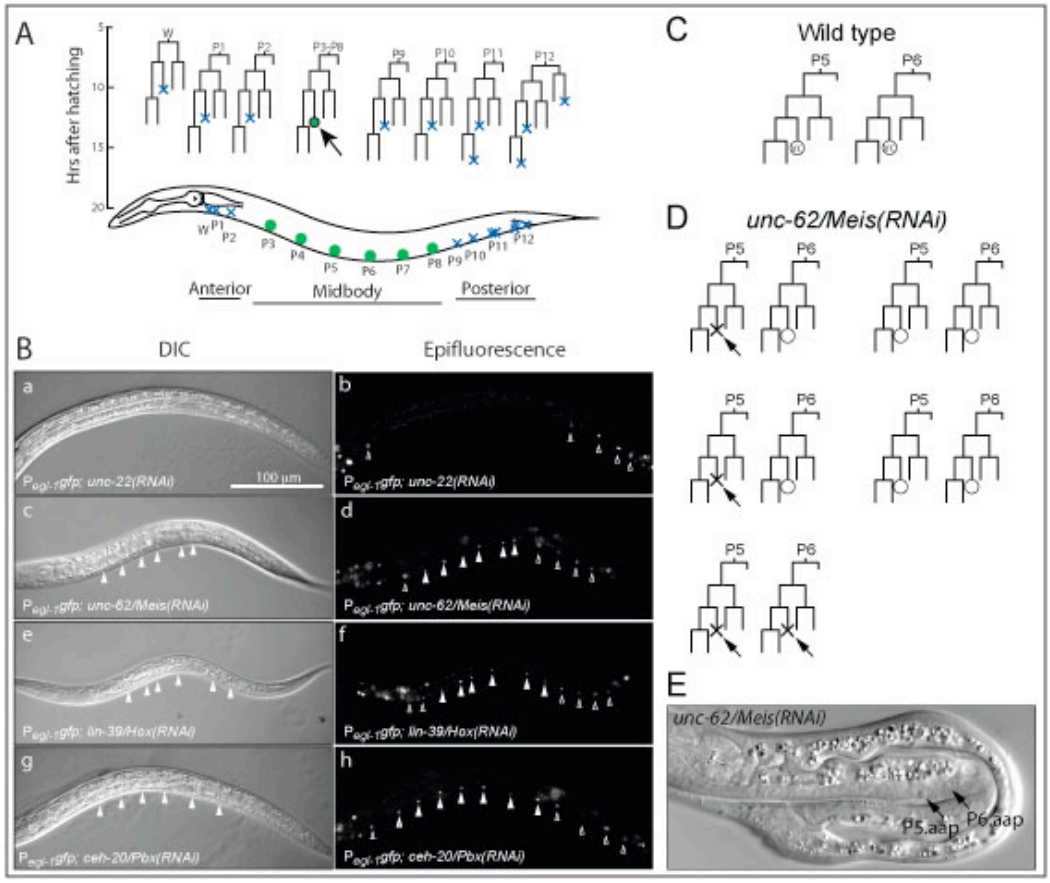


Figure 4-1. *unc-62/Meis*, *lin-39/Hox*, and *ceh-20/Pbx* are required for *egl-1/BH3* repression and survival of the VC neurons.
(A) Lineages of the ventral nerve cord are shown schematically, with horizontal lines representing cell divisions and vertical lines representing time. The anterior, midbody, and posterior regions are indicated. X: cells that undergo programmed cell death. Circles: VC motor neurons. (B) DIC (a,c,e,g) and epifluorescence (b,d,f,h) images of L3-stage hermaphrodites carrying the $P_{egl\text{-}1}gfp$ reporter and exposed to the indicated RNAi. Open arrowheads: cells that die in wild type animals. Filled arrowheads: cells that survive in wild type animals. Only fluorescent cells of the ventral nerve cord are highlighted. Scale bar in (a) applies to all panels. Anterior is leftwards, ventral is downwards. (C) P5 and P6 cell lineages of wild-type animals, in which P5.aap (the posterior daughter of the anterior daughter of the anterior daughter of P5) and P6.aap survive and differentiate into VC neurons (Sulston and Horvitz, 1977a). (D) Lineages of the P5 and P6 cells of five N2 animals exposed to *unc-62/Meis(RNAi)*. Cells that underwent programmed cell death are indicated by an X and highlighted by arrows. (E) DIC image animal in which both P5.aap and P6.aap died. Arrows highlight the two corpses.

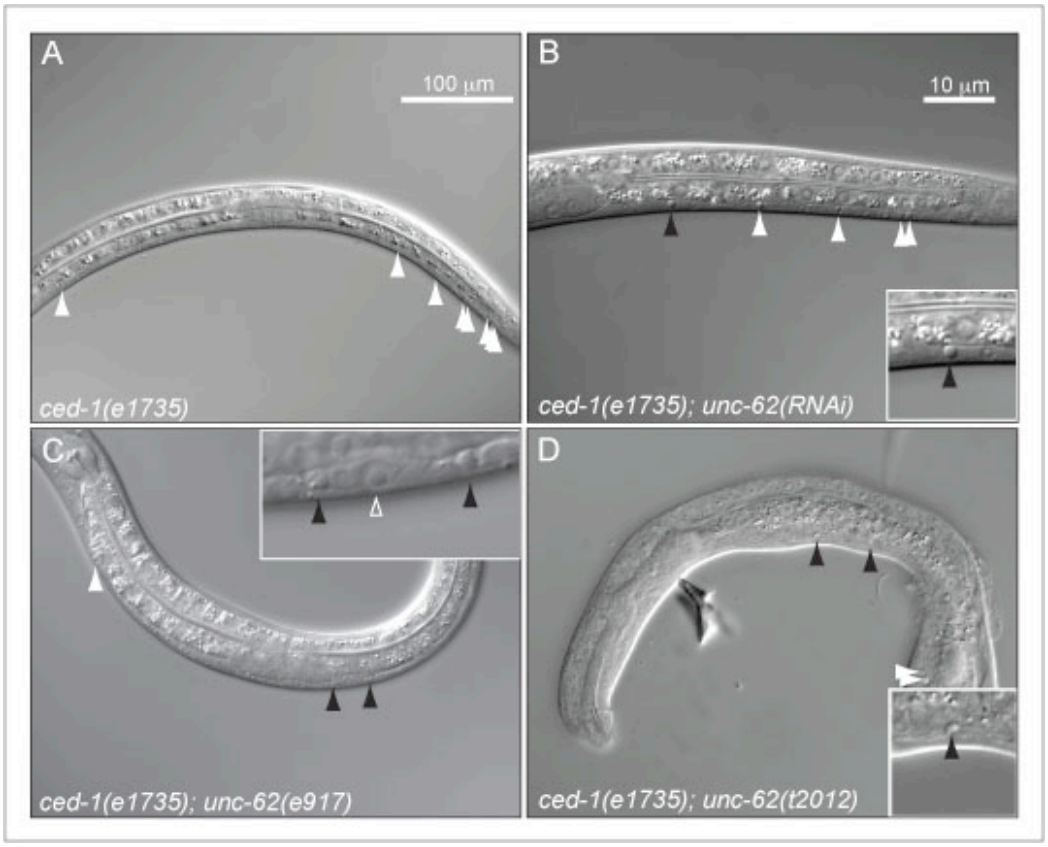


Figure 4-2. *unc-62/Meis* is required for survival of cells in the midbody region of the ventral nerve cord.
White arrowheads indicate corpses of cells that die in the wild type in the anterior and posterior regions of the ventral nerve cord. Black arrowheads indicate ectopic corpses in the midbody region of the ventral cord, where no cell death occurs in wild-type animals. (A) In *ced-1(e1735)* mutants, cell deaths produce corpses in the anterior and posterior regions of the ventral nerve cord, but all midbody P cell descendants survive and corpses are never observed in the midbody region. (B) *unc-62(RNAi)* results in the presence of corpses in the midbody region of the ventral nerve cord. Black arrow highlights a corpse in the P8 cell lineage, which in the wild type never has a cell that undergoes programmed cell death. (C,D) Two alleles of *unc-62/Meis*, *e917* (in panel C) and *t2012* (in panel D), also show corpses in the midbody region of the ventral nerve cord. In (C), the open arrowhead indicates a healthy hypodermal nucleus with pronounced nucleolus, not a cell corpse. Scale bar in (B) applies to (C) and (D) as well.

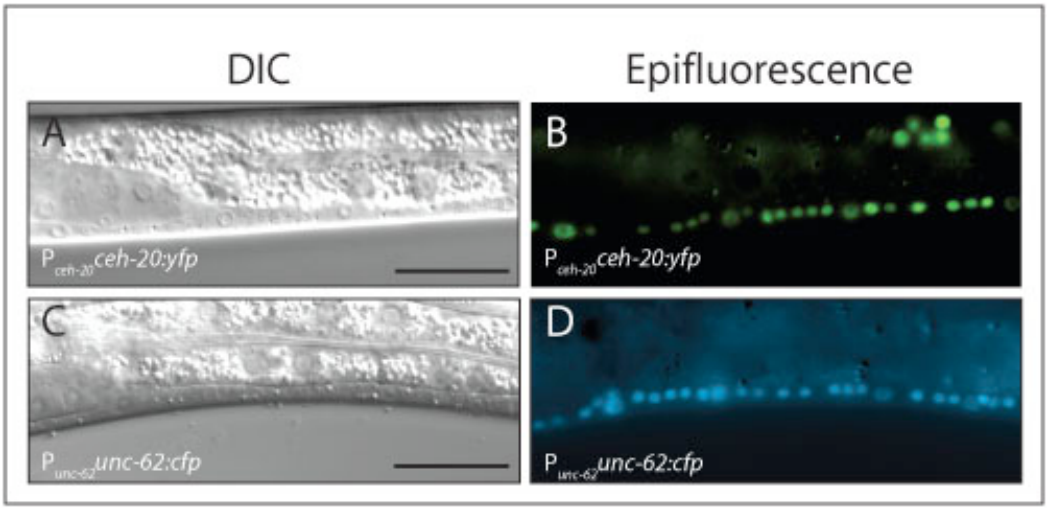


Figure 4-3. *ceh-20/Pbx* and *unc-62/Meis* are expressed in the VC neurons and many other cells.
L3-stage hermaphrodites expressing rescuing translational reporters for CEH-20/Pbx (A,B) or UNC-62/Meis (C,D) were photographed using DIC (A,C) or epifluorescence (B,D) optics. Both *ceh-20/Pbx* (A,B) and *unc-62/Meis* (C,D) are expressed in all nuclei in the ventral nerve cord, including the VC neurons. Scale bars represent 10 μm.

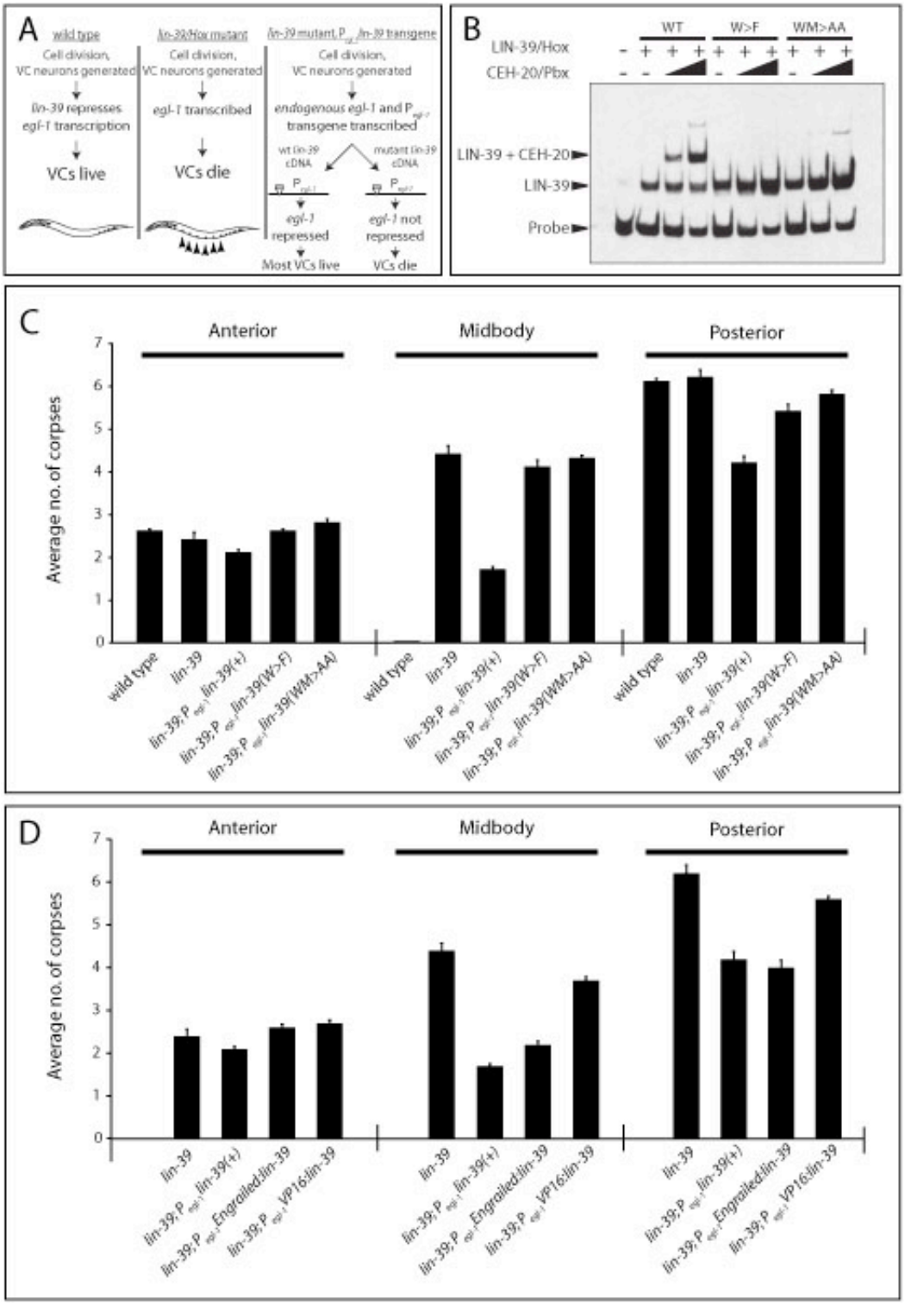

A
wild type
lin-39/Hox mutant
lin-39 mutant, P egl-1 lin-39 transgene
Cell division, VC neurons generated
lin-39 represses egl-1 transcription
VCs live
egl-1 transcribed
VCs die
endogenous egl-1 and P egl-1 transgene transcribed
wt lin-39 cDNA
mutant lin-39 cDNA
egl-1 repressed
egl-1 not repressed
Most VCs live
VCs die
B
WT
W>F
WM>AA
LIN-39/Hox
CEH-20/Pbx
LIN-39 + CEH-20
LIN-39
Probe
C
Anterior
Midbody
Posterior
Average no. of corpses
wild type
lin-39
lin-39; P egl-1 lin-39(+)
lin-39; P egl-1 lin-39(W>F)
lin-39; P egl-1 lin-39(WM>AA)
D
Anterior
Midbody
Posterior
Average no. of corpses
lin-39
lin-39; P egl-1 lin-39(+)
lin-39; P egl-1 Engrailed:lin-39
lin-39; P egl-1 VP16:lin-39

Figure 4-4. LIN-39/Hox cooperates with CEH-20/Pbx and represses transcription to promote VC survival.
(A) Schematic of *lin-39/Hox* rescue assay. In wild-type animals (left column), *lin-39/Hox* represses *egl-1/BH3* in the VC neurons, and the cells live. In *lin-39/Hox* mutants (middle column), *egl-1/BH3* transcription is initiated and the VC neurons die shortly after being born. To rescue VC death in the *lin-39/Hox* mutant animals (right column), we introduced P_{egl-1}*lin-39* to drive *lin-39/Hox* cDNA from the *egl-1/BH3* promoter. In these worms, *lin-39/Hox* transcription (from the transgene) and *egl-1/BH3* transcription (from the endogenous locus) are concurrently initiated in the VC neurons, but subsequent production of functional LIN-39/Hox leads to repression of *egl-1/BH3* and a reduction in VC cell death. (B) Electrophoretic mobility shift assay (EMSA) was performed using a Pbx/Hox consensus probe and purified LIN-39/Hox and CEH-20/Pbx protein fragments. Increasing quantities of CEH-20/Pbx were added to a fixed amount of LIN-39/Hox. (C) Number of corpses was scored in three regions of the ventral nerve cord in *ced-1(e1735)* animals; anterior (W, P1, and P2 descendants), midbody (P3-P8 descendants) and posterior (P9-P12 descendants). The ability of wild-type LIN-39, LIN-39(W>F) and LIN-39(WM>AA) to rescue VC death in the *lin-39/Hox* mutant was examined. Bars represent corpse number ± s.e.m. (D) Using the same assay, the rescuing ability of Engrailed:LIN-39 and VP16:LIN-39 was assayed. For C and D, at least three independent lines (20 worms per line) were scored per transgene (see Supplemental Figure 3 for data from individual lines).

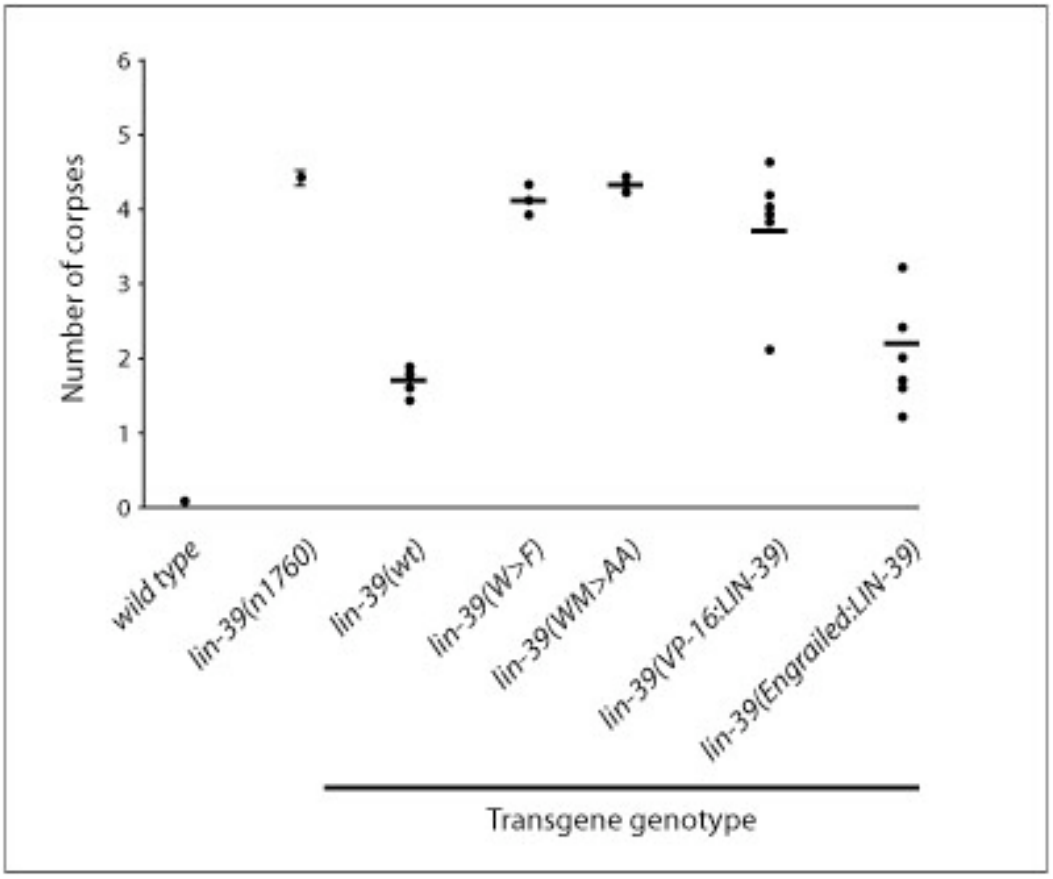


Figure 4-5. To inhibit death of the VC neurons, LIN-39/Hox binds CEH-20/Pbx cooperatively and acts as a transcriptional repressor.
Corpses in the midbody region of the ventral nerve cord were scored in *ced-1* mutants of the indicated genotypes. For the transgenic lines, we scored multiple lines for each genotype, and the average number of corpses for each transgenic line is indicated by a dot. Horizontal bars represent the average number of corpses for each genotype. For *lin-39(VP-16:LIN-39),* one transgenic line was different from all the others, and rescued for unclear reasons. A minimum of 20 animals was scored per line.

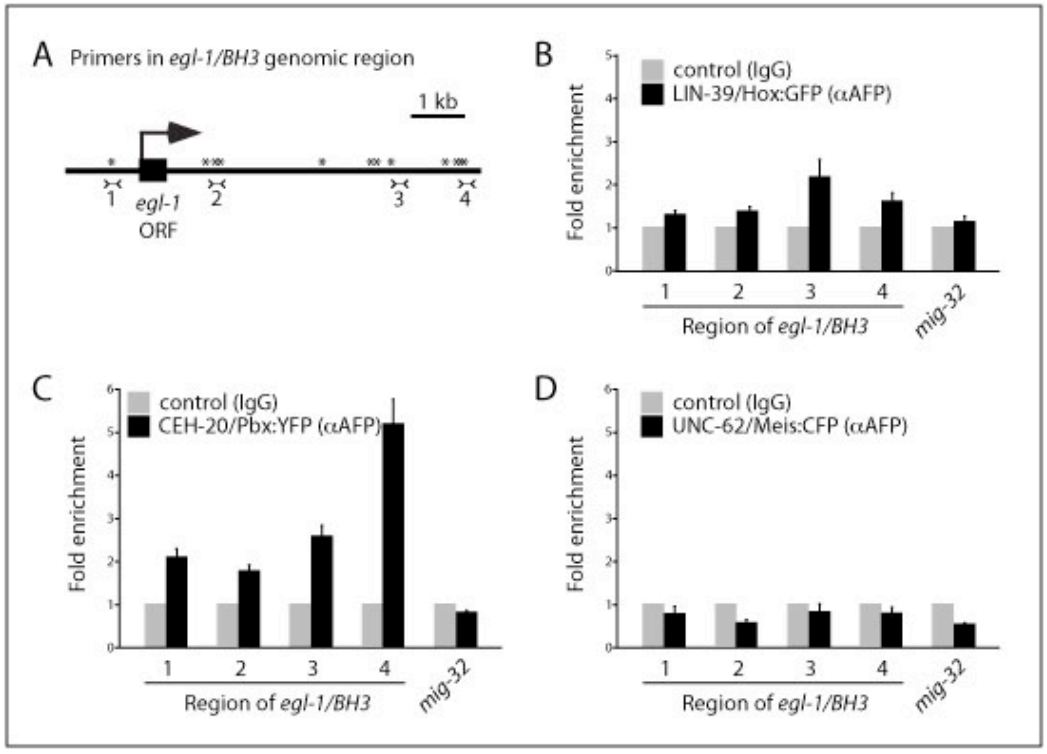


Figure 4-6. LIN-39/Hox and CEH-20/Pbx bind to the *egl-1/BH3* locus at multiple sites *in vivo*.

(A) Four primer sets (1-4) were designed to recognize widely-spaced regions of the *egl-1/BH3* genomic locus. Their location within the 7.66 kb minimal rescuing fragment of *egl-1/BH3* is shown. Asterisks denote evolutionarily conserved consensus Hox-Pbx binding sites (see Supplemental Results) (B) Chromatin immunoprecipitation (ChIP) was performed on animals carrying the P_{lin-39}*lin-39:gfp* construct, using anti-AFP antibody or control IgG. Immunoprecipitated DNA was quantitated by real-time PCR using the indicated *egl-1/BH3* primer sets or an irrelevant control (*mig-32)*. Results are plotted as fold-enrichment of anti-AFP IP versus control IgG IP. ChIP was repeated as in (B) using worms carrying the P_{ceh-20}*ceh-20:yfp* rescuing reporter (C) and the P_{unc-62}*unc-62:cfp* rescuing reporter (D). The average fold-enrichment from at least three independent experiments ± s.e.m is shown. The experiments shown in this figure were performed by David Wang.

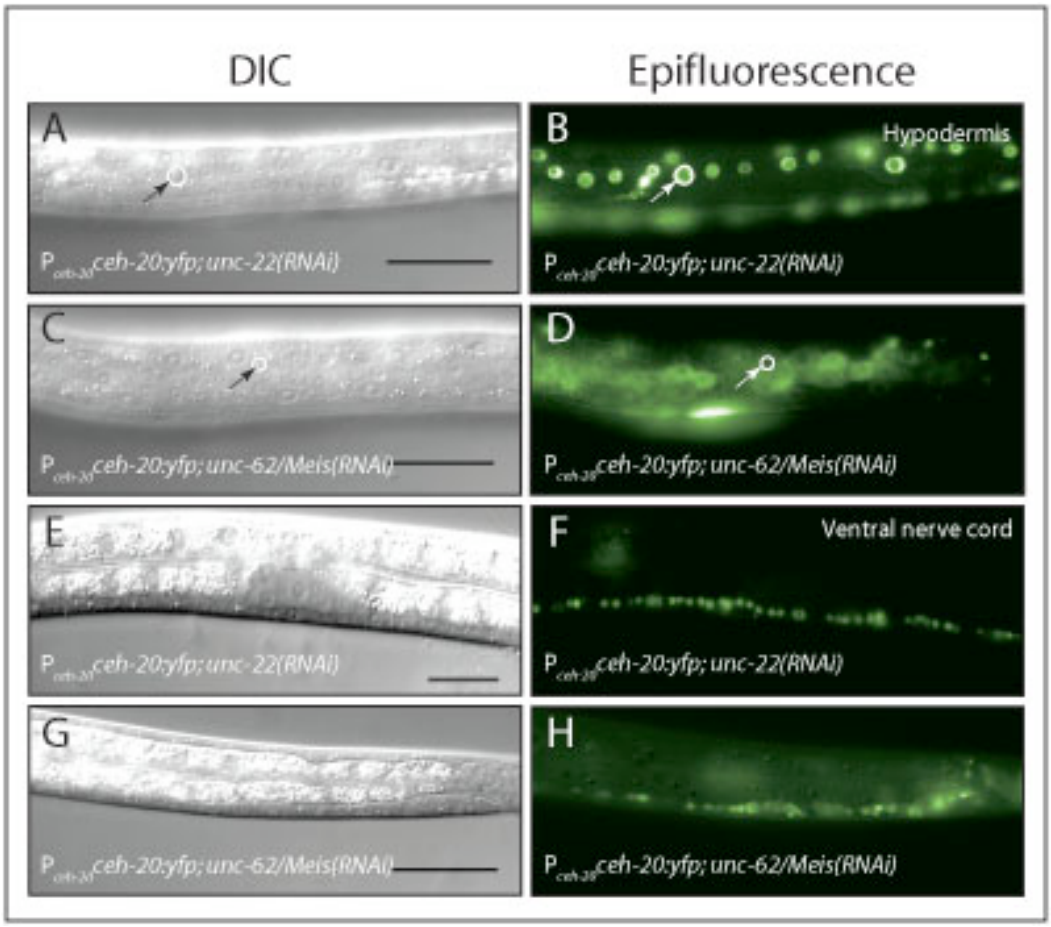


Figure 4-7. *unc-62/Meis* is required for nuclear localization of CEH-20/Pbx. L3-stage hermaphrodites expressing a rescuing translational reporter for CEH-20/Pbx were photographed using DIC (A,C,E,G) or fluorescence (B,D,F,H) optics. (A,B) CEH-20/Pbx is highly enriched in the nuclei of lateral hypodermal cells in animals fed control *unc-22(RNAi)*. A single nucleus is outlined in white and highlighted by an arrow. Nucleoli are prominent in the hypodermal cells and CEH-20/Pbx is relatively excluded from them. (C,D) *unc-62/Meis(RNAi)* results in loss of nuclear localization of CEH-20/Pbx in the lateral hypodermal cells. A single nucleus is outlined in white and highlighted by an arrow. (E,F) CEH-20/Pbx shows nuclear localization in the ventral nerve cord of worms fed control *unc-22(RNAi)*. (G,H) In worms fed RNAi to *unc-62/Meis*, nuclear CEH-20/Pbx is variably lost in neurons and hypodermal cells of the ventral nerve cord. 97 of 97 neuronal nuclei in the ventral nerve cord of P_{ceh-20}*ceh-20:yfp*; *unc-22(RNAi)* animals were obviously fluorescent, whereas eight of 104 neuronal nuclei of P_{ceh-20}*ceh-20:yfp; unc-62/Meis(RNAi)* lacked nuclear fluorescence completely and most others showed diminished nuclear fluorescence. Scale bars represent 10 µm.

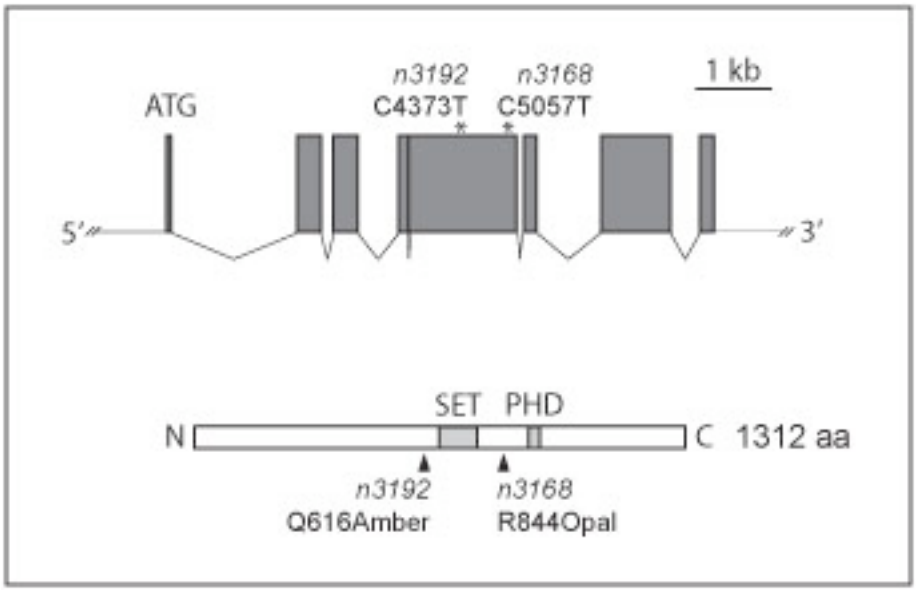


Figure 4-8. Description of *lin-59/Ash1* alleles.
Both *n3192* and *n3168* are predicted to produce premature stop codons in Exon 5 of *lin-59/Ash1* as indicated. If transcribed and translated, *n3192* would truncate the LIN-59 protein just N-terminal to the SET domain, and *n3168* would truncate LIN-59 between the SET and PHD domains.

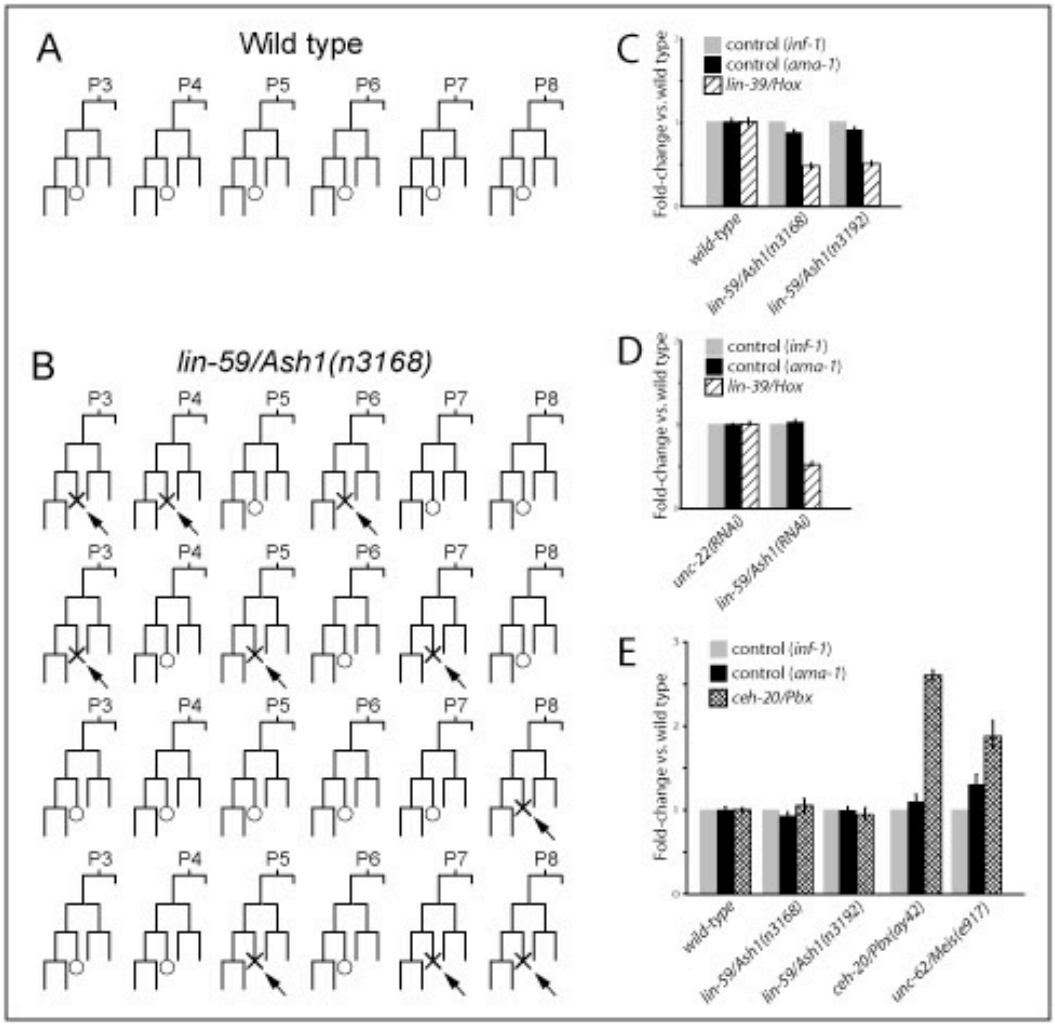


Figure 4-9. The trithorax group gene *lin-59/Ash1* is required for VC survival and maintenance of *lin-39/Hox* mRNA levels.
(A) The P3-P8 cell lineages of wild-type animals (Sulston and Horvitz, 1977a). Pn.aap cells survive and become VC neurons, indicated by open circles. (B) Cell lineages of four *lin-59(n3168)* mutants. Arrows highlight programmed cell deaths (X). Open circles indicate Pn.aap cells that survive. (C) qRT-PCR was used to quantitate mRNA levels of *lin-39/Hox* and two control genes from animals of the indicated genotypes. Error bars represent s.e.m. (D) Relative mRNA levels from control *unc-22(RNAi)* and *lin-59/Ash1(RNAi)* animals. (E) Relative mRNA levels of *ceh-20/Pbx* and two controls from mutants of the indicated genotypes. In (C-E), data are normalized to *inf-1* and expressed as average fold-change versus wild type.

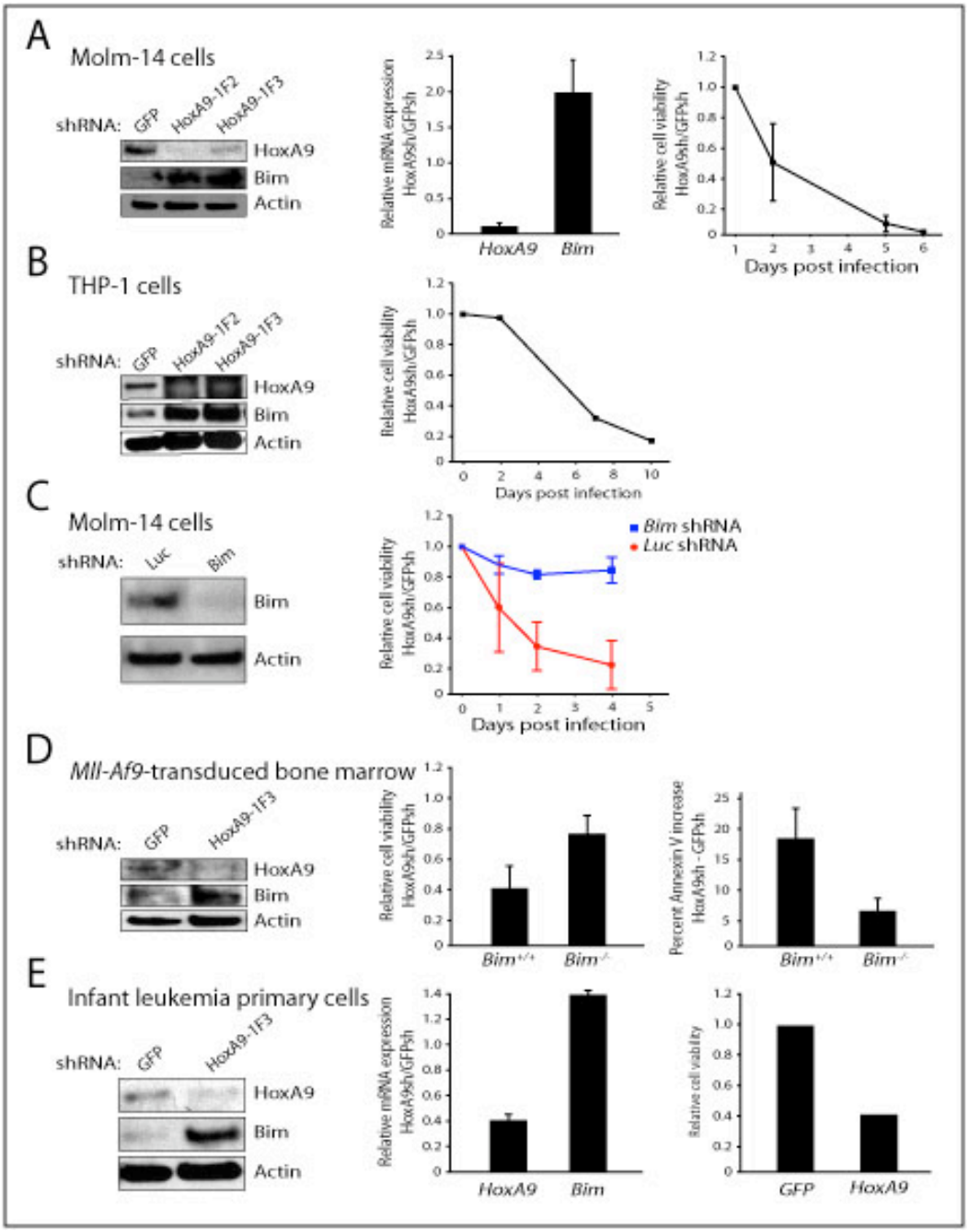


Figure 4-10. HoxA9 represses transcription of the BH3-only gene *Bim* and promotes survival in mammalian cells transformed by MLL fusions.
(A) Molm14 cells were transduced with lentivirus expressing shRNAs targeting GFP (control) or HoxA9 (1F2 and 1F3) and harvested after 72 hours. Left panel shows

western blot analyses for HoxA9 and Bim levels, with Actin loading control. Middle panel shows quantitative RT-PCR for HoxA9 and Bim transcript levels (HoxA9 shRNA/control) standardized to GAPDH. Right panel shows time course survival of infected Molm14 cells as determined by Annexin V staining. (B) THP-1 cells were transduced as in A. Left panel shows western blots for protein levels. Right panel shows time course survival of infected THP-1 cells as determined by Annexin V staining. (C) Molm14 cell lines were transduced with lentiviral shRNA constructs targeting *Luciferase* (*Luc*) or *Bim*. Left panel shows western blots for protein levels. The cells were then transduced with retroviruses expressing shRNA targeting *GFP* or *HoxA9*. Cell counts using trypan blue were performed at indicated time points. The graph represents survival of *HoxA9* shRNA-transduced cells relative to *GFP* shRNA-transduced controls. (D) Bone marrow from B6/129 $Bim^{+/+}$ and $Bim^{-/-}$ mice was transduced with *MLL-AF9* retrovirus. Transformed cells were then transduced with *GFP* shRNA or *HoxA9* shRNA lentiviruses. Left panel shows western blots for protein levels. Middle panel shows survival of cells at four days, determined by trypan blue exclusion and cell counting. Right panel shows percentage of Annexin V-positive cells at four days. (E) Primary *MLL*-rearranged infant leukemic blast cells were transduced with *GFP* shRNA or *HoxA9* (1F3) shRNA lentiviruses. Cells were harvested at 48 hours after infection. Left panel shows western blots for protein levels. Middle panel shows quantitative RT-PCR for *HoxA9* and *Bim* transcript levels. Right panel shows cell viability using trypan blue of the cells 24 hours after infection. The experiments shown in this figure were performed by Matthew Stubbs and Joerg Faber in Scott Armstrong's laboratory.

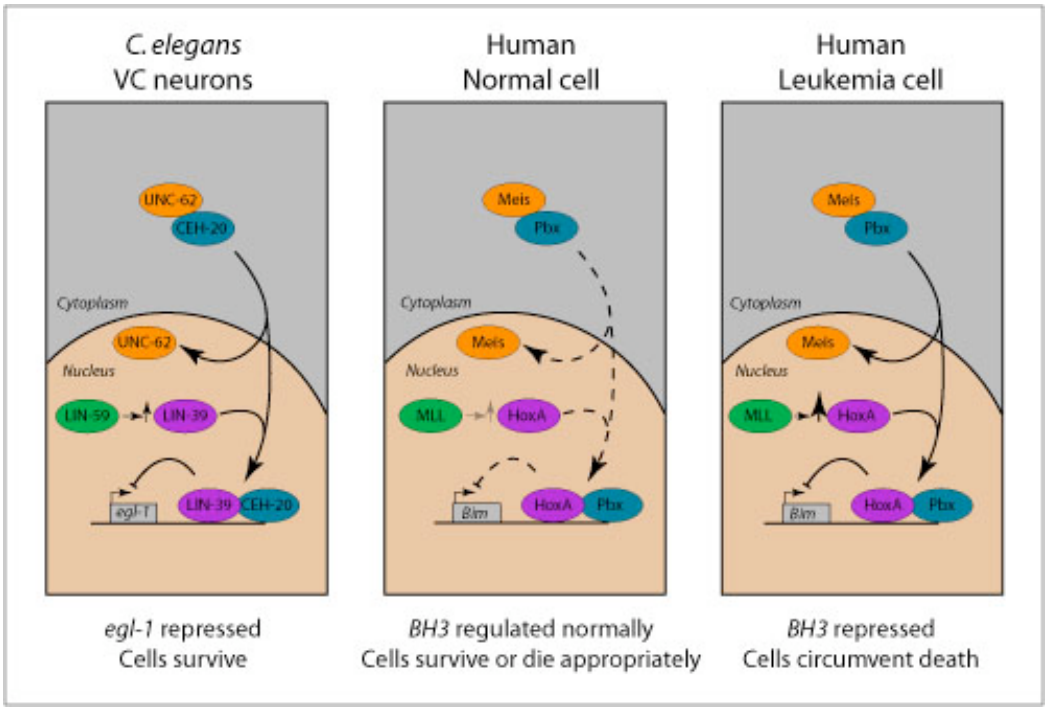


Figure 4-11. In the VC neurons of *C. elegans* (left panel), survival depends on the activities of four proteins.
UNC-62/Meis promotes nuclear localization of CEH-20/Pbx. LIN-59/Ash1 maintains transcription of *lin-39/Hox*. LIN-39/Hox and CEH-20/Pbx bind the *egl-1/BH3* promoter and repress transcription. This prevents the initiation of programmed cell death, and the VC neurons survive. A similar mechanism may operate in human cells during normal development (middle panel), where it could prevent death of specific cells during development, but allow death when the mechanism is appropriately inactivated developmentally. However, when translocations or other mutations upregulate Hox activity and prevent inactivation of this mechanism (right panel), Hox-mediated repression of BH3 genes provides a survival advantage to the leukemic cells.

A *egl-1/BH3* transcription

Genotype	Number of nuclei expressing $P_{egl-1}gfp$		
	Anterior†	Midbody	Posterior
unc-22(RNAi)	3.9 ± 0.2	0.0 ± 0.0	6.8 ± 0.1
lin-39/Hox(RNAi)	4.3 ± 0.2	4.8 ± 0.2	6.9 ± 0.1
ceh-20/Pbx(RNAi)	4.9 ± 0.2	4.8 ± 0.3	6.0 ± 0.2
unc-62/Meis(RNAi)	5.0 ± 0.2	3.2 ± 0.3	6.0 ± 0.2
lin-39/Hox(n1760)	4.2 ± 0.2	6.0 ± 0.0	6.8 ± 0.1
ceh-20/Pbx(ay9)	4.6 ± 0.2	5.0 ± 0.2	6.4 ± 0.1
ceh-20/Pbx(ay42)	2.8 ± 0.2	5.7 ± 0.1	4.3 ± 0.1
unc-62/Meis(e644)	2.6 ± 0.2	0.2 ± 0.1	6.6 ± 0.1
unc-62/Meis(e917)	4.5 ± 0.3	1.3 ± 0.2	6.7 ± 0.2
*unc-62/Meis(t2012)**	1.9 ± 0.2	0.7 ± 0.1	4.4 ± 0.2

B Cell death

Genotype	Number of corpses		
	Anterior†	Midbody	Posterior
unc-22(RNAi)	2.4 ± 0.2	0.0 ± 0.0	5.1 ± 0.2
unc-22(RNAi); egl-1/BH3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
lin-39/Hox(RNAi)	3.0 ± 0.1	3.3 ± 0.3	5.2 ± 0.2
lin-39/Hox(RNAi); egl-1/BH3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
ceh-20/Pbx(RNAi)	2.8 ± 0.1	1.8 ± 0.3	4.7 ± 0.2
ceh-20/Pbx(RNAi); egl-1/BH3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
unc-62/Meis(RNAi)	2.2 ± 0.2	0.9 ± 0.2	4.8 ± 0.2
unc-62/Meis(RNAi) egl-1/BH3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
lin-59/Ash1(RNAi)	2.6 ± 0.1	2.8 ± 0.2	3.4 ± 0.3
lin-59/Ash1(n3192)	2.5 ± 0.1	1.4 ± 0.2	4.4 ± 0.2
lin-59/Ash1(n3168)	2.9 ± 0.1	1.7 ± 0.2	4.4 ± 0.1

Table 4-1 Hox and Hox cofactor genes repress *egl-1/BH3* transcription and are required for cell survival in the midbody.

(A) Numbers of nuclei expressing $P_{egl-1}gfp$; mean ± s.e.m. Complete genotypes were as indicated plus *ced-3(n717);* $P_{egl-1}gfp$. Rows one to five also included *ced-1(e1735)*, row seven included *unc-36(e251)*, and row ten included *dpy-11(e224)*. Animals were scored as L3 larvae. n=30 for *unc-22(RNAi)*; n=50 for all others. *Animals of this genotype exhibited extremely abnormal and variable body morphology; as a result, nuclei numbers may be artificially low because the ventral nerve cord was difficult to identify. (B) Number of corpses; mean ± s.e.m. All strains were homozygous for *ced-1(e1735)*. The allele of *egl-1* used was *n1084n3082*. n=18 for *lin-59(RNAi)*, n=30 for all others. †The anterior ventral nerve cord includes the retrovesicular ganglion, in which some cells die embryonically (Sulston et al., 1983b). The persistence of corpses in this region through to the L3 larval stage is variable.

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

REGULATION OF PROGRMMED CELL DEATH IN *C. ELEGANS*

Background

The genetic pathway by which cells undergo PCD, or apoptosis, was initially discovered in *C. elegans* (Metzstein et al., 1998). It quickly became apparent that this pathway was evolutionarily conserved and important for human health (Horvitz, 2003). The core PCD pathway in *C. elegans* consists of the products of four genes. CED-3 is a caspase that, when activated, kills the cell by cleaving multiple protein targets (Ellis and Horvitz, 1986; Yuan et al., 1993). CED-4, an Apaf-1 ortholog, facilitates activation of CED-3 (Chinnaiyan et al., 1997; Seshagiri and Miller, 1997; Zou et al., 1997). CED-9, an anti-apoptotic member of the Bcl-2 protein family, inhibits CED-4 to prevent activation of CED-3 (Hengartner et al., 1992; Hengartner and Horvitz, 1994; Spector et al., 1997). EGL-1, a pro-apoptotic BH3-only member of the Bcl-2 protein family, disengages CED-9, freeing CED-4 to facilitate activation of CED-3 and cell death (Conradt and Horvitz, 1998). Organismal survival, as well as survival of many (if not all) somatic cells in the animal, depends on the activity of CED-9 (Hengartner et al., 1992). All somatic cell deaths depend on the activities of EGL-1, CED-4, and CED-3 (Conradt and Horvitz, 1998; Ellis and Horvitz, 1986). Thus the fate of each of the 1090 somatic cells born during the development of a *C. elegans* hermaphrodite depends on proper regulation of these four components of the PCD pathway.

So how is it that 131 cells die and 959 survive in every wild-type hermaphrodite? How are the core components of the PCD pathway regulated to instruct each individual cell to adopt its proper fate? One could imagine numerous scenarios through which this specification might occur. For example, the pro-apoptotic proteins CED-3, CED-4, and EGL-1 could be absent from all somatic cells except those instructed to die, in which a transcriptional or post-transcriptional program could be initiated, leading to production or stabilization of these proteins. Alternatively, these proteins could be present in all cells but inhibited by CED-9 and some as yet unknown inhibitor of EGL-1 function, and a death signal could inactivate these inhibitors of apoptosis to allow PCD to occur in specific cells. Perhaps the "ground state" is death, and survival signals are necessary to initiate pro-survival signaling in cells that live. Another possibility is that CED-9, CED-4, and CED-3 are ubiquitously expressed, while the cell-specific transcriptional status of *egl-1* determines the fate of each individual cell. The expression patterns of these four proteins have been extraordinarily difficult to determine, so distinguishing between these various possibilities has not been trivial.

When my dissertation research began, two cell-specific PCD regulatory pathways had been described, and both involved transcriptional regulation of *egl-1*. In the HSN neurons of hermaphrodites, high levels of the transcription factor TRA-1 maintained *egl-1* repression and cell survival through direct transcriptional regulation of *egl-1 (Conradt and Horvitz, 1999)*. In a few specific neurons of the pharynx, the competition between activating and repressing transcription factors for binding sites on *egl-1* determined the gene's transcriptional status and the fate of the cells (Thellmann et al.,

2003). These examples suggested that the cell-specific transcriptional status of *egl-1* might determine the fate of individual cells in *C. elegans*. However, it was not clear whether the life vs. death decisions of other cells in *C. elegans* were also determined by a similar mechanism.

Cell fate in the ventral nerve cord

One key problem with the hypothesis that *egl-1* transcriptional status determines whether each cell lives or dies was the fact that only two transcriptional regulators of *egl-1* had been found, despite enormous genetic screening efforts designed to detect mutants with abnormal patterns of PCD. If transcription factors regulate the life vs. death decision of each cell, why had these screens only uncovered genes that regulate this decision in the HSNs and NSM sisters? Why could genes regulating death and survival of the rest of the 1090 cells not be found also? One reason is because people have not looked carefully for mutations specifically affecting PCD of other cells, because these screens are very difficult to do. Also, Scott Cameron and I hypothesized that such genes might exist, but that altered function of such genes might have a pleiotropic effect on the organism. This could result in sterility or lethality, in which case mutations in these genes would not have been isolated from the cell death screens previously performed. My limited experience with standard mutagenesis screens searching for novel regulators of PCD is consistent with the suggestion that these types of screens have reached their limits. Although all information suggested that one hit from such a screen, *n3393*, contained a mutation in a novel gene necessary for correct determination of the life-vs.-death decision

of specific cells, my work and Matthew Jamison's showed that *n3393* was merely a novel allele of the core PCD gene *ced-4* (Chapter 2). PCD screens in our lab and others were simply returning more alleles of the same known genes, rather than novel information.

To overcome this issue, I created the P_{egl-1}*histone:gfp* reporter strain and compared the *egl-1* expression pattern in wild-type animals to the pattern in animals exposed to RNAi against 122 candidate genes. Fourteen hits from this screen reproducibly altered the pattern of *egl-1* transcription (Chapter 3). I chose to analyze one hit, *unc-62,* in detail to determine the mechanisms through which it regulated *egl-1* transcription and PCD (Chapter 4).

These investigations led to a greater understanding of how PCD is regulated in the ventral nerve cord of *C. elegans* hermaphrodites, as well as providing insight into the pathogenesis of MLL-rearranged leukemias, which I will discuss in the second half of this chapter. Notably, Hox transcription factors and cofactors play essential roles in sculpting the ventral nerve cord, and they do so by regulating the transcription of *egl-1* in a cell-specific manner (Chapters 3 and 4). The Hox transcription factor MAB-5 cooperates with the cofactor CEH-20 to directly activate *egl-1* transcription in a particular cell (P11.aaap) in the posterior of the ventral nerve cord. In the absence of MAB-5, CEH-20, or Site 1 where these proteins bind to the *egl-1* promoter, *egl-1* is not transcribed in this cell, and PCD does not occur. Another Hox transcription factor, LIN-39, cooperates with CEH-20 to bind to the *egl-1* promoter and repress transcription in the VC neurons in the midbody region of the ventral nerve cord. This activity is

necessary for survival of these cells. UNC-62 and LIN-59 are also required for survival of these cells, presumably through nuclear localization of CEH-20 and transcriptional activation of *lin-39*, respectively. Thus CEH-20 can act as a direct activator or repressor of *egl-1* transcription through partnering with different Hox proteins in different cells, and this protein is necessary for death in one case and survival in another. To complicate matters further, there is a cell in the posterior ventral cord (P12.pp) that depends on Site 1 but not MAB-5 or CEH-20 to undergo PCD, and yet another cell (P12.aaap) whose death depends on MAB-5 and CEH-20 but not Site 1. Although this work has made huge progress in understanding regulation of PCD in the ventral nerve cord by Hox proteins, this is clearly a complicated process that is not yet fully understood, but is clearly important from the perspectives of basic science as well as clinical impact.

To take a step back, my research has shown that the neurons of the ventral nerve cord, like the HSNs and NSM sister cells, depend on the transcriptional status of *egl-1* to instruct their life-vs.-death cell fate decision. At the same time, work from others has shown that other cells depend on transcriptional control of other genes in the PCD pathway, such as *ced-9* and *ced-3*, to decide their fate (Maurer et al., 2007; Park et al., 2006). Taken together, these findings suggest an emerging picture in which the various somatic cells of *C. elegans* utilize a wide array of mechanisms to decide whether to survive or undergo PCD. In fact, there appears to be almost no common theme, as each cell type uses vastly different strategies to control the balance of anti- and pro-apoptotic proteins it contains and thus its propensity to undergo PCD. All mechanisms found to date involve transcriptional regulation of the core PCD machinery, but the mechanisms

regulating PCD in most cells still remain to be discovered, so it is certainly possible that post-transcriptional regulatory mechanisms are also involved.

Remaining questions

As I mentioned, many questions remain as to how PCD is regulated in the ventral nerve cord. For example, why does removal of LIN-39 result in PCD of the VC neurons, but not the neighboring VA, VB, and VD neurons? There could be an activator of *egl-1* transcription in the VC neurons that is absent from the other cells, or a repressor in the VA, VB, and VD neurons that is redundant with LIN-39 and absent from the VCs. Screens using the P_{egl-1}*histone:gfp* reporter in a *lin-39* mutant background are being carried out by my labmates now to answer this question. Why is Site 1 in *egl-1* necessary for PCD of the P12.pp cell? Perhaps some unknown factor or factors bind that site to activate *egl-1* transcription in that cell. How do MAB-5 and CEH-20 promote death of P12.aaap without binding Site 1? These proteins are not present in P12.aaap, but are expressed in its ancestors earlier in the lineage. Perhaps they act indirectly by influencing the level of another as-yet-undiscovered direct transcriptional regulator of *egl-1*. Since I only screened 122 candidate genes and successfully identified novel regulators of PCD, I believe that a genome-wide screen using the same strategy would likely lead to the identification of other novel PCD regulators that might answer some of these questions.

Another related question involves regulation of the Hox transcription factors and cofactors in *C. elegans*. Although polycomb-group and trithorax-group genes are important repressors and activators of Hox transcription in other organisms, their role is less clear in *C. elegans*. I found that the trithorax-group gene *lin-59* is necessary for wild-type levels of *lin-39* transcription and for VC survival, but I did not investigate the expression levels of other important genes such as *egl-1* and *unc-62* in the absence of *lin-59*. My results suggest the hypothesis that *lin-59* promotes VC survival by maintaining *lin-39* levels in the VC neurons that are sufficient to repress *egl-1* transcription in those cells. It is equally plausible that *lin-59* maintains *unc-62* levels instead of (or in addition to) *lin-39*, or that it affects *egl-1* transcription directly. Interestingly, the polycomb-group gene *mig-32* does not appear to affect *lin-39* transcript levels, but may affect the pattern of PCD in the ventral nerve cord. Further investigation is needed to clarify the roles of polycomb- and trithorax-group genes in regulation of PCD in *C. elegans*.

It is interesting that the Hox cofactor CEH-20 can either activate or repress *egl-1* directly, depending on context. In the former case, it partners with MAB-5 to bind to a single site on *egl-1* in P11.aaap. In the latter, it partners with LIN-39 to bind multiple redundant sites on *egl-1* in the VC neurons. What determines whether CEH-20 activates or represses *egl-1* transcription upon binding with a Hox transcription factor? It is conceivable that the protein sequence of the Hox, the number of sites bound, or the presence of other cell-specific interacting factors could play critical roles. The ventral

nerve cord provides a good experimental system in which to parse out these different possibilities.

Finally, the largest unanswered question remains: how do the rest of the 1090 cells in the worm decide whether to undergo PCD or survive? Characterization of the other 13 hits from my screen would probably provide some insight into this problem, as would expanding the screen to genome-wide coverage. For those cells which do not depend on transcriptional regulation of *egl-1* to determine their fate, alternative strategies will have to be employed to determine the mechanisms that decide whether they survive or undergo programmed cell death.

HOX-MEDIATED INHIBITION OF APOPTOSIS IN ONCOGENESIS

Background

As described in detail in Chapters 1 and 4, the functions of trithorax-group, Hox, and TALE-class cofactor genes contribute to oncogenesis in humans. Most notably, chromosomal translocations involving the trithorax-group gene *MLL* are common in acute lymphocytic leukemia (ALL) and acute myelogenous leukemia (AML), where they contribute to the oncogenic phenotype by causing overexpression of particular Hox and Meis genes (Armstrong et al., 2002; Ernst et al., 2002; Grier et al., 2005; Krivtsov et al., 2006; Wong et al., 2007). Although it is clear that Hox-mediated transcriptional activity plays a critical role in leukemogenesis, the mechanisms and critical target genes involved remain almost entirely unknown. Some suggestion that MLL fusions contributed to

leukemia cell survival had been observed, but again the mechanistic details had not been elucidated (Kawagoe et al., 2001).

Lessons from *C. elegans*

My research into the regulation of PCD in the ventral nerve cord of *C. elegans* showed that a Hox-Pbx complex binds the promoter of *egl-1* to directly repress its transcription, inhibiting PCD and promoting survival of the VC neurons. This activity required the Meis homolog *unc-62* and the trithorax-group gene *lin-59* as well. Based on these results, Scott Cameron and I hypothesized that this mechanism might be conserved in mammals and important for Hox-mediated leukemogenesis. If so, the BH3-only pro-apoptotic genes might represent critical targets of Hox transcriptional regulation. The hypothesis was that MLL fusions activate certain Hox transcription factors and cofactors, which directly bind and repress transcription of pro-apoptotic BH3-only genes, promoting abnormal cell survival and contributing to oncogenesis. To begin testing this hypothesis, we enlisted the help of Scott Armstrong, a collaborator who specializes in studying MLL-dependent leukemia. He was able to show that HoxA9 represses transcription of the BH3-only gene *Bim* in leukemia cell lines, MLL-fusion-immortalized bone marrow from mice, and primary human patient samples. In each case, knock-down of HoxA9 by shRNA results in *Bim* upregulation and cell death (Chapter 4). This suggests that Hox-mediated repression of BH3-only genes is conserved in mammals and important for leukemia cell survival. HoxA9 does bind the *Bim* promoter and thus probably represses *Bim* transcription directly in mammals, just as LIN-39 directly

represses *egl-1* in *C. elegans* (Scott Armstrong, personal communication). What roles the Meis and Pbx cofactors play in this process remains to be determined. Our data predict a model where HoxA9 binds the *Bim* promoter at multiple redundant sites in a complex with a mammalian Pbx protein, and mammalian Meis proteins are required for nuclear localization of Pbx. It would also be interesting to explore whether Hox-mediated repression of BH3-only genes is important for normal development in mammals as it is in *C. elegans*, perhaps during normal hematopoiesis where BH3-only genes are known to play important roles. Furthermore, current evidence indicates that this mechanism is critical for survival of leukemia cells, but whether (and at what stage) Hox-mediated repression of BH3-only genes contributes to the process by which normal cells are transformed into leukemia cells remains to be determined.

Although Hox-mediated oncogenesis is most well-studied in leukemias, upregulation and mutation of Hox transcription factors and cofactors have been observed in other types of cancer as well. Thus Hox-mediated inhibition of apoptosis may be a widespread mechanism that cancer cells can use to gain a survival advantage. To examine this possibility, David Wang and I examined Hox, Pbx, and Meis mRNA levels in a panel of lung and breast cancer tissues, as well as normal controls. This was performed with the assistance of Luc Girard in John Minna's lab, utilizing microarray data that lab had previously obtained. We found that several Hox genes and cofactors were overexpressed in cancer samples compared to controls, some with differences that were statistically significant. In addition, HoxB8 and HoxD1 expression levels correlated significantly with resistance to Doxorubicin and the Smac Mimetic, respectively, across several cancer

cell lines examined. These findings, taken together with the results from *C. elegans* and leukemias, suggest that Hox overexpression may promote cell survival in breast and lung cancers, perhaps through transcriptional repression of BH3-only genes. Vera Paulson will explore this hypothesis further in collaboration with Luc Girard and the Minna lab. Future efforts could also determine whether this mechanism is utilized by other cancer types where Hox genes and cofactors are overexpressed.

The research I performed in *C. elegans* has advanced the understanding of the molecular mechanisms driving leukemia cell survival. The most desirable future outcome of these studies would be the development of novel therapeutics to treat cancer. This mechanism provides a very promising new target, because disruption of Hox-mediated repression of BH3-only genes would be expected to result in death of cancer cells. Indeed, the *in vivo* LIN-39 rescue experiments I did showing that LIN-39 can only promote cell survival if it is able to bind CEH-20 demonstrate that therapeutically disrupting this particular interaction should result in BH3-only upregulation and cell death. Targeting this interaction might be challenging, however, because it involves protein-protein interactions rather than enzymatic activity. Historically, these types of interactions have been difficult to disrupt by therapeutic intervention. Because of the promise this mechanism offers as a therapeutic target, future efforts should be directed toward novel drug design strategies that can target protein-protein interactions as well as further mechanistic characterization, which may uncover more traditionally "druggable" molecules involved in Hox-mediated inhibition of apoptosis.

APPENDIX A: CANDIDATE GENE LIST FOR RNAI SCREEN

ID	Gene	Source	Screened	Primary	Secondary
B0025.3	*csn-2*	Ahr2003	Yes		
B0280.4	*odd-1*	CRZ1	Yes		
B0286.5	*fkh-6*	Ahr2003	Yes		
B0304.1	*hlh-1*	Chr I	Yes		
B0350.2	*unc-44*	other	Yes		
C01G8.9		Ahr2003	Yes		
C01H6.5	*nhr-23*	Ahr2003	Yes		
C04A2.3	*egl-27*	Ahr2003	Yes		
C04G2.6	*dis-3*	Ahr2003	Yes	Hit?	Hit
C07H6.7	*lin-39*	other	Yes		
C08B11.2	*hda-2*	HDAC	Yes		
C08B11.3		Ahr2003	Yes		
C08C3.1	*egl-5*	other	Yes		
C08C3.3	*mab-5*	other	Yes		
C08F8.8	*nhr-67*	*rrf-3*	Yes		
C09G12.8	*ced-10*	other	No		
C09G4.5	*mes-6*	other	Yes		
C10A4.8		CRZ1	No		
C10E2.3	*hda-4*	HDAC	Yes		
C14B1.4	*tag-125*	other	Yes		
C14B9.6	*gei-8*	other	Yes		
C17C3.7	*hlh-25*	*rrf-3*	No		
C17G1.4		NFAT	Yes	Hit?	
C24A8.3	*pqn-15*	NFAT	Yes		
C27A12.3	*tag-146*	CRZ1	Yes	Hit?	
C27C12.2		CRZ1	Yes		
C27H6.2	*ruvb-1*	rep	Yes		
C28A5.4	*ceh-43*	*rrf-3*	Yes		
C28C12.8	*hlh-12*	Ahr2003	Yes		
C30A5.7	*unc-86*	other	Yes		
C32A3.1	*sel-8*	Ahr2003	Yes		
C32F10.6	*nhr-2*	Chr I	Yes		
C33D3.1	*elt-2*	Ahr2003	Yes		
C34H3.2	*odd-2*	CRZ1	No		
C35A5.9	*hda-11*	HDAC	Yes		
C35C5.1	*sdc-2*	Ahr2003	Yes	Hit?	
C38D4.6	*pal-1*	Ahr2003	Yes		
C47C12.3	*ref-2*	CRZ1	No		
C48E7.3	*lpd-2*	*rrf-3*	Yes	Hit?	Hit
C50B8.2	*bir-2*	other	Yes		
C52B9.8		rep	Yes		

C53A5.3	*hda-1*	HDAC	Yes	Hit?	
C54H2.3	*tag-294*	other	Yes		
C55B7.12	*che-1*	CRZ1	Yes		
D2045.1b	*atx-2*	NFAT	Yes		
F02E9.4	*pqn-28*	rep	Yes		
F11C1.6	*nhr-25*	Chr I	Yes	Hit?	Hit
F13D11.2	*hbl-1*	Ahr2003	Yes		
F17A2.5	*ceh-40*	other	Yes		
F18A1.2	*lin-26*	Ahr2003	Yes		
F18A1.3	*lir-1*	Ahr2003	Yes		
F23B12.6	*tag-114*	other	Yes		
F25D7.3	*blmp-1*	CRZ1	Yes		
F26F12.7	*let-418*	rep	No		
F28F9.1	*zag-1*	CRZ1	Yes		
F29B9.2		other	Yes		
F29C4.5	*duo-2*	NFAT	Yes		
F29F11.5	*ceh-22*	other	Yes		
F29G9.4		Ahr2003	Yes		
F31E3.1	*ceh-20*	*rrf-3*	Yes		
F34D10.5	*lin-48*	CRZ1	No		
F36F2.3a	*tag-214*	rep	Yes	Hit?	Hit
F38A6.1	*pha-4*	Ahr2003	Yes		
F39D8.2	*psa-3*	other	No		
F41H10.6	*hda-6*	HDAC	Yes		
F43E2.11		rep	No		
F43E2.7		rep	Yes		
F43G6.4	*hda-5*	HDAC	Yes		
F43G6.6		other	Yes		
F43G9.11	*ces-1*	CRZ1	Yes		
F43G9.12		Ahr2003	Yes	Hit?	Hit
F44A6.2	*sex-1*	Ahr2003	Yes	Hit?	
F45B8.4	*pag-3*	CRZ1	No		
F46G10.7	*sir-2.2*	HDAC	Yes		
F47A4.2	*dpy-22*	NFAT	Yes	Hit?	
F48D6.1	*taf-11.1*	rep	Yes		
F48D6.3	*hlh-13*	*rrf-3*	Yes		
F49E10.5	*ctbp-1*	other	Yes		
F53C3.11		*rrf-3*	Yes	Hit?	Hit
F54C1.3	*mes-3*	other	Yes		
F54H5.4	*mua-1*	CRZ1	No		
F55A3.3		rep	Yes	Hit?	Hit
F55F8.4	*cir-1*	Ahr2003	Yes	Hit?	Hit
F57B10.1		Ahr2003	Yes		
F57C9.4B		CRZ1	Yes		

H28G03.6	*mtm-5*	rep	Yes		
K01C8.7		rep	Yes		
K01G5.2a	*hpl-2*	rep	Yes		
K02B12.1	*ceh-6*	Chr I, *rrf-3*	Yes		
K02B12.3		Ahr2003	Yes		
K02D7.2		CRZ1	No		
K04G11.4		other	No		
K06A9.1b.p		NFAT	Yes		
K07A1.11	*rba-1*	other	No		
K07A1.12	*lin-53*	other	No		
K08B4.1	*lag-1*	Ahr2003	No		
K08H2.6	*hpl-1*	other	Yes		
K10D3.3	*taf-11.2*	rep	Yes		
K10G6.1	*lin-31*	other	Yes		
K11D2.4a		CRZ1	Yes		
K12H4.1	*ceh-26*	Ahr2003	Yes		
M05B5.5	*hlh-2*	Ahr2003	No		
R02D3.5		other	Yes		
R03E9.1	*mdl-1*	*rrf-3*	No		
R06C1.1	*hda-3*	HDAC	Yes		
R06C7.7	*lin-61*	rep	Yes	Hit?	Hit
R07B1.1	*vab-15*	Chr I	Yes		
R07E5.10	*pdcd-2*	Ahr2003	Yes	Hit?	Hit
R119.6	*taf-4*	rep	Yes		
R11H6.5		NFAT	No		
R13A5.5	*ceh-13*	Chr I	Yes		
T04D1.4	*tag-192*	NFAT	No		
T07G12.12	*him-8*	other	Yes		
T12D8.7	*taf-9*	rep	Yes		
T13H2.4	*pqn-65*	NFAT	No		
T13H2.5		rep	No		
T14G8.1	*chd-3*	rep	Yes		
T19E7.2	*skn-1*	Ahr2003	Yes		
T22C8.5		CRZ1	Yes		
T22D1.10	*ruvb-2*	rep	Yes	Hit?	Hit
T26A5.5		other	Yes		
T27F2.1	*skp-1*	Ahr2003	Yes	Hit?	Hit
T27F2.3	*bir-1*	other	No		
T28F12.2a	*unc-62*	Ahr2003	Yes	Hit?	Hit
W01D2.2	*nhr-61*	Ahr2003	No	Hit?	
W02D3.9	*unc-37*	Ahr2003	No		
W02H5.7	*sknr-1*	Ahr2003	No		
W03D2.1	*pqn-75*	NFAT	No		
W04A8.7	*taf-1*	rep	Yes		

W05E10.3	*ceh-32*	*rrf-3*	Yes		
W09B6.2	*taf-6.1*	rep	Yes		
W09C2.1	*elt-1*	Ahr2003	No		
W10C8.2	*pop-1*	Chr I	No		
Y2H9A.1	*mes-4*	other	Yes		
Y37E11.b4		rep	No		
Y37E11AL.8	*taf-6.2*	rep	No		
Y38H8A.5		CRZ1	Yes		
Y40B1A.4		*rrf-3*, CRZ1	Yes	Hit?	
Y47D3A.6A	*tra-1*	CRZ1	No		
Y47D3B.7	*sbp-1*	Ahr2003	Yes		
Y49E10.14	*pie-1*	Ahr2003	Yes		
Y50E8A.9		NFAT	No		
Y51H1A.5	*hda-10*	HDAC	Yes		
Y53C10A.12	*hsf-1*	*rrf-3*	Yes	Hit?	
Y54E10BR.8		CRZ1	No		
Y54E5B.3	*let-49*	Ahr2003	Yes	Hit?	
Y55F3AM.14		CRZ1	No		
Y66A7A.8	*tbx-33*	Ahr2003	No		
Y75B8A.1	*php-3*	other	Yes		
Y75B8A.2	*nob-1*	Ahr2003	Yes		
Y94H6A.11		CRZ1	No		
Y95B8A.7		NFAT	No		
ZC123.3		*rrf-3*	Yes	Hit?	
ZC328.2		CRZ1	Yes		
ZC64.3	*ceh-18*	Chr I	Yes	Hit?	Hit
ZK1037.5		Ahr2003	Yes		
ZK1193.5		Ahr2003	Yes		
ZK1236.2	*cec-1*	rep	Yes		
ZK337.2		CRZ1	Yes		
ZK84.1		NFAT	No		
ZK858.4	*mel-26*	Chr I	Yes		

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