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Six and Eya promote apoptosis through direct transcrip
tional activation of the proapoptotic BH3-only gene egl-1 in Caenorhabditis elegans

Takashi Hirose, Brendan D. Galvin, and H. Robert Horvitz

Howard Hughes Medical Institute and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

Contributed by H. Robert Horvitz, July 14, 2010 (sent for review April 30, 2010)

The decision of a cell to undergo programmed cell death is tightly regulated during animal development and tissue homeostasis. Here, we show that the Caenorhabditis elegans Six family homeodomain protein C. elegans homeobox (CEH-34) and the Eyes absent ortholog EYA-1 promote the programmed cell death of a specific pharyngeal neuron, the sister of the M4 motor neuron. Loss of either ceh-34 or eya-1 function causes survival of the M4 sister cell, which normally undergoes programmed cell death. CEH-34 physically interacts with the conserved EYA domain of EYA-1 in vitro. We identify an egl-1 1′ cis-regulatory element that controls the programmed cell death of the M4 sister cell and show that CEH-34 binds directly to this site. Expression of the proapoptotic gene egl-1 in the M4 sister cell requires ceh-34 and eya-1 function. We conclude that an evolutionarily conserved complex that includes CEH-34 and EYA-1 directly activates egl-1 expression through a 1′ cis-regulatory element to promote the programmed cell death of the M4 sister cell. We suggest that the regulation of apoptosis by Six and Eya family members is conserved in mammals and involved in human diseases caused by mutations in Six and Eya.

Apoptosis, also referred to as programmed cell death, plays fundamental roles in animal development and tissue homeostasis (1). The misregulation of apoptosis is associated with many human disorders, including cancer and neurodegenerative and autoimmune diseases (2). Determining how particular cells are specified to live or die is critical to understanding both normal animal development and human diseases associated with the misregulation of apoptotic cell death.

During the development of the Caenorhabditis elegans hermaphrodite, 131 somatic cells undergo programmed cell death (3, 4). Genetic studies of programmed cell death in C. elegans have defined an evolutionarily conserved pathway that executes this process (5). This pathway consists of four genes, egl-1 [egg-laying defective (egl)], ced-9 [cell-death abnormal (ced)], ced-4, and ced-3, all of which are conserved from C. elegans to mammals. Although much is understood about the pathway responsible for the execution of programmed cell death in both C. elegans and other animals, less is known about the mechanisms that control how specific cells decide whether to survive or die by programmed cell death. In C. elegans, most of the genes identified to control cell-death specification encode transcription factors, some of which are known to directly regulate the transcription of cell-death genes (6–11). For example, the Snail family transcription factor CES-1 [cell-death specification (ces)] can directly repress expression of the BH3-only proapoptotic gene egl-1 and prevent the deaths of the NSM sister cells (6, 7). This regulatory mechanism is conserved in mammals and has been implicated in human cancer: acute lymphoblastic leukemia results from an overexpression of the CES-1 homolog Slug, which directly represses expression of the BH3-only proapoptotic gene Puma, preventing apoptosis of hematopoietic progenitor cells (12, 13).

By contrast, the C. elegans Bar homeodomain transcription factor CEH-30 [C. elegans homeobox (ceh)] protects specific neurons from undergoing programmed cell death by acting independently of egl-1 (14); ceh-30 also regulates egl-1 transcription (15). The antiapoptotic function of ceh-30 likely explains the previously observed loss of sensory hair cells of the inner ear and progressive hearing loss of mice deficient in the function of the ceh-30 homolog Barhl1 (16).

The identification and characterization of additional factors that regulate cell-type-specific apoptosis in C. elegans promise to reveal both conserved molecular mechanisms that control cell-death specification and how misregulated apoptosis can cause human disease. Here, we show that the C. elegans Six family homeodomain protein CEH-34 and the Eyes absent ortholog EYA-1 directly activate expression of the proapoptotic BH3-only gene egl-1 in the M4 sister cell to promote programmed cell death. We suggest that, in mammals, Six family members and Eya control apoptosis by directly regulating the transcription of BH3-only genes and mutations in Six and Eya genes perturb normal apoptosis by misregulating the transcription of BH3-only genes.

Results

Mutants Defective in the Programmed Cell Death of the M4 Sister Cell. The C. elegans M4 motor neuron is located on the dorsal side of the anterior bulb of the pharynx and regulates pharyngeal muscle contraction during feeding (17, 18). The mother of the M4 neuron divides to generate an anterior daughter that survives and becomes the M4 neuron and a posterior daughter, the M4 sister cell, which dies by programmed cell death during embryogenesis (Fig. 1A) (3). To identify genes that control the specification of the M4 sister-cell death, we performed a genetic screen for mutants with a surviving M4 sister cell. We used a Pceh-28::4xNLS::gfp reporter transgene that is specifically expressed in the M4 neuron in wild-type animals and in both the M4 neuron and the surviving M4 sister cell in ced-3 mutants defective in programmed cell death (Fig. 1A and B) (19). Thus, this reporter can be used to detect M4 sister-cell survival.

We screened the F2 progeny of hermaphrodites mutagenized with ethyl methanesulfonate (EMS) and, from a total of 72,000 haploid genomes screened, isolated 59 mutants with a surviving M4 sister cell, which was located variability as in ced-3 mutants (Fig. 1B and Fig. S1). From these 59 mutants, we identified 38 alleles of ced-3, 13 alleles of ced-4, 3 alleles of egl-1, and 5 mutants that seemed not to carry alleles of any known cell-death genes. Here, we focus on two mutants that affected the deaths of both the M4 sister cell and the pharyngeal I1 sister cells, n4820 and n4796.

In n4820 mutants, 80% of the M4 sister cells (n = 120) and 10% of the I1 sister cells (n = 240) survived (Fig. 1B and C and Table S1). In n4796 mutants, 38% of the M4 sister cells (n = 140) and 1% of the I1 sister cells (n = 240) survived.

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tation but not the region of the G-to-C mutation (Fig. 2). Genomic deletion, fi

Zero of B and C-to-T mutations were rescued for the phenotype of M4 sister cells. To determine which mutation caused survival of the M4 sister cell and the I1 sister cells, we performed rescue experiments for the phenotype of M4 sister-cell survival. The results of these rescue experiments are indicated. (C) DNA sequence surrounding the site altered by the n4820 mutation is shown for C. elegans and related species. Nucleotides identical to those in C. elegans are indicated by bold, and the nucleotide mutated in n4820 animals is highlighted in red.

deviations of M4 sister survival in the indicated strains are shown.

47% of the I1 sister cells (n = 240) survived (Fig. 1 B and C and Table S1). By contrast, the n4820 and n4796 mutations did not affect the programmed cell deaths of the NSM sister cells or the VC homologs of the ventral nerve cord (Table S1). Thus, n4820 and n4796 affect M4 sister-cell and I1 sister-cell deaths but do not affect all programmed cell deaths.

n4820 Is a Noncoding Allele of the Proapoptotic BH3-Only Gene egl-1. The mutation n4820 complemented ced-3(n717) and ced-4 (n1162) but failed to complement egl-1(n1084 n3082) for M4 sister-cell survival (Table S2). We mapped n4820 to chromosome V, the same chromosome as egl-1. Whereas the egl-1(n4820) mutation caused survival of the M4 sister cell and the I1 sister cells but not of other cells fated to die (Table S1), an egl-1 null allele causes survival of essentially all somatic cells that die by programmed cell death (Table S1) (20). These data indicate that egl-1(n4820) is an atypical egl-1 allele that specifically affects the programmed cell deaths of the M4 sister cell and the I1 sister cells.

We determined the sequence of the egl-1 locus of egl-1(n4820) mutants and found two mutations. One is a C-to-T mutation located 4.9 kb upstream of the egl-1 start codon, and the other is a G-to-C mutation located 4.2 kb upstream of the egl-1 start codon (Fig. 2A). To determine which mutation caused survival of the M4 sister cell, we performed rescue experiments for the phenotype of M4 sister survival of egl-1(n4820) mutants by injecting egl-1 genomic fragments carrying the G-to-C mutation. No myoblast fates of M4 sister survival in the indicated strains are shown.

deads of the NSM sister cells (0% NSM sister survival; n = 240) or the VC homologs (0.0 extra VC homologs/animal; n = 120).

Together, these results establish that the C-to-T mutation was responsible for the survival of the M4 sister cell and the I1 sister cells in egl-1(n4820) mutants. To distinguish the n4820 C-to-T mutation, which causes survival of the M4 sister cell and the I1 sister cells, from the G-to-C mutation, we designated the G-to-C mutation n5334 (Fig. 2A). The DNA sequence immediately surrounding the n4820 mutation is highly conserved in other nematode species (Fig. 2C). Based on these results, we suggest that the n4820 region defines a binding site for a transcriptional activator that promotes egl-1 expression in the M4 sister cell and the I1 sister cells and that this binding site is defective in n4820 and n5334 mutants.

n4796 Is an Allele of ceh-34, a Six Family Homeodomain Gene. We mapped n4796 to a 150-kb interval of chromosome V almost completely covered by six cosmids (Fig. S3). We performed rescue experiments for the phenotype of M4 sister survival of n4796 animals by injecting cosmids in this interval. The cosmids C10G8 rescued the phenotype of M4 sister survival of n4796 animals (Fig. S3). C10G8 encodes eight predicted genes, including ceh-34. A genomic fragment that contains only ceh-34 rescued the phenotype of M4 sister survival of n4796 animals (Fig. S3). n4796 animals have a G-to-A mutation in the splice acceptor site of the second exon of ceh-34 (Fig. 2A). RNAi of ceh-34 caused survival of the M4 sister cell (58% of M4 sisters survived; n = 123). These results indicate that n4796 is a reduction-of-function allele of ceh-34.

ceh-34 encodes a 256 amino acid protein that is similar to Six family homeodomain proteins, which typically function as transcription factors (21). Six family homeodomain proteins contain a conserved Six domain that mediates protein-protein interactions and a homeodomain that binds DNA (21) (Fig. 2B). Both domains are evolutionarily conserved from C. elegans to humans (Fig. S4). The CEH-34 protein is 48% and 63% identical to the human protein SIX1 within the Six domain and the homeo-domain, respectively.

We tested whether the functions of C. elegans CEH-34 and human SIX are evolutionarily conserved. We showed that expression of a ceh-34 cDNA under the control of the ceh-34 promoter in a ceh-34(n4796) mutant rescued the defect in M4 sister-cell death (Table S3). We then substituted a human SIX1 cDNA for the ceh-34 cDNA in this construct and found that expression of SIX1 also rescued the defect in M4 sister-cell death of ceh-34(n4796).
CEH-34 Is Expressed Predominantly in the Nuclei of Pharyngeal Cells.

To determine the expression pattern of ceh-34, we constructed a translational ceh-34::gfp reporter transgene and showed that it can rescue the phenotype of M4 sister survival of ceh-34(n4796) mutants. Expression of this ceh-34::gfp transgene began during embryogenesis (Fig. 3 C and D). CEH-34::GFP was localized to the nuclei of expressing cells (Fig. 3 C–F), consistent with its likely function as a transcription factor. During embryonic morphogenesis and larval development and throughout adulthood, expression of the ceh-34::gfp transgene was seen predominantly in pharyngeal cells (Fig. 3 D and E). A similar expression pattern for a ceh-34::gfp transgene has been independently observed (22). We observed that our ceh-34::gfp transgene was expressed in all pharyngeal neurons (M4, I1, MI, I3, M3, NSM, MC, I2, I4, I5, I6, M1, M2, and M5), some pharyngeal muscle cells (pm1 and pm2) and pharyngeal epithelial cells (e1 and e3), and some body wall muscles around the anterior pharynx (Fig. 3F).

Eyes Absent Ortholog EYA-1 Is Required for M4 Sister- and I1 Sister-Cell Death. Six family homeodomain genes were originally identified in studies of Drosophila eye development (23). The Drosophila Six family homeodomain gene sine oculis is required for proper eye specification and functions in a conserved transcriptional network that includes the paired-box (Pax) homeodomain gene eyeless (eya) and the transcriptional cofactors eyes absent (eya) and dachshund (dac) (24). We tested whether the C. elegans orthologs of these three genes promote M4 sister-cell death. We found that a deletion allele of eya-1, the C. elegans eyes absent ortholog, caused survival of both the M4 sister cell (49% of M4 sisters survived; n = 120) and the I1 sister cells (19% of I1 sisters survived; n = 240) but did not affect the NSM sister cells or the VC homologs (Fig. 4A and Table S1). Null mutations in vab-3 and dac-1, the C. elegans orthologs of eyeless and dachshund, respectively, did not cause survival of the M4 sister cell (Fig. 4A). These results indicate that the C. elegans eyes absent ortholog eya-1 is required for the deaths of the M4 sister cell and the I1 sister cells and suggest that eya-1 might function with the Six family homeodomain gene ceh-34 to promote programmed cell death.

EYA-1 Forms an Evolutionarily Conserved Complex with the Six Family Homeodomain Protein CEH-34. The Six family homeodomain proteins of Drosophila and mammals physically interact with the conserved C-terminal Eya domains of Eya proteins (25, 26). Because mutations of ceh-34 and eya-1 caused the same phenotype of M4 sister-cell and I1 sister-cell survival, we examined whether CEH-34 physically interacts with EYA-1. We fused GST to the C-terminal Eya domain of EYA-1 (GST-EYA-1C) and found that this protein specifically bound CEH-34 (Fig. 4B). CEH-34 and EYA-1 also have been shown by others to both act to specify coelomocyte cell fate and interact physically (22).

If CEH-34 physically interacts and functions with EYA-1 in vivo, the two proteins must be expressed in at least some of the same cells. Indeed, the expression pattern of an eya-1::gfp transgene capable of rescuing the phenotype of eya-1 mutants is similar to that of our ceh-34::gfp transgene; eya-1 is predominantly expressed in pharyngeal cells (27). We observed expression of both the

![Diagram](image-url)
ceh-34::gfp transgene and an eya-1::gfp transgene in the M4 sister cell in ced-3(n717) mutants (Fig. 4 C–H). These results suggest that CEH-34 and EYA-1 form an evolutionarily conserved complex in the M4 sister cell to promote programmed cell death.

CEH-34 binds to the egl-1 5′ cis-regulatory element defined by egl-1(n4820). Because egl-1(n4820) and ceh-34(n4796) both specifically affected the programmed cell deaths of the M4 sister cell and the I1 sister cells, we examined whether egl-1 and ceh-34 might functionally interact to regulate these programmed cell deaths. Interestingly, the DNA sequence surrounding the egl-1(n4820) mutation is similar to the consensus-binding sequence of Sine Oculis (SO), the Drosophila homolog of CEH-34 (Fig. 5A) (28).

To test if CEH-34 can bind directly to this presumptive egl-1 cis-regulatory element, we performed gel-shift assays. We observed that CEH-34 indeed binds to this sequence (Fig. 5B).

To test the specificity of this interaction, we performed competition experiments with unlabeled competitors. Both the wild-type competitor and competitors with mutations outside the SO consensus-binding sequence (wild type, I, and V in Fig. 5A) competed with the labeled wild-type probe for CEH-34 binding (Fig. 5C). By contrast, competitors with mutations in the SO consensus-binding sequence (II, III, IV, and scrambled in Fig. 5A) did not efficiently compete with the labeled wild-type probe for CEH-34 binding (Fig. 5C). These results indicate that CEH-34 binds to the SO consensus-binding sequence of an egl-1 cis-regulatory element surrounding the egl-1(n4820) mutation.

Because the egl-1(n4820) mutation is located within the SO consensus-binding sequence, we examined whether the egl-1(n4820) mutation affects CEH-34 binding. Whereas wild-type competitor efficiently competed for CEH-34 binding, a competitor containing the egl-1(n4820) mutation did not compete for CEH-34 binding as efficiently as the wild-type competitor (Fig. 5D). These results indicate that the egl-1(n4820) mutation reduces CEH-34 binding to this egl-1 cis-regulatory element.

egl-1 expression in the M4 sister cell requires ceh-34 and eya-1 function. Given that CEH-34 directly binds to the egl-1 cis-regulatory element in vitro and that CEH-34 forms a complex with EYA-1, we examined if ceh-34 or eya-1 mutations alter egl-1 expression in the M4 sister cell. In ced-3 mutants, a P3::4xNLS::gfp reporter transgene that contains the egl-1 cis-regulatory element required for CEH-34 binding was expressed in the M4 sister cell (100% of animals; n = 119) but not in the M4 neuron (0% of animals; n = 119), strongly suggesting that egl-1 expression regulates the death of the M4 sister cell (Fig. 6, A, B, C, and G). We observed that in 55% of ced-3; ceh-34(n4796) animals (n = 97) and 64% of eya-1(ok654); ced-3 animals (n = 89), the M4 sister cell did not express the P3::4xNLS::gfp transgene (Fig. 6, D–G). These results establish that ceh-34 and eya-1 are necessary for normal egl-1 expression in the M4 sister cell and suggest that ceh-34 and eya-1 activate egl-1 expression to promote M4 sister-cell death.

Discussion

ceh-34 and eya-1 promote M4 sister-cell death by directly activating transcription of the proapoptotic gene egl-1. From a genetic screen for mutants defective in the death of the M4 sister cell and a subsequent candidate-gene approach, we identified two genes that specify the cell-death fate of the M4 sister cell: the Six family homeodomain gene ceh-34 and the eyes absent ortholog eya-1. Based on our findings, we propose a model for the regulation of M4 sister-cell death (Fig. 6F). In wild-type animals, a complex consisting of the CEH-34 and EYA-1 proteins binds to the egl-1 5′ cis-regulatory element and activates egl-1 expression in the M4 sister cell, causing the M4 sister cell to die. In ceh-34 or eya-1 mutants, a functional CEH-34-EYA-1 complex is not formed, and egl-1 is not expressed, causing the M4 sister cell to survive. In egl-1(n4820) mutants, the CEH-34-EYA-1 complex cannot bind to the egl-1 5′ cis-regulatory element, and egl-1 is not expressed, causing the M4 sister cell to survive. The same transcriptional regulatory mechanism likely, at least in part, promotes I1 sister-cell death, because ceh-34(n4796), eya-1(ok654), and egl-1(n4820) also caused survival of the I1 sister cells.

We propose that CEH-34 and EYA-1 function cell autonomously in the M4 sister cell to activate egl-1 expression and promote programmed cell death. Consistent with this hypothesis, we observed that ceh-34 and eya-1 were expressed in the M4 sister cell. Whereas ceh-34 and eya-1 were expressed in both the M4
The 5μm arrowheads indicate the M4 sister cell. Both cells are circled. (Scale bar, control of M4 sister-cell death by CEH-34 and EYA-1 is shown for the wild-

Fig. 6. egl-1 expression in the M4 sister cell requires ceh-34 and eya-1. (A–F) The P EGL-1::4xNLS::gfp and PCEH-28::4xNLS::mCherry transgenes were coexpressed in ced-3(n717) animals (A–C) and ced-3(n717); ceh-34(n4796) animals (D–F). (A and D) Expression of the P EGL-1::4xNLS::gfp transgene. (B and E) Expression of the PCEH-28::4xNLS::mCherry transgene in the M4 neuron and M4 sister cell. (C and F) Merged images. Arrows indicate the M4 neuron; arrowheads indicate the M4 sister cell. Both cells are circled. (Scale bar, 5 μm.) (G) Percent of animals lacking expression of the P EGL-1::4xNLS::gfp transgene in the M4 sister cell is indicated for ced-3(n717), ced-3(n717); ceh-34(n4796), and eya-1(ok654); ced-3(n717) animals. (H) A model for the control of M4 sister-cell death by CEH-34 and EYA-1 is shown for the wild-type, ceh-34, eya-1, or egl-1(n4820) mutants.

neuron and M4 sister cell, egl-1 was expressed only in the M4 sister cell destined to die but not in the M4 neuron destined to survive. This observation suggests that ceh-34 and eya-1 are necessary but not sufficient to activate egl-1 expression in the M4 sister cell. Other factors that activate egl-1 expression in the M4 sister cell or that repress egl-1 expression in the M4 neuron likely also interact with CEH-34 and EYA-1 in causing the M4 sister cell to die or the M4 neuron to survive (Fig. 6H).

ceh-34 and eya-1 Function More Broadly in Pharyngeal Development. Although ceh-34 and eya-1 regulate the programmed cell deaths of the M4 sister cell and the I1 sister cells, these genes also function in other aspects of development. ceh-34(tm591Δ) deletion mutants arrest as early larvae and exhibit abnormal pharyngeal morphology with decreased expression of the ceh-28 reporter transgene that we used as a reporter for the M4 neuron and M4 sister cell. eya-1 deletion mutants exhibit various defects, including abnormal pharyngeal morphology (27). Both eya-1 (27) and ceh-34 (our results) are predominantly expressed in pharyngeal cells. These observations suggest that ceh-34 and eya-1 are involved in aspects of pharyngeal development besides the regulation of the programmed cell deaths of the M4 sister cell and I1 sister cells.

Functions of ceh-34 and eya-1 in Regulating Programmed Cell Death Are Evolutionarily Conserved. CEH-34 family Six proteins and EYA-1 family Eya proteins function in a diversity of organisms in a variety of developmental processes, including apoptosis, cell survival, and cell differentiation (24). We observed that human SIX1 can rescue the defect in M4 sister-cell death of ceh-34 (n4796) mutants. This result suggests that the mechanisms of Six family protein function in regulation of apoptosis are evolutionarily conserved between C. elegans and humans.

Eya function in apoptosis is also likely conserved from C. elegans to mammals. Just as eya-1 promotes M4 sister-cell death in C. elegans, the overexpression of mammalian Eya proteins promotes apoptosis of murine myeloid cells (29). By contrast, during embryonic development, C. elegans eya-1 mutants have excessive programmed cell deaths (27), suggesting a protective function of eya-1. Such a protective function of eya-1 might also be evolutionarily conserved, because loss of Eya function causes increased apoptosis in Drosophila (30), zebrafish (31), and mice (32). Eya proteins act as protein phosphatases (33) as well as transcriptional cofactors, and the phosphatase activity of Eya proteins on histone variant H2AX is required to protect mammalian kidney embryonic cells from apoptosis after DNA damage (34). These observations indicate that Eya proteins can function in either promoting or inhibiting apoptosis, depending on cellular context, and more generally, that the function of Eya in regulating apoptosis is evolutionarily conserved from C. elegans to mammals.

Human Diseases Caused by Mutations in Six Family Homeodomain Genes or eyes absent Homologs Likely Result in Part from Misregulation of Transcription of BH3-Only Genes and Apoptosis. Six family homedomain genes and eyes absent genes have been implicated in mammalian apoptosis and various human diseases, including cancer (35, 36), cardiomyopathy (37), and developmental disorders (38-40). Mutations in SIX3 were identified in human patients with holoprosencephaly (HPE), a severe malformation of the forebrain characterized by an incomplete segregation of the cerebral hemispheres (38). In mice, a reduction of Six3 function can cause an HPE-like phenotype as well as decreased apoptosis along the ventral midline of the telencephalon and in optic stalks and increased apoptosis in the lateral dorsal telencephalon of embryos of a phenotypically normal heterozygous Sonic hedgehog mutant (41). Mutations in SIX1 and EYA1 were identified in human patients with branchio-oto-renal (BOR) syndrome, which is characterized by craniofacial abnormalities, hearing loss, and kidney defects (39, 40). Six1- or Eya1-deficient mice showed increased apoptosis in the inner ear and kidney, likely causing a lack of inner ear structure and failure of kidney development (32, 42, 43). In these mice, increased or decreased apoptosis is seen in tissues in which abnormalities also are observed in human HPE or BOR patients. These observations suggest that the misregulation of apoptosis caused by a mutation in Six or Eya is likely involved in these diseases. Because Six and Eya likely function in transcriptional regulation, transcriptional misregulation of target genes likely causes altered apoptosis in mice with a mutation in Six or Eya. Based on our findings, we suggest that mammalian Six and Eya proteins directly regulate transcription of BH3-only genes, that altered apoptosis in mammals carrying Six or Eya mutations is caused by misregulation of transcription of BH3-only genes, and that, in humans, such altered apoptosis is at least in part responsible for the HPE and BOR syndromes.

Materials and Methods
See SI Materials and Methods for a full description.
Strains and Genetics. C. elegans strains were cultured and maintained on NGM plates at 20 °C as described (44). The Bristol strain N2 was the standard wild-type strain, and the Hawaiian strain CB4856 was used for SNP mapping.

In Vitro GST Fusion Protein-Binding Experiments. In vitro GST fusion protein-binding experiments were performed essentially as described (25).

Gel Mobility Shift Assays. Gel mobility shift experiments were performed essentially as described (28).

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Supporting Information

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SI Materials and Methods

We used the following mutations and integrants, which were described by Riddle et al. (1) unless otherwise noted. LGI: eya-1 (ok654Δ, m759Δ), nls177[P_ceh-28::4xNLS::gfp; lin-15AB(+)] (this study), nls226[chc-34::gfp; lin-15AB(+)] (this study); LGII: nls343 [P_egl-1′::4xNLS::gfp; lin-15AB(+)] (this study), nls352[eya-1::3-gfp; rol-6(su1006)] (this study); LGIII: ced-3(n1612), ced-9(n1950), daf-1(gk211A, gk213D), nls176[P_ceh-28::4xNLS::gfp; lin-15AB(+)] (this study); LGIV: ced-3(n717), nls175[P_ceh-28::4xNLS::gfp; lin-15AB(+)] (this study); LGV: ceh-34(n4796, m591A) (this study), dpy-11(e224),egl-1(n1084 n3082, n4820 n5334, n5347a) (2) (this study); LGX: bch24 [P_ghb-13::3-gfp; lin-15AB(+)] (3), lin-15(n765a, n106 [P_lwm-11::gfp; lin-15AB(+)] (4), nls283[P_gcy-10::4xNLS::gfp; lin-15AB(+)] (this study), nls349[P_ceh-28::4xNLS::mCherry; lin-15AB(+)] (this study), vab-3(e648, jn608) (5); extrachromosomal arrays: tje21[eyu-1(+); rol-6(su1006); sur-5::gfp] (6), tje25[eya-1::gfp; rol-6(su1006)] (6). Information about the animals was described (8).

Genetic screens were performed using ethyl methanesulfonate (EMS) mutagenesis as described (7). SNP mapping was performed to map ceh-34(n4796) and eql-1(n4820) essentially as described (8).

Phenotypic Analysis and Microscopy. Animals were examined for gross developmental defects using a dissecting microscope and a compound microscope equipped with Nomarski optics. Programmed cell deaths were scored using gfp reporters and a compound microscope equipped with fluorescence optics. M4 sister-cell death was scored using nls175, nls176, or nls177 at the L1 stage. I1 sister-cell death was scored using nls283 at the L4 stage. NSM sister-cell death was scored using bch24 at the L1 stage (3), and the deaths of the VC homologs were scored using nls106 at the L4 stage (4). To score expression of the P_egl-1::4xNLS::gfp reporter transgene in the M4 sister cell, gravid animals carrying nls343 and nls349 were put on NGM plates. After 24 h, L1 larvae were scored for expression of the P_egl-1::4xNLS::gfp transgene using a compound microscope equipped with fluorescence optics.

Germline Transformation. Germ-line transformation experiments were performed as described (9). gfp or mCherry transgenes were injected at 50–100 µg/mL into lin-15(n765a) or ced-3(717); lin-15(n765a) animals with 50 µg/mL of pL15EK as a coinjection marker (10). For ceh-34 rescue experiments, cosmids or genomic constructs were injected at 10 µg/mL into ceh-34(n4796) animals with 50 µg/mL of P_lwm-11::gfp as a coinjection marker (11). For egl-1 (n4820 n5334) rescue experiments, the egl-1 transgenes were injected at 2 µg/mL into eql-1(n4820 n5334) animals with 50 µg/mL P_lwm-11::gfp as a coinjection marker (11).

Molecular Biology and Transgenes. The P_ceh-28′:4xNLS::gfp and P_ceh-28′:4xNLS::mCherry transgenes contained 2.4 kbp of 5′ promoter of ceh-28 in pPD122.56 carrying either gfp or mCherry. The P_gcy-10′:4xNLS::gfp transgene contained 1.8 kbp of 5′ promoter of gcy-10 in pPD122.56. The ceh-34′-rescuing transgene contained 4.0 kbp of 5′ promoter, the coding region, and 0.5 kbp 3′ of the stop codon in pGEM-T Easy vector. The ceh-34′:gfp transgene contains 3.8 kbp of 5′ promoter and the coding region in pPD95.77. The eql-1′-rescuing transgene contained 6.5 kbp of 5′ promoter, the coding region, and 2.2 kbp 3′ of the eql-1 stop codon. The QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) was used to make eql-1 constructs containing one or both of the point mutations n4820 or n5334. The P_egl-1′::4xNLS::gfp transgene contained 6.5 kbp of the 5′ promoter of eql-1 in pPD122.56. For in vitro transcription and translation, ceh-34 cDNA fragments containing their entire coding regions were cloned in pGEM-T Easy. For a GST-EYA-1C fusion protein, the C-terminal region of EYA-1 corresponding to amino acids 220–503 was cloned in pGEX-4T-3.

In Vitro GST Fusion Protein-Binding Experiments. GST-EYA-1C fusion proteins were expressed in Escherichia coli strain BL21 and were purified using glutathione Sepharose 4B (Amersham Biosciences). Full-length [32P]methionine-labeled CEH-34 and luciferase were expressed using a reticulocyte lysate in vitro transcription and translation system (Promega). [32P]methionine-labeled CEH-34 and luciferase were incubated with GST-EYA-1C bound to glutathione Sepharose 4B for 2 h at 4 °C in binding buffer containing 20 mM Heps (pH 7.7), 150 mM NaCl, 0.1% Nonidet P-40, 2.5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol. The beads were washed three times with binding buffer, and the bound proteins were eluted by boiling in SDS/PAGE sample buffer for 5 min. The samples were subjected to 6–18% gradient Tris-HCl polyacrylamide gel electrophoresis (BioRad) followed by autoradiography.

Gel Mobility Shift Assays. A 30-bp double-stranded radioactive probe was generated by annealing two partially overlapping 26-bp oligonucleotides and filling in a 5′ overhang using α-32P-CTP and Klenow polymerase; unincorporated α-32P-CTP was removed using a gel-filtration column packed with Sephadex G-25 (Amersham Biosciences). Full-length CEH-34 protein was expressed by in vitro transcription and translation using a reticulocyte lysate (Promega); 0.5–2 µL reticulocyte lysate was incubated with 10,000 cpm 32P-labeled probe in 20 mM Heps (pH 7.9), 100 mM KCl, 1 mM DTT, 0.3% BSA, 0.01% Nonidet P-40, and 20% glycerol for 1 h at room temperature. The reactions were analyzed by electrophoresis using non-denatured 6% polyacrylamide gels in 0.5% TBE buffer followed by autoradiography. For competition experiments, CEH-34 protein was preincubated with unlabeled competitors for 20 min at room temperature followed by incubation with 32P-radioactive probes.


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**Fig. S1.** The merged epifluorescence of the *Pceh-28::4xNLS::gfp* transgene and Nomarski images are presented. Genotypes are (A) *ced-3*(n717), (B) *egl-1*(n4820), and (C) *ceh-34*(n4796). The surviving M4 sister cell is similarly located at the ventral side of the posterior bulb in these mutants. The arrows indicate the M4 neuron, and the arrowheads indicate the surviving M4 sister cell in the posterior bulb. (Scale bar, 20 μm.)

**Fig. S2.** An *egl-1*(n4820) C-to-T mutation is responsible for survival of the M4 sister cell. The transgenes containing the *egl-1* genomic fragment of the wild type *egl-1*(+), *egl-1*(n5334), *egl-1*(n4820), and *egl-1*(n4820 n5334) were used to transform *egl-1*(n4820 n5334) animals, and the animals carrying transgenes were assayed for the phenotype of M4 sister-cell survival; 60 animals were scored for each strain.

**Fig. S3.** SNP mapping of *ceh-34*(n4796); *n4796* was mapped between nucleotides 4549 on C52A10 (map position −2.4) and 38015 on F08F3 (map position −1.9) on chromosome V. Cosmid C10GB, highlighted in red, rescued the phenotype of M4 sister-cell survival of *ceh-34*(n4796) mutants. A fragment of C10GB containing only *ceh-34* rescued the phenotype of M4 sister-cell survival. The number of rescued lines is indicated.

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Fig. S4. Alignment of *C. elegans* CEH-34 protein sequence with that of *Drosophila* sine oculis (SO) and human SIX1. Accession numbers: NP_504419 for CEH-34, NP_476733 for SO, and CAA62974 for human SIX1. The CLUSTAL W algorithm was used to align the amino acids. Identical amino acids and similar amino acids are indicated with inverted boxes and gray boxes, respectively. A yellow underline indicates the extent of the Six domain, and the orange underline indicates the extent of the homeodomain.

Table S1. *egl-1(n4820), ceh-34(n4796), and eya-1(ok654Δ) promote survival of the M4 sister cell and I1 sister cells but not of the NSM sister cells or the VC homologs*

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>Percent M4 sister survival†</th>
<th>Percent I1 sister survival‡</th>
<th>Percent NSM sister survival‡</th>
<th>Extra VC homologs§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>ced-3(n717)</td>
<td>94</td>
<td>57</td>
<td>100</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>*egl-1(n1084 n3082)</td>
<td>97</td>
<td>52</td>
<td>100</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>*egl-1(n4820)</td>
<td>80</td>
<td>10</td>
<td>0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>*ceh-34(n4796)</td>
<td>38</td>
<td>47</td>
<td>0</td>
<td>0.0 ± 0.1</td>
</tr>
<tr>
<td>*eya-1(ok654Δ)¶</td>
<td>49</td>
<td>19</td>
<td>1</td>
<td>0.0 ± 0.1</td>
</tr>
</tbody>
</table>

*These strains contained integrated gfp reporter transgenes to score survival of the indicated cell type as described in Materials and Methods.
†One hundred twenty animals were scored.
‡Two hundred forty cells were scored.
§These strains contained *dpy-11(e224)*, which does not affect programmed cell death.
¶eya-1(ok654Δ) animals were the progeny of *eya-1(ok654Δ); tjEx21[eya-1(+); rol-6(su1006); sur-5::gfp]* animals. eya-1(ok654Δ) animals that lose the *eya-1(+)-rescuing extrachromosomal array were scored.

Table S2. *n4820 is an allele of egl-1*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percent M4 sister survival</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0</td>
<td>120</td>
</tr>
<tr>
<td>*egl-1(n1084 n3082)</td>
<td>97</td>
<td>120</td>
</tr>
<tr>
<td>*egl-1(n1084 n3082)+</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>*egl-1(n4820)</td>
<td>80</td>
<td>120</td>
</tr>
<tr>
<td>*egl-1(n4820)+</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>*egl-1(n4820)+egl-1(n1084 n3082)</td>
<td>71</td>
<td>24</td>
</tr>
</tbody>
</table>

All strains contained integrated gfp reporter transgenes to score survival of the M4 sister cell.

Table S3. *Human SIX can rescue the defect in M4 sister-cell death of ceh-34(n4796) mutants*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percent M4 sister survival</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0</td>
<td>120</td>
</tr>
<tr>
<td><em>ceh-34(n4796)</em></td>
<td>38</td>
<td>140</td>
</tr>
<tr>
<td>*ceh-34(n4796); Ex[P..ceh-34::ceh-34 cDNA] line 1</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>*ceh-34(n4796); Ex[P..ceh-34::ceh-34 cDNA] line 2</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>*ceh-34(n4796); Ex[P..ceh-34::SIX1 cDNA] line 1</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>*ceh-34(n4796); Ex[P..ceh-34::SIX1 cDNA] line 2</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

All strains contained integrated gfp reporter transgenes to score survival of the M4 sister cell. For each transgene, *P..ceh-34::ceh-34 cDNA* and *P..ceh-34::SIX1 cDNA*, two independent transgenic lines, were scored.