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

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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* 5′ *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 5′ *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*.

cell death | sine oculis | eyes absent | transcription

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. 1*A*) (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 P_{ceh-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. 1 *A* 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 variably as in *ced-3* mutants (Fig. 1*B* 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. 1 *B* and *C* and Table S1). In *n4796* mutants, 38% of the M4 sister cells (*n* = 140) and
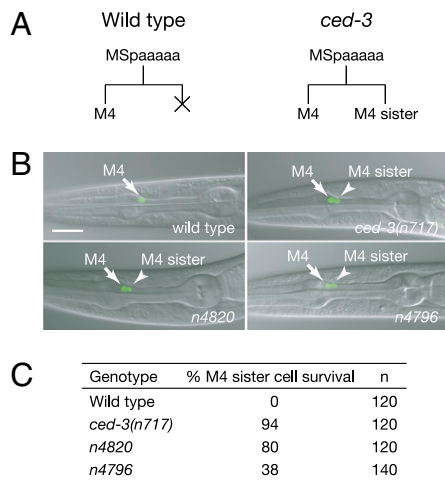
Fig. 1. Mutants defective in M4 sister-cell death. (*A*) A schematic representation of the M4 lineage in the wild-type and *ced-3* mutants is shown. The M4 sister cell undergoes programmed cell death in the wild-type and survives in *ced-3* animals. (*B*) Merged epifluorescence and Nomarski images of the pharynx in the wild-type, *ced-3(n717)*, *n4820*, and *n4796* animals carrying the $P_{ceh-28}::4xNLS::gfp$ transgene are shown. Arrow, the M4 neuron; arrowhead, the surviving M4 sister cell. (Scale bar, 20 μm.) (*C*) The penetrances 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. 2*A*). 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 an *egl-1* genomic fragment containing 6.5 kb upstream of the *egl-1* start codon, the *egl-1* coding region, and 2.2 kb downstream of the *egl-1* stop codon. Four of five transgenic lines that contained a wild-type *egl-1* genomic fragment and three of six transgenic lines that contained an *egl-1* genomic fragment carrying the G-to-C mutation were rescued for the phenotype of M4 sister survival (i.e., the M4 sister cells died in the rescued lines) (Fig. 2*B* and Fig. S2). Zero of five transgenic lines that contained an *egl-1* genomic fragment carrying either the C-to-T mutation or both the G-to-C and C-to-T mutations were rescued for the phenotype of M4 sister survival (Fig. 2*B* and Fig. S2). Furthermore, we isolated a 268-bp genomic deletion, *n5347Δ*, lacking the region of the C-to-T mutation but not the region of the G-to-C mutation (Fig. 2*A*). In *n5347Δ* mutants, 90% of the M4 sister cells (*n* = 118) and 39% of the I1 sister cells (*n* = 240) survived; *n5347Δ* did not affect the
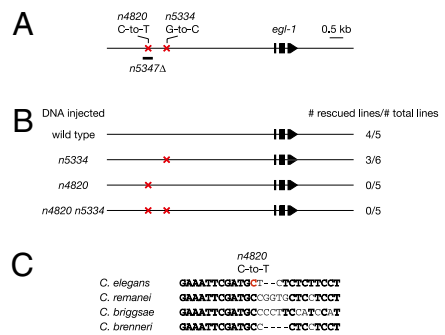

Fig. 2. *n4820* is an *egl-1* noncoding allele. (*A*) The *egl-1(n4820)* isolate contained two mutations in the *egl-1* 5′ upstream noncoding region: C-to-T (*n4820*) and G-to-C (*n5334*). The extent of the *n5347Δ* deletion is shown. The exons of *egl-1* are shown as black boxes. (*B*) The indicated *egl-1* genomic fragments were injected into *egl-1(n4820 n5334)* animals to test for rescue of 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.

deaths 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. 2*A*). The DNA sequence immediately surrounding the *n4820* mutation is highly conserved in other nematode species (Fig. 2*C*). 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 *n5347Δ* 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 cosmid 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. 3*A*). RNAi of *ceh-34* caused survival of the M4 sister cell (38% 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. 3*B*). 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 homeodomain, 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)*
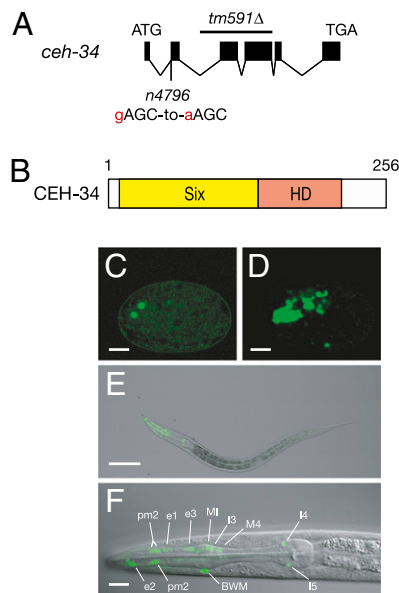
Fig. 3. *n4796* is an allele of the Six family homeodomain gene *ceh-34*. (*A*) Genomic organization of the *ceh-34* locus. The location and nature of the *n4796* mutation are indicated. The red lowercase letter indicates the splice acceptor site of the *n4796* mutation in the first intron, and the uppercase letters indicate DNA sequences in the second exon. The extent of the *tm591Δ* deletion is indicated. (*B*) Representation of the domain structure of the CEH-34 protein. The yellow box indicates the Six domain (Six), and the orange box indicates the homeodomain (HD). (*C–F*) Epifluorescence images showing expression of the *ceh-34::gfp* transgene. (*C*) The early embryo, (*D*) 1.5-fold stage embryo, (*E*) first-stage (L1) larva, and (*F*) the pharynx of an L1 larva at higher magnification. Cells expressing the *ceh-34::gfp* transgene are identified in *F*. BWM, body-wall muscle. (Scale bars: *C*, *D*, and *F*, 10 μm; *E*, 50 μm.)

mutants (Table S3). We conclude that the functions of *C. elegans ceh-34* and human *SIX* are evolutionarily conserved.

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. 3*F*).

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* (*ey*) 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. 4*A* 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. 4*A*). 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. 4*B*). 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
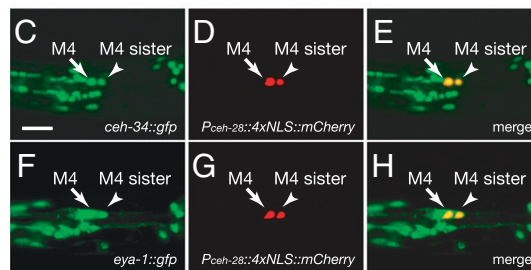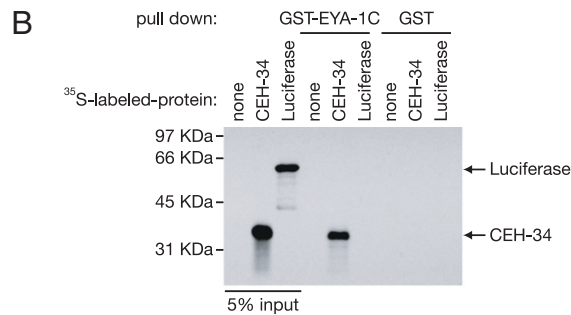

Fig. 4. (*A*) The penetrances of M4 sister-cell survival in the indicated mutants are shown. *Pax6*, *paired-like* homeodomain gene; *Eya*, mammalian *eyes absent* homolog; *Dach*, mammalian *dachshund* homolog. (*B*) CEH-34 binds the C-terminal domain of EYA-1 (EYA-1C) in vitro; 5% of input proteins and bound proteins were subjected to electrophoresis and visualized using autoradiography. The positions of CEH-34 and luciferase are indicated by arrows. (*C–H*) *ceh-34::gfp* and *eya-1::gfp* transgenes are expressed in the M4 sister cells in *ced-3* mutants. (*C* and *F*) Expression of the *ceh-34::gfp* and *eya-1::gfp* transgenes, respectively. (*D* and *G*) Expression of the M4 neuron and the M4 sister-cell marker *Pceh-28::4xNLS::mCherry*. (*E* and *H*) Merged images. Arrows indicate the M4 neurons, and arrowheads indicate the M4 sister cells. (Scale bar, 10 μm.)

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. 5*A*) (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. 5*B*).

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. 5*A*) competed with the labeled wild-type probe for CEH-34 binding (Fig. 5*C*). By contrast, competitors with mutations in the SO consensus-binding sequence (II, III, IV, and scrambled in Fig. 5*A*) did not efficiently compete with the labeled wild-type probe for CEH-34 binding (Fig. 5*C*). 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. 5*D*). 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 $P_{egl-1}::4xNLS::gfp$ 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 (ok654Δ); ced-3* animals ($n = 89$), the M4 sister cell did not express the $P_{egl-1}::4xNLS::gfp$ 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. 6*H*). 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(ok654Δ)*, 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
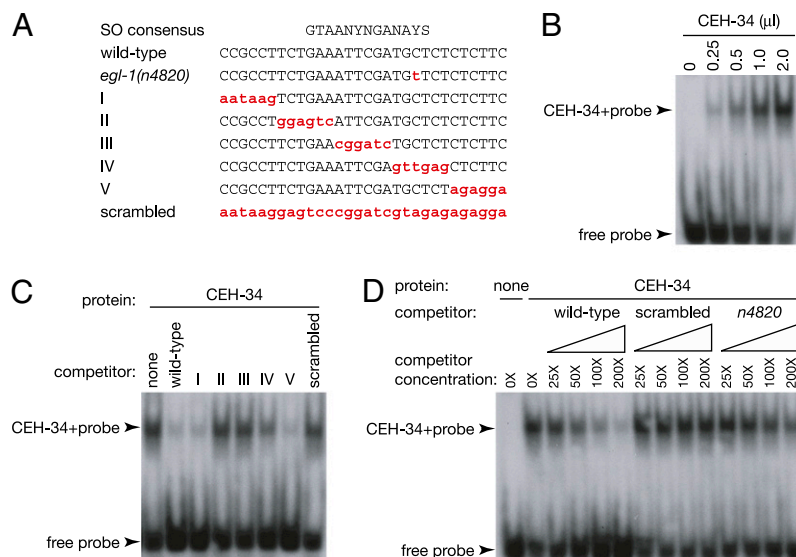

Fig. 5. CEH-34 binds to the *egl-1* 5′ *cis*-regulatory element defined by *egl-1(n4820)*. (*A*) Sine Oculis (SO) consensus-binding sequence and the DNA sequences of oligonucleotide probes used in the gel-shift assays are shown. Mutated bases are shown in red lowercase letters. Y indicates C or T, S indicates G or C, and N indicates A, C, G, or T. (*B*) The indicated amount of reticulocyte lysate expressing CEH-34 was mixed with the wild-type radioactive probe. The shifted band and free probe are indicated by arrowheads. (*C*) The indicated unlabeled competitors were preincubated in 200-fold molar excess to the radiolabeled probe. The shifted band and free probe are indicated by arrowheads. (*D*) The indicated unlabeled competitors were preincubated in 0- to 200-fold molar excess to the radiolabeled probe. The shifted band and free probe are indicated by arrowheads.
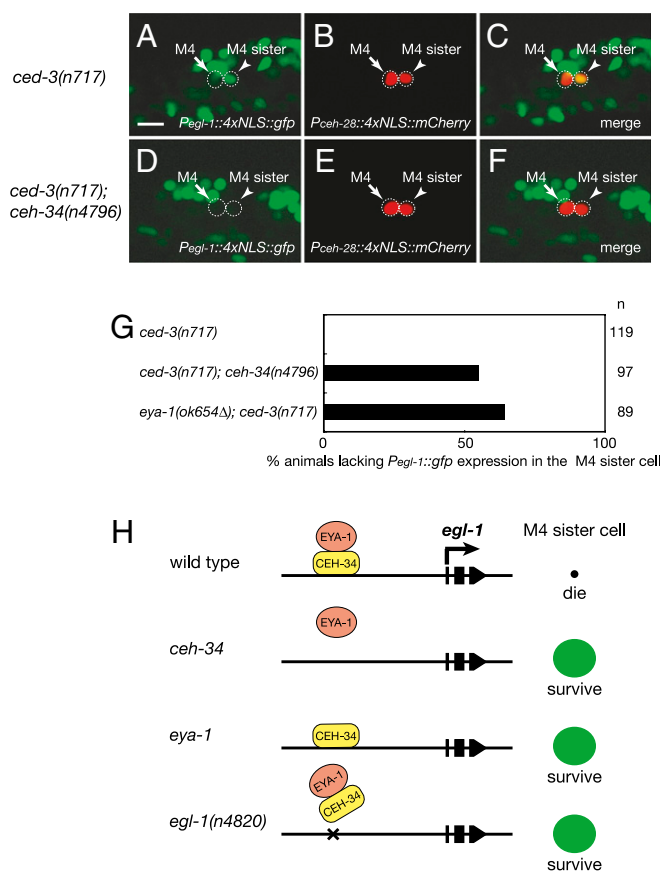
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Fig. 6. egl-1 expression in the M4 sister cell requires ceh-34 and eya-1. (A–F) The P_{egl-1}::4xNLS::gfp and P_{ceh-28}::4xNLS::mCherry transgenes were coexpressed in ced-3(n717) animals (A–C) and ced-3(n717); ceh-34(n4796) animals (D–F). (A and D) Expression of the P_{egl-1}::4xNLS::gfp transgene. (B and E) Expression of the P_{ceh-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(ok654Δ); 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 regu-

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 homeodomain 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.

DEVELOPMENTAL BIOLOGY

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