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The *C. elegans* protein CEH-30 protects male-specific neurons from apoptosis independently of the Bcl-2 homolog CED-9

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The developmental control of apoptosis is fundamental and important. We report that the *Caenorhabditis elegans Bar* homeodomain transcription factor CEH-30 is required for the sexually dimorphic survival of the male-specific CEM (cephalic male) sensory neurons; the homologous cells of hermaphrodites undergo programmed cell death. We propose that the cell-type-specific anti-apoptotic gene *ceh-30* is transcriptionally repressed by the TRA-1 transcription factor, the terminal regulator of sexual identity in *C. elegans*, to cause hermaphrodite-specific CEM death. The established mechanism for the regulation of specific programmed cell deaths in *C. elegans* is the transcriptional control of the BH3-only gene *egl-1*, which inhibits the *Bcl-2* homolog *ced-9*; similarly, most regulation of vertebrate apoptosis involves the *Bcl-2* superfamily. In contrast, *ceh-30* acts within the CEM neurons to promote their survival independently of both *egl-1* and *ced-9*. Mammalian *ceh-30* homologs can substitute for *ceh-30* in *C. elegans*. Mice lacking the *ceh-30* homolog *Barhl1* show a progressive loss of sensory neurons and increased sensory-neuron cell death. Based on these observations, we suggest that the function of Bar homeodomain proteins as cell-type-specific inhibitors of apoptosis is evolutionarily conserved.

[*Keywords*: Apoptosis; cell death; cell fate; *elegans*; Bar homeodomain; sex determination]

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Programmed cell death, or apoptosis, is a widespread feature of animal development. Abnormalities in apoptosis can have pathological consequences. Reduced cell death can cause the survival of unnecessary or unwanted cells, such as neurons that have not made appropriate synaptic connections (for review, see Yeo and Gautier 2004) or cells that could be dangerous if they were to survive, such as immune cells that recognize self-antigens (Bidere et al. 2006) and cells that have escaped controls on their proliferation (Weaver and Cleveland 2005). Increased programmed cell death is associated with a broad variety of human disorders, including immunodeficiency and neurodegenerative diseases (for review, see Rathmell and Thompson 2002; Krantic et al. 2005).

A core pathway for the cell-killing step of apoptosis is conserved from nematodes to humans; key insights concerning this pathway have come from investigations of programmed cell death in *Caenorhabditis elegans* (Metzstein et al. 1998). In this core pathway, cells are killed by a cysteine protease called a caspase; in *C. elegans*, this caspase is CED-3 (*ced*, cell death abnormal) (Yuan et al. 1993). Caspase activity is promoted by an adaptor molecule, called CED-4 in *C. elegans* (Yuan and Horvitz 1992; Shaham and Horvitz 1996) and Apaf-1 in mammals (Zou et al. 1997). CED-4 and Apaf-1 activation is regulated by multidomain members of the CED-9 Bcl-2 superfamily. CED-9 is the sole multidomain *Bcl-2* family member in *C. elegans* and provides both anti-apoptotic and, to a lesser extent, proapoptotic activities (Hengartner and Horvitz 1994a,b). Multidomain members of the Bcl-2 superfamily are regulated by BH3-only proteins; in *C. elegans*, the BH3-only protein EGL-1 (*egl*, egg-laying defective) is required for essentially all somatic programmed cell deaths (Conradt and Horvitz 1998).

Despite a detailed knowledge of this core pathway for apoptosis, less is known about how cells are developmentally determined to die. In mammals, the regulation of programmed cell death in the developing immune system by recognition of self-antigens (for review, see Bidere et al. 2006) and in the developing nervous system by

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neurotrophic signals has been described (for review, see Weaver and Cleveland 2005). In *C. elegans*, 131 cells die during hermaphrodite development; during male development, another 21 cells die that in hermaphrodites either do not die or are never generated (Sulston and Horvitz 1977; Kimble and Hirsh 1979; Sulston et al. 1983). Nine genes that exert cell-specific control over eight of these 152 cell deaths have been described (Ellis and Horvitz 1991; Metzstein et al. 1996; Conradt and Horvitz 1999; Metzstein and Horvitz 1999; Thellmann et al. 2003; Hoeppner et al. 2004; Liu et al. 2006). These nine genes are believed to regulate cell death by cell-specific transcriptional regulation of the BH3-only killer gene *egl-1*, which acts to inhibit the Bcl-2 homolog CED-9. Mutations in human counterparts of these cell-specific regulators of apoptosis can contribute to disease, particularly to cancer. For example, *ces-1* and *ces-2* (*ces*, cell death specification), *C. elegans* genes that specifically regulate the deaths of the sister cells of the NSM neurons, have human homologs that can regulate B-cell survival in humans, and translocations altering the *ces-2* homolog *HLF* cause Acute Lymphoblastic Leukemia (Inaba et al. 1996; Wu et al. 2005).

We studied the survival decision of one of the two classes of neurons sexually dimorphic for programmed cell death in *C. elegans*, the CEM (cephalic male) neurons. The presumptive CEM neurons die during hermaphrodite embryogenesis but survive in males to become sensory neurons (Sulston et al. 1983; Chasnov et al. 2007). We report that the gene *ceh-30* encodes a cell-type-specific anti-apoptotic homeodomain transcription factor. *ceh-30* is directly regulated by the sex determination pathway to control the sex-specific survival of the CEMs. The anti-apoptotic function of *ceh-30* involves a novel mechanism that is independent of the Bcl-2 homolog CED-9 and the BH3-only cell-killing gene *egl-1*. The anti-apoptotic function of *ceh-30* is conserved in its mammalian counterparts *Barhl1* and *Barhl2*; mice lacking *Barhl1* suffer from progressive deafness, likely because of increased apoptosis of sensory hair cells of the inner ear.

Results

ceh-30 *gain-of-function mutations cause CEM neuron survival in hermaphrodites and act downstream from sex determination*

The cell-fate reporter *pkd-2*::*gfp* is expressed in the male-specific CEM neurons as well as in some male-specific tail neurons (Fig. 1A,B; Barr and Sternberg 1999). We found that *pkd-2*::*gfp* was expressed in hermaphrodite CEM neurons when the programmed deaths of these cells were prevented either by weak masculinization or by a defect in cell death (Fig. 1C,D; Table 1A). We used *pkd-2*::*gfp* as a marker of CEM survival in screens for mutant hermaphrodites in which the CEM neurons survived (H.T. Schwartz and H.R. Horvitz, unpubl.). Among the isolates we recovered were three mutations—*n3713*, *n3714*, and *n3720*—each of which semidominantly causes CEM survival in hermaphrodites (Table 1; Fig. 1E). No other defects were seen in these three mutant strains. The *pkd-2*::*gfp*-expressing CEM neurons of these mutant hermaphrodites more closely resembled those seen in cell death-defective hermaphrodites than those seen in weakly masculinized hermaphrodites: The intensity of GFP expression and the process morphologies and nuclear positions of the CEMs were more variable in hermaphrodites defective in cell death than in males or in partially masculinized hermaphrodites (data not shown); this latter observation is consistent with previous electron microscopic examination of *ced-3(n717)* hermaphrodites, which found variability in the processes of undead CEM neurons (White et al. 1991). We mapped the three mutations to the left end of a six-map-unit interval on LGX and found that the three mutations acted similarly (Table 1A; data not shown). We showed that the three mutations are in the same gene, which we later determined to be *ceh-30* (*ceh*, *C. elegans* homeobox) (see below).

We performed gene dosage experiments and concluded that *n3713* and *n3714* are gain-of-function (gf) mutations (Table 1B; Supplementary Table S1). Adding either of two large genomic duplications *mnDp57* and *yDp14*, each of which carries an extra copy of the *ceh-30* region, did not cause increased CEM survival in wild-type hermaphrodites, and adding wild-type *ceh-30* copies to *n3713* or *n3714* mutants neither enhanced nor suppressed their semidominant CEM survival phenotypes. The latter result indicates that *n3713* and *n3714* do not cause loss-of-function (lf) or dominant-negative activity and must therefore cause a gain-of-function activity, resulting in either altered or ectopic gene function. Data presented below concerning the wild-type function of *ceh-30* and the nature of the *ceh-30(*gf*)* mutations are consistent with the hypothesis that the gain-of-function mutations cause ectopic expression of the wild-type gene product in the CEMs of hermaphrodites.

One way mutations can cause CEM survival in hermaphrodites is by partial masculinization, so that the CEMs adopt their male fate of survival in animals that are nonetheless predominantly hermaphrodites. Unlike the mutation *sel-10(n1077)*, which causes CEM survival by partial masculinization (Desai and Horvitz 1989; Jager et al. 2004), *ceh-30(*gf*)* mutations did not enhance the somatic masculinization caused by the weak *tra-2* (*tra*, sexual transformer) alleles *e1875* and *n1106* (data not shown). Thus, *ceh-30(*gf*)* does not act broadly to promote masculinization. Both *n3713*gf and *n3714*gf protected the CEMs of animals completely feminized by null mutations in the *fem* genes, which are the final genes required for masculinization in the sex determination pathway (Table 1C; Supplementary Table S2; Hodgkin 2002). Therefore, *ceh-30(*gf*)* mutations act to cause CEM neuron survival downstream from or in parallel to all genes required for masculinization.

Loss of ceh-30 *function causes CEM neurons in males to undergo programmed cell death*

We sought intragenic suppressors of *ceh-30(n3714*gf*)* and recovered one mutation, *n4111*, that proved to be an al-

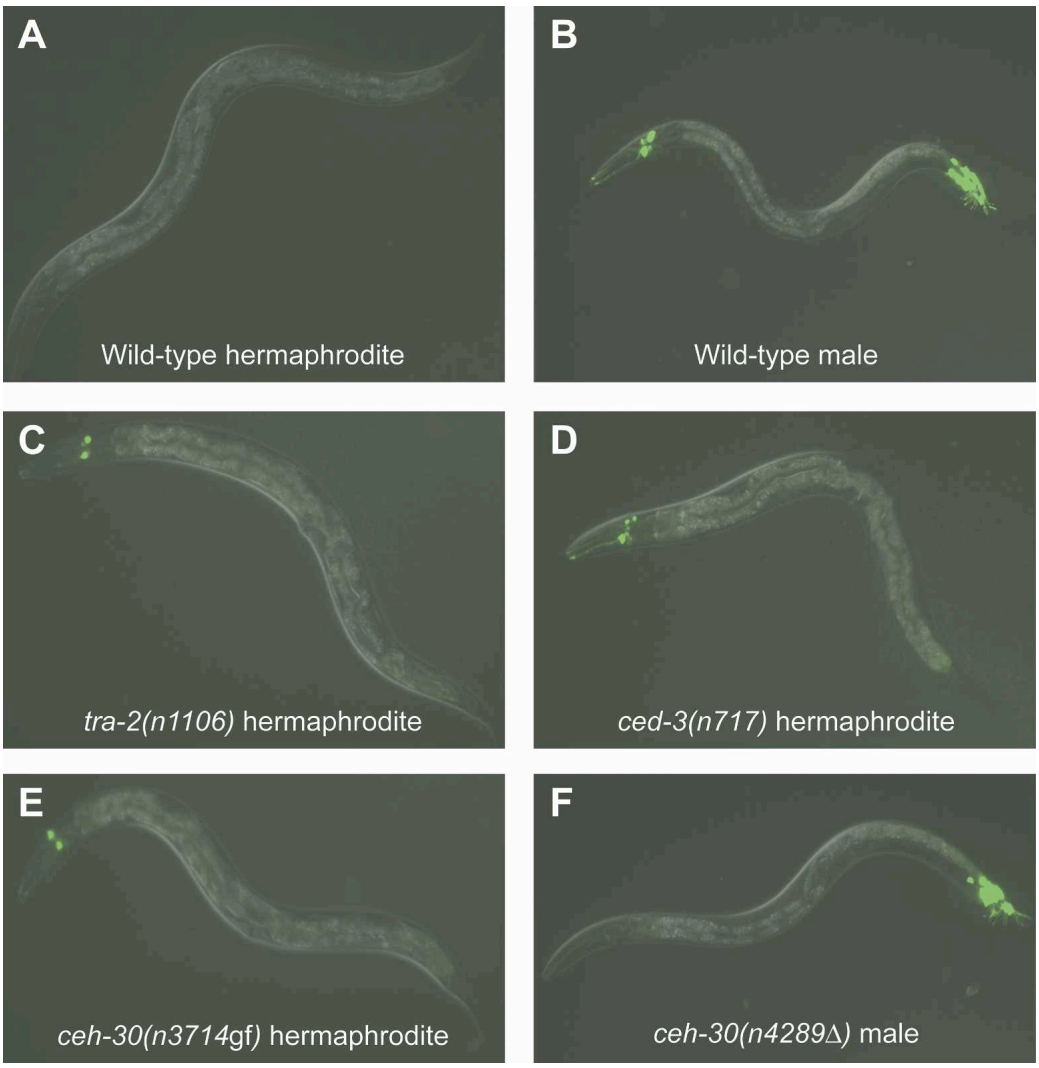

Figure 1. *pkd-2*::*gfp* is expressed in the CEM neurons and is a marker of CEM survival. Merged fluorescence and visible light images. (*A*) Wild-type hermaphrodite. (*B*) Wild-type male. (*C*) *tra-2(n1106)* partially masculinized hermaphrodite. (*D*) *ced-3(n717)* cell death-defective hermaphrodite. (*E*) *ceh-30(n3714*gf*)* hermaphrodite. (*F*) *ceh-30(n4289*Δ*)* male. All strains are homozygous for the *pkd-2*::*gfp* reporter *nIs133* and *him-5(e1467)*. Genotypes are otherwise as indicated. Anterior is to the left, and ventral is down.

lele of *ceh-30* (Table 2A; see Materials and Methods; see below). *ceh-30(n4111 n3714)* caused an effect opposite to that caused by *ceh-30(*gf*)*: Whereas *ceh-30(n3714*gf*)* hermaphrodites have normally male-specific CEM neurons, *ceh-30(n4111 n3714)* males lack CEM neurons, as do normal hermaphrodites (Table 2B). The CEM-deficient phenotype of *ceh-30(n4111 n3714)* males is recessive: *tra-1(*lf*)*; *ceh-30(n4111 n3714/+)* males did not lack CEMs [*tra-1(*lf*)* can be used to cause animals with two X chromosomes to develop as males], and the CEM-deficient phenotype of *ceh-30(n4111 n3714)* males was complemented by the genomic duplications *mnDp57* and *yDp14* (Supplementary Table S3; data not shown). Experiments detailed below demonstrated that *n4111* causes a loss of gene function. The missing CEM neurons of *ceh-30(n4111 n3714)* males were not restored by a null mutation in *tra-1* (Table 2B; Supplementary Table S4). *tra-1* is the most downstream gene in the sex determination pathway (Hodgkin 2002), indicating that *ceh-30* does not act within the sex determination pathway to cause CEMs to adopt their male fate of survival. The missing CEM neurons of *ceh-30(n4111 n3714)* males were completely restored by loss of function of *egl-1*, *ced-4*, or *ced-3* genes required for programmed cell death (Table 2B; Supplementary Table S4). The missing CEMs of *ceh-30(*lf*)* males are therefore generated as in wild-type males and then inappropriately undergo programmed cell death.

CEH-30 is an evolutionarily conserved Bar homeodomain transcription factor

We mapped *n3714*gf to a 25-kb interval on the cosmid C33D12 and established an overlapping but less well-defined position for *n4111* (see Materials and Methods). *ceh-30(n4111 n3714)* males transformed with the over-

Table 1. *A* ceh-30 *gain-of-function mutation causes survival of the sexually dimorphic CEM neurons by acting downstream from or in parallel to the sex determination pathway*

(A) Mutations in the *ceh-30* locus semidominantly cause CEM survival

Genotype	Sex	CEM survival (%)			
		None	D or V	D and V	*n* =
Wild type	Herm.	100	0	0	60
Wild type	Male	0	0	100	60
tra-2(n1106)	Intersex	0	8	92	60
egl-1(n1084 n3082)	Herm.	0	7	93	60
ced-4(n1162)	Herm.	0	6	94	63
ced-3(n2427)	Herm.	85	15	0	113
ced-3(n717)	Herm.	0	10	90	60
*ceh-30(n3713*gf*)/+*	Herm.	22	72	7	60
*ceh-30(n3714*gf*)/+*	Herm.	22	70	8	60
*ceh-30(n3720*gf*)/+*	Herm.	25	67	8	60
*ceh-30(n3713*gf*)*	Herm.	0	47	53	60
*ceh-30(n3714*gf*)*	Herm.	0	42	58	60
*ceh-30(n3720*gf*)*	Herm.	0	48	52	60
*ceh-30(n3713*gf*/n3714*gf*)*	Herm.	0	50	50	60
*ceh-30(n3713*gf*/n3720*gf*)*	Herm.	2	47	52	60

(B) The *ceh-30* mutation *n3714* acts by gain of function to cause CEM neuron survival

Genotype	*ceh-30* locus	CEM survival in hermaphrodites (%)			
		None	D or V	D and V	*n* =
Wild type	+/+	100	0	0	60
yDp14	+/+/+/+	100	0	0	60
ceh-30(n3714)/+	gf/+	28	68	3	60
yDp14/+; ceh-30(n3714)/+	gf/+/+	30	65	5	60
ceh-30(n3714)	gf/gf	0	48	52	100
yDp14/+; ceh-30(n3714)	gf/gf/+	3	53	44	68
yDp14; ceh-30(n3714)	gf/gf/+/+	2	57	42	60

(A) In this and other tables, CEM survival was scored using a *pkd-2*::*gfp* reporter as described in Materials and Methods. When CEM survival was scored using the dissecting microscope, the left and right ventral CEMs could not be distinguished readily from each other and the left and right dorsal CEMs could not be distinguished readily from each other; CEM survival was therefore assessed for ventral CEMs and for dorsal CEMs. The resulting numbers were found to be reproducible and sensitive to changes in the degree of CEM death or survival. In this and in other tables, "D or V" denotes animals in which dorsal or ventral CEMs, but not both, were observed and indicates animals displaying only weak CEM survival; "D and V" denotes animals in which both dorsal and ventral CEMs were observed and indicates animals showing strong CEM survival. All animals were homozygous for *nIs133*. Otherwise, the genotypes of the animals analyzed were as listed, except for the heterozygotes, which were heterozygous for *unc-2(e55)* and *lon-2(e678)*. Maternal genotype did not affect the results concerning the heterozygotes (data not shown). *tra-2(n1106)* hermaphrodites are weakly masculinized (Desai and Horvitz 1989). *ced-3(n2427)* and *ced-3(n717)* animals are weakly and strongly defective in programmed cell death, respectively (Shaham et al. 1999). (Herm.) Hermaphrodite. *tra-2(n1106)* XX animals are largely hermaphroditic with some male characteristics, including CEM survival.
(B) All animals were homozygous for *nIs128*. Otherwise, genotypes were as listed, except that when the chromosomal duplication *yDp14* was present the strain was also homozygous for *unc-2(e55)* and *lon-2(e678)*, and the *ceh-30(n3714)/+* strain was heterozygous for *unc-2(e55)* and *lon-2(e678)*. (*ceh-30* locus) Number of wild-type (+) and mutant (gf) copies of the locus in each strain. An expanded version of this table, examining all of the same dosage conditions using the duplication *mnDp57* and using the allele *ceh-30(n3713*gf*)*, is shown in Supplementary Table S1.

continued on next page

lapping cosmids C13G6 and C33D12 were rescued for CEM survival (Fig. 2A; data not shown). Examination of the genomic sequence corresponding to these cosmids revealed that an intron of the gene *ceh-30* contains a consensus binding site for the transcription factor TRA-1, which is required to repress male sexual fates (see Fig. 2D; Zarkower and Hodgkin 1993). We determined the DNA sequence of *ceh-30* in our mutants and found that *n4111 n3714* animals but not the *n3714* parental strain had a mutation in the predicted *ceh-30* coding sequence, changing codon 21 from glutamine to an ochre stop codon. All three independently isolated *ceh-30(*gf*)* mu-

Table 1. *(continued)*

(C) Gain of *ceh-30* function promotes CEM survival downstream from or parallel to the *fem* genes

Genotype	CEM survival in hermaphrodites (%)			
	None	D or V	D and V	*n* =
Wild type	100	0	0	60
*ceh-30(n3714*gf*)*	0	45	55	60
fem-1(e1965)	98	2	0	60
*fem-1(e1965); ceh-30(n3714*gf*)*	5	55	40	60
fem-2(e2105)	99	1	0	139
*fem-2(e2105); ceh-30(n3714*gf*)*	3	84	14	110
fem-3(e1996)	100	0	0	60
*fem-3(e1996); ceh-30(n3714*gf*)*	12	43	45	60

(C) All animals were homozygous for *nIs133*. Otherwise, the genotypes of the animals analyzed were as listed. The *fem-1(e1965)* and *fem-3(e1996)* homozygotes scored were the progeny of crosses between *fem/nT1*[*qIs51*] males and *fem* homozygous females; thus, half were XX females and half X0 females. *fem-2(e2105)* homozygotes scored were the progeny of maternally rescued *fem-2(e2105)* homozygotes. An expanded version of this table, including similar results obtained using *ceh-30(n3713*gf*)*, is shown in Supplementary Table S2.

tants had an identical mutation altering an evolutionarily conserved predicted TRA-1-binding site in the second intron of *ceh-30* (Fig. 2D). This mutation is equivalent to one known to prevent TRA-1 from binding to a regulatory site in vitro and to prevent transcriptional repression by *tra-1* in vivo (Conradt and Horvitz 1999). Similarly, we found that TRA-1A could bind the site mutated by *n3714*gf in gel-shift experiments and that this binding was prevented by the addition of excess unlabeled wild-type probe, but was very poorly competed by excess unlabeled probe containing the *n3714*gf mutation (Fig. 2E). The nature of the *ceh-30* gain-of-function mutations is consistent with the results of our gene-dosage experiments and suggests a model in which the gain-of-function mutations release *ceh-30* from a negative regulation that represses *ceh-30* expression in hermaphrodites.

Our isolation of a *ceh-30* deletion mutation, *n4289*Δ, confirmed that *n4111* causes loss of *ceh-30* gene function. *ceh-30(n4289*Δ*)* removes the second exon, which encodes most of the predicted homeodomain (see below), and is predicted to cause a frameshift after amino acid 61 if the first and third exons are spliced together (Fig. 2B). We found that *ceh-30(n4289*Δ*)* caused males to lack CEM neurons (Fig. 1F; Table 2B; Supplementary Table S4) and failed to complement *ceh-30(n4111 n3714)* for CEM survival in *tra-1* XX males (Supplementary Table S3). The transgene BSK-*ceh-30*, which contains the *ceh-30* genomic locus (see Supplemental Material), complemented the CEM survival defects of both *ceh-30(*lf*)* mutants (Fig. 2A; Table 2C; data not shown). A version of the rescuing transgene modified to contain the *n3714*gf mutation in the TRA-1-binding site of *ceh-30* [BSK-*ceh-30(n3714)*] caused CEM survival in hermaphrodites (data not shown).

We performed a *cis–trans* test to confirm our hypothesis that *n3714*gf is an allele of *ceh-30*. Specifically, we asked if the noncoding mutation *n3714*gf causes CEM survival by activating *ceh-30* in *cis*. We found that the *n3714*gf semidominant phenotype of CEM survival in hermaphrodites required only one functional copy of *ceh-30* and that this functional wild-type copy of *ceh-30* must be the copy in *cis* to *n3714*gf: Seventy-six percent of *n3714*gf/+ and 78% of *n3714*gf/*n4289*Δ hermaphrodites had surviving CEMs, but only 4% of *n4111 n3714*gf/+ hermaphrodites had surviving CEMs ($n \geq 100$) (Supplementary Table S5). *n3714*gf therefore affects the same gene as the *ceh-30* loss-of-function mutation *n4111*, proving that *n3714*gf is an allele of *ceh-30*.

ceh-30 encodes a homolog of the *Drosophila* homeodomain transcription factors *BarH1* and *BarH2* (Kojima et al. 1991; Higashijima et al. 1992a) and their murine counterparts *Barhl1* and *Barhl2* (Bulfone et al. 2000). The 237-amino-acid predicted CEH-30 protein is 64% identical to human Barhl1 from amino acids 85–180 of CEH-30 (Fig. 2C). This homology includes both the homeodomain, which contains a phenylalanine-to-tyrosine substitution characteristic of the Bar subclass of homeodomains (Kojima et al. 1991), and a 22-amino-acid motif immediately C-terminal of the homeodomain; our BLAST searches indicated that homologs of this motif are found only in Bar homeodomain proteins, and we named it the BARC motif (Bar homeodomain C-terminal motif) (see Supplementary Table S6). A *ceh-30* minigene including *ceh-30* 5′ and 3′ sequences and 625 bp of *ceh-30* intron 2 (see Supplemental Material) rescued the defect in CEM survival of *ceh-30(n4289*Δ*)* males as effectively as did the original genomic construct (Table 2C). We tested whether the protective function of CEH-30 is evolutionarily conserved with that of its mammalian homologs by replacing the *ceh-30* cDNA of this construct with murine *Barhl1* or *Barhl2* cDNAs. Both of the resulting transgenes rescued the CEM survival defect of *ceh-30(n4289*Δ*)* males (Table 2C).

ceh-30 *acts cell-autonomously in the CEM neurons to promote their survival*

A *ceh-30* genomic construct into which *gfp* had been inserted (see Supplemental Material) rescued *ceh-*

30(n4289Δ) for CEM survival (Supplementary Table S7) and caused GFP expression in the nuclei of many neurons in the threefold embryo and in as many as a dozen neurons in larvae. We did not observe GFP expression in the CEMs of male larvae, indicating that any *ceh-30* expression in the CEM neurons is likely to be weak or transient. The ventral CEM neurons of 1.5-fold stage masculinized embryos, which can be identified by their positions within the embryo, expressed *ceh-30*::*gfp* (see Supplementary Fig. S1). Expression in the dorsal CEMs cannot be as readily examined. We conclude that *ceh-30* is expressed transiently in embryonic CEMs.

We tested whether *ceh-30* acts cell-autonomously in the CEMs to promote their survival by examining animals that developed from zygotes carrying an extrachromosomal *ceh-30(n3714*gf*)* transgene marked with a pan-neuronal *unc-119*::*mStrawberry* reporter to identify neurons containing the transgene. In males, in which the transgene is not required for CEM neurons to survive, 18.9% (n = 477) of CEMs lacked the transgene as a result of mitotic loss. In hermaphrodites, only one of 719 surviving CEMs lacked the transgene, indicating that the CEMs die unless prevented from doing so by a *ceh-30(*gf*)* transgene. (The single hermaphrodite CEM that did not contain the transgene can be explained by the low frequency of spontaneous CEM survival that occurs in wild-type hermaphrodites: In 185 hermaphrodites, one surviving CEM of a possible 740 was seen using *pkd-2*::*gfp* expression as an assay.) Thus, *ceh-30* protected only those CEMs that retained the transgene, indicating that *ceh-30* functions in the CEM neurons.

ceh-30 *acts specifically to control the life-versus-death decision of the CEM neurons*

We tested the ability of *ceh-30* mutations to modify the deaths of cells other than CEMs. The partial loss-of-function alleles *ced-4(n3158)* and *ced-3(n2427)* each causes a

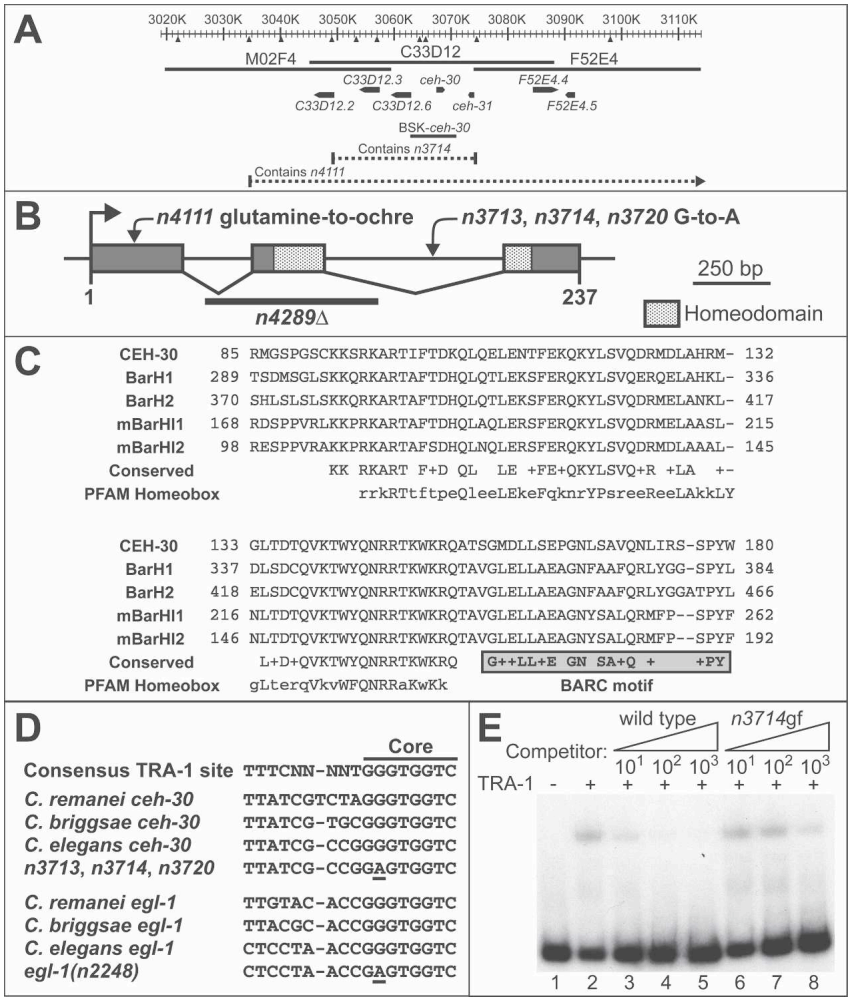

Figure 2. The *ceh-30* locus. (*A*) High-resolution genetic mapping of *ceh-30* mutations. Scale indicates location on LGX. Arrowheads indicate polymorphisms used to map *ceh-30(n3714*gf*)* and *ceh-30(n4111 n3714*lf*)*. Coding regions for *ceh-30* and nearby genes are shown according to Wormbase (http://WS160.wormbase.org). The sequenced cosmid M02F4 is similar to the cosmid C13G6. The *ceh-30* genomic rescuing construct is shown as a horizontal bar labeled "BSK-*ceh-30*." The intervals containing *n3714*gf and *n4111*lf are indicated with dotted lines (see Materials and Methods). (*B*) Genomic organization of the *ceh-30* locus. Locations and natures of *ceh-30* mutations are indicated. The portion of the *ceh-30* locus encoding the homeodomain is indicated by the split dotted box. (*C*) Alignment of the indicated sections of the predicted CEH-30 protein with its *Drosophila* homologs BarH1 (accession no. AAA28382) and BarH2 (accession no. M82884) and mouse homologs mBarhl1 (accession no. AAH55731) and mBarhl2 (accession no. AAH55789). On the line labeled "Conserved," residues identical among all five proteins are named and residues similar among all five proteins are indicated with a plus symbol (+). The PFAM consensus homeobox domain (accession no. PH00046) is shown. A black box surrounds the conserved residues that define the BARC motif we identified as specific to Bar-subclass homeodomain proteins (see Supplementary Table S6). (*D*) The putative TRA-1A-binding sites of *ceh-30* and *egl-1* in *C. elegans* and related nematodes are shown (http://genome.wustl.edu), with *ceh-30(*gf*)* mutations *n3713*, *n3714*, and *n3720* and the homologous *egl1(n2248*gf*)* mutation underlined. The core of the TRA-1 consensus binding site (Zarkower and Hodgkin 1993) is indicated. (*E*) TRA-1A binds to the wild-type but not to the *n3714*gf mutant predicted TRA-1A-binding site in the second intron of *ceh-30*. One nanogram of labeled probe was shifted by in vitro-translated TRA-1A (lane *2*) but not by in vitro translation products made without a *tra-1* template (lane *1*). This binding was competed by excess unlabeled wild-type probe (lanes *3–5*) but only poorly by excess *n3714*gf mutant probe (lanes *6–8*).

Table 2. *The* BarH *homeodomain gene* ceh-30 *protects CEM neurons from apoptosis, and this function is evolutionarily conserved*

(A) *ceh-30(n4111)* is a linked suppressor of *ceh-30(n3714*gf*)*

Genotype	CEM survival in hermaphrodites (%) None	D or V	D and V	*n* =
Wild type	98	2	0	100
*ceh-30(n3714*gf*)*	0	33	67	60
ceh-30(n4111 n3714)	98	2	0	60
*ceh-30(n3714*gf*)/+*	15	67	19	81
*ceh-30(n3714*gf*/n4111 n3714)*	13	68	19	120
ceh-30(n4111 n3714)/+	97	3	0	120

(B) The CEM neurons of *ceh-30(*lf*)* males inappropriately undergo programmed cell death

Genotype	CEM survival in males (%) None	D or V	D and V	*n* =
Wild type	0	0	100	60
*ceh-30(n3714*gf*)*	0	0	100	61
*ceh-30(n4111 n3714*lf*)*	44	33	23	66
*ceh-30(n4289*Δ*)*	83	14	3	71
tra-1(e1099)	0	0	100	46
tra-1(e1099); *ceh-30(n4111 n3714*lf*)*	51	47	2	45
egl-1(n1084 n3082)	0	0	100	62
egl-1(n1084 n3082); *ceh-30(n4111 n3714*lf*)*	0	0	100	65
ced-3(n717)	0	0	100	60
ced-3(n717); *ceh-30(n4111 n3714*lf*)*	0	0	100	60

(C) The CEH-30 homologs mBarhl1 and mBarhl2 can rescue *ceh-30(n4289*Δ*)* for CEM survival

*ceh-30(n4289*Δ*)* ± transgene:	CEM survival in males (%) None	D or V	D and V	*n* =
No transgene	93	7	0	42
ceh-30 genomic locus	4	19	77	26
No cDNA insert	86	14	0	43
ceh-30 cDNA	0	11	89	36
mBarhl1 cDNA	13	51	36	53
mBarhl2 cDNA	31	40	29	48

(A) All animals were homozygous for *nIs133*. Otherwise, the genotypes of the animals analyzed were as listed, except for the heterozygotes, which were heterozygous for *unc-2(e55)* and *lon-2(e678)*.
(B) All animals were homozygous for *nIs133*, and all animals not homozygous for *tra-1(e1099)* were homozygous for *him-5(e1467)* or, in the case of strains containing *egl-1(n1084 n3082)*, for *him-8(e1489)*. The genotypes of the animals analyzed were otherwise as listed. *tra-1(e1099)* homozygous animals were the self-progeny of *tra-1(e1099)/qC1* heterozygotes. An expanded version of this table, including *ced-4(n1162)* and *ceh-30(n4289*Δ*)*, is shown in Supplementary Table S4.
(C) All animals were homozygous for *nIs133*, *him-5(e1467)*, and *unc-76(e911)*. Transgenic animals rescued for *unc-76* were scored for CEM survival. Constructs are described in the Supplemental Material. "*ceh-30* genomic locus" is pBSK-*ceh-30*.

weak defect in cell death and provides a sensitized genetic background in which weak effects on apoptosis can readily be detected (Reddien et al. 2001). In these sensitized backgrounds, we found no effect of *ceh-30* mutations on programmed cell deaths in the anterior pharynx or of the Pn.aap cells (Table 3A,B). To assess more broadly the extent of cell death in *ceh-30* mutants, we used a *ced-1(*lf*)* mutation to cause persistence of cell corpses (Hedgecock et al. 1983). *ceh-30(*lf*)* did not change the number of persistent cell corpses in the head, while *ceh-30(*gf*)* might have caused a slight reduction in corpse number (Table 3C); a reduction of about one corpse would be consistent with the only effect of *ceh-30(*gf*)* on cell death being that of CEM survival, given that there are four CEM neurons and our observation that *ced-1(*lf*)* causes 24% (n = 10) of embryonic cell deaths to persist into larval development as corpses. The numbers of neurons in the male and hermaphrodite ventral nerve cords were also unaffected by mutations in *ceh-30* (Table 3C). In short, these assays did not demonstrate any function outside the CEM neurons for *ceh-30* in the regulation of either cell death or cell number.

The sex determination pathway and ceh-30 *act independently of* ced-9 *Bcl-2 to control CEM neuron survival*

To examine how *ceh-30* protects the CEM neurons and, in particular, where *ceh-30* interfaces with the evolutionarily conserved core cell-killing pathway, we tested whether the anti-apoptotic gene *ced-9* is required for CEM survival in *ceh-30(n3714*gf*)* hermaphrodites. Specifically, we asked if the putative null allele *ced-9(n2812)*, a Q46amber mutation (Hengartner and Horvitz 1994b), would suppress the CEM survival caused by *ceh-30(n3714*gf*)*. Loss of *ced-9* Bcl-2 function causes ectopic activation of programmed cell death, resulting in lethality. Mutations that block the cell death pathway downstream from *ced-9*, such as in the *ced-3* caspase, can suppress this lethality. The weak mutation *ced-3(n2427)*, which suppresses *ced-9(n2812)* lethality and slightly reduces the amount of programmed cell death but allows many programmed cell deaths to occur normally (Hengartner and Horvitz 1994a), can be used to examine the regulation of cell death in strains lacking all *ced-9* Bcl-2 function. We found that in a *ced-9(n2812)*; *ced-3(n2427)* background, the CEMs showed nearly normal sexually dimorphic regulation of their decision to undergo programmed cell death decision: Most CEMs survived in males (no males lacked CEMs, and only 23% showed partial CEM survival; n = 71), and most CEMs died in hermaphrodites [none showed strong CEM survival, and only 20% showed partial CEM survival; this survival can be attributed to the weak protective effect of *ced-3(n2427)*; n = 89] (Table 4; data not shown). In the absence of *ced-9* function, as in a wild-type background, *ceh-30(n3714*gf*)* caused CEM survival in hermaphrodites (40% showed strong CEM survival, and 52% showed partial CEM survival; n = 60), and *ceh-30(*lf*)* mutations caused the CEM neurons of males to die (22% lacked

Table 3. *The cell death-protective function of* ceh-30 *is specific to the CEM neurons*

(A) *ceh-30* mutations do not modify programmed cell death in the anterior pharynx

	Extra cells in the anterior pharynx		
Genotype	Wild type	*ced-3(n2427)*	*ced-4(n3158)*
Wild type	0 ± 0	1.2 ± 0.2	4.2 ± 0.2
*ceh-30(n3714*gf*)*	0 ± 0	1.3 ± 0.2	4.3 ± 0.3
*ceh-30(n4111 n3714*lf*)*	0 ± 0	1.1 ± 0.2	4.1 ± 0.2
*ceh-30(n4289*Δ*)*	0 ± 0	1.2 ± 0.2	4.0 ± 0.4

(B) *ceh-30* mutations do not modify programmed cell death in the Pn.aap lineage

	Extra *lin-11*::*gfp*-expressing cells in the ventral nerve cord		
Genotype	Wild type	*ced-3(n2427)*	*ced-4(n3158)*
Wild type	0 ± 0	2.4 ± 1.1	4.5 ± 0.6
*ceh-30(n3714*gf*)*	0 ± 0	2.5 ± 1.1	4.7 ± 0.6
*ceh-30(n4289*Δ*)*	0 ± 0	2.7 ± 0.9	4.6 ± 0.6

(C) Persistent cell corpses in a *ced-1* background and cell number in the ventral nerve cord are inconsistent with nonspecific effects of *ceh-30* on cell death

		Neurons in the ventral nerve cord	
Genotype	Cell corpses in L1 heads in a *ced-1(e1735)* background	Hermaphrodite	Male
Wild type	26.1 ± 1.7	56.2 ± 1.1	56.4 ± 1.0
*ceh-30(n3714*gf*)*	24.7 ± 2.7	55.7 ± 0.8	56.4 ± 1.3
*ceh-30(n4289*Δ*)*	26.0 ± 2.0	55.9 ± 0.9	57.4 ± 1.7

(A) The number of cells in the anterior pharynx was determined as described in Materials and Methods. Twenty animals of each genotype were counted. Error is standard deviation. Genotypes were as listed.
(B) The number of Pn.aap cells was determined using a modified *nIs106 lin-11*::*gfp* reporter for the VC neuron cell fate as described in Materials and Methods. Fifty animals of each genotype were counted. Error is standard deviation. All animals were homozygous for *nIs106*. Genotypes were otherwise as listed.
(C) The numbers of persistent cell corpses in the heads of L1 larvae and of neuronal nuclei in the ventral nerve cords of males and hermaphrodites were determined as described in Materials and Methods. Ten animals of each genotype were counted. Error is standard deviation. Genotypes are as listed, except that ventral nerve cord neuronal nuclei were counted in animals homozygous for *him-5(e1467)*.

CEMs, and 75% showed only partial CEM survival; $n = 63$). Similar results were observed using a second putative null allele of *ced-9*, *n3400*, and using other weak alleles of *ced-3* (*n2446*, *n2447*, and *n2923*) (data not shown). Loss of *egl-1* BH3-only function, which prevents CEM death in wild-type animals, had no effect on CEM death in the absence of *ced-9* function (Table 4). This result is consistent with previous findings indicating that *egl-1* acts through *ced-9* Bcl-2 to perform its cell-killing function (Conradt and Horvitz 1998). The sexually dimorphic control of CEM survival therefore does not require regulation of *ced-9* or of *egl-1*.

Discussion

The Bar-class homeodomain gene ceh-30 *acts as a switch for the sexually dimorphic survival of the CEM neurons*

Our studies of the genetic control of the death of the sexually dimorphic CEM sensory neurons of *C. elegans* identified a novel mechanism by which sex determination regulates specific programmed cell deaths during nervous system development. This regulation depends on the control by sex determination of a previously uncharacterized gene, the evolutionarily conserved Bar homeodomain gene *ceh-30*, that acts as a genetic switch in determining the survival decision of the CEM neurons (shown diagrammatically in Fig. 3). *ceh-30* gain-of-function mutations cause the CEMs of hermaphrodites to survive as they do in males. These *ceh-30* gain-of-function mutations disrupt a binding site for TRA-1, the terminal regulator of sexual identity in *C. elegans*, and likely prevent the TRA-1-mediated transcriptional repression of *ceh-30* in hermaphrodites. A model for how TRA-1 regulates *ceh-30* expression and CEM neuron survival is shown in Figure 3A.

ceh-30 *functions in the CEM neurons to promote their survival*

We observed expression of a rescuing *ceh-30*::*gfp* transgene only in neurons. Bar homeodomain proteins in

other organisms are similarly expressed primarily in the nervous system (Higashijima et al. 1992b; Saito et al. 1998). Most cells that express *ceh-30* do so only transiently: Many more neurons show detectable *ceh-30*::*gfp* expression in embryos than in larvae or adults. We observed *ceh-30*::*gfp* expression in the CEMs of embryos, but not in those of larvae, indicating that *ceh-30* expression in the CEMs is transient and occurs during embryonic development, the stage at which CEM neurons of hermaphrodites die.

Consistent with our observation that *ceh-30* is expressed in the CEM neurons, we found strong indications that *ceh-30* functions cell-autonomously in the CEMs: CEM survival in hermaphrodites caused by a *ceh-30* gain-of-function transgene was dependent on the presence of the transgene in the surviving CEMs. The mechanism by which *ceh-30* gain of function protects hermaphrodite CEMs conceivably could differ from that by which *ceh-30* normally protects male CEM neurons; experiments to test whether *ceh-30* acts in the CEMs of males were not feasible because of background survival of CEM neurons in *ceh-30(n4289Δ)* males. It seems likely that the requirements for *ceh-30(gf)* function in hermaphrodite CEMs are similar to those for normal *ceh-30* function in male CEMs. From these considerations and from our observation of the expression of *ceh-30* in the CEMs of embryos, we conclude that *ceh-30* likely acts cell-autonomously to cause CEM survival.

ceh-30 *acts specifically to control the survival of the CEM neurons*

The effects of *ceh-30* on CEM survival could reflect a specific function for *ceh-30* in the CEM neurons or a particular sensitivity of the CEMs to a general defect in the regulation of programmed cell death. We therefore tested for effects of *ceh-30* on other programmed cell deaths, using assays known to be sensitive to subtle defects in programmed cell death and assays that examined the consequences of the generation and survival decisions of a large number of cells (see Table 3). These experiments did not reveal any function for *ceh-30* in the regulation of cell death or cell number other than for the CEM neurons. Nonetheless, *ceh-30*::*gfp* is expressed in and *ceh-30* might regulate the fates of neurons other than the CEMs, especially if such a role were to be concealed by redundancy. We found no additional defects in *ceh-30* mutants or in animals doubly mutant for *ceh-30* and for *ces-1*, *tra-1*, *eor-1*, or *eor-2*, genes known to regulate other specific programmed cell deaths (Ellis and Horvitz 1991; Conradt and Horvitz 1999; Hoeppner et al. 2004).

The sex determination pathway regulates ceh-30 *to control sensitivity to programmed cell death independently of the Bcl-2 homolog CED-9*

Animals lacking the Bcl-2 homolog *ced-9* showed essentially wild-type regulation of the CEM survival decision, specified by sexual identity and mediated by the control of *ceh-30*. This *ced-9*-independent regulation of CEM survival differs from the regulation of other specific cell deaths in *C. elegans*, which are controlled by the transcriptional regulation of the BH3-only killer gene *egl-1*, which in turn acts through *ced-9* (Conradt and Horvitz 1998; Metzstein and Horvitz 1999; Thellmann et al. 2003; Hoeppner et al. 2004; Liu et al. 2006). Previous results indicated that regulation of programmed cell death can occur independently of *ced-9*: Animals lacking *ced-9* function and weakly defective in the downstream killer gene *ced-3* show significant cell death, restricted almost completely to cells that normally die in the wild type (Hengartner and Horvitz 1994a). How cell-specific regulation of programmed cell death can occur independently of *ced-9* has been completely unknown until very recently. Besides *ceh-30*, the only gene demonstrated to

Table 4. ceh-30 *and sexual identity regulate CEM survival downstream from or in parallel to* egl-1 *and* ced-9

Genotype in the presence of *nIs133*; *ced-9(n2812)*; *ced-3(n2427)*	Sex	CEM survival (%)			
		None	D or V	D and V	*n* =
Wild type	Herm.	80	20	0	89
ceh-30(n3714gf)	Herm.	0	63	37	63
ceh-30(n4289Δ)	Herm.	84	16	0	74
egl-1(n1084 n3082)	Herm.	83	17	0	103
egl-1(n1084 n3082); ceh-30(n3714gf)	Herm.	0	73	27	77
egl-1(n1084 n3082); ceh-30(n4289Δ)	Herm.	85	15	0	100
Wild type	Male	0	23	77	71
ceh-30(n3714gf)	Male	0	10	90	70
ceh-30(n4289Δ)	Male	22	75	3	63
egl-1(n1084 n3082)	Male	1	14	85	101
egl-1(n1084 n3082); ceh-30(n3714gf)	Male	0	7	93	71
egl-1(n1084 n3082); ceh-30(n4289Δ)	Male	28	71	1	92

All animals except those containing *egl-1(n1084 n3082)* were homozygous for *him-5(e1467)*. Animals containing *egl-1(n1084 n3082)* were homozygous for *him-8(e1489)*. Genotypes were otherwise as listed. Note that the apparent lessening of the *ceh-30(n4289Δ)* phenotype in these animals is a result of the presence of the defect in cell killing caused by the weak mutation *ced-3(n2427)*. (Herm.) Hermaphrodite.

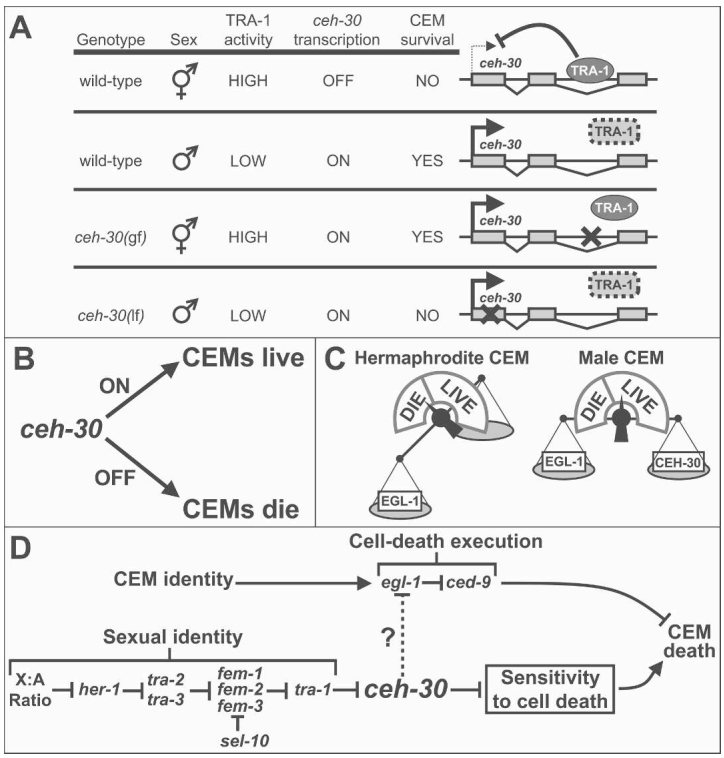

Figure 3. (*A*) A model for the regulation of CEM neuron survival by the sex determination pathway and by *ceh-30*. In the wild-type hermaphrodite, TRA-1 is active and binds an intronic regulatory site of *ceh-30*, preventing *ceh-30* transcription and thereby preventing CEM survival. In the wild-type male, TRA-1 is inactive; *ceh-30* is therefore expressed and protects the CEM neurons from programmed cell death. In a *ceh-30* gain-of-function hermaphrodite, TRA-1 is active but is unable to bind the mutated regulatory site, so *ceh-30* is expressed and protects the CEMs. In a *ceh-30* loss-of-function male, *ceh-30* is expressed but is nonfunctional and unable to protect the CEM neurons from programmed cell death. (*B*) *ceh-30* acts as a genetic switch for CEM neuron survival: If *ceh-30* is active, CEMs live; if *ceh-30* is inactive, CEMs die. Normally, *ceh-30* is active in males and inactive in hermaphrodites. (*C*) A representation of the sensitivity to programmed cell death controlled by *ceh-30*. In hermaphrodites, the CEM neurons express the killer gene *egl-1*, which promotes their deaths. In males, the CEM neurons have *ceh-30* activity, which desensitizes these cells to programmed cell death by acting downstream from or in parallel to *egl-1* and *ced-9*. Such a CEH-30-protected CEM neuron will live even when EGL-1 is expressed. (*D*) A model for the genetic pathway for the regulation of CEM neuron death by the determination of sexual identity. See text for details.

act in this process is the transcriptional regulator *pal-1*, recently shown to control the programmed cell death of the tail spike cell (Maurer et al. 2007).

Other evidence indicates that the regulation of CEM neuron survival is atypically independent of *ced-9*. The *ced-9* gain-of-function mutant *n1950*, which in other assays is nearly completely defective in programmed cell death in the soma (Hengartner and Horvitz 1994a), caused only moderate CEM survival (Supplementary Table S8). Also, the cell-killing function of *ced-9* (Hengartner and Horvitz 1994a; Reddien et al. 2001) appears not to significantly affect CEM survival, as *ced-9(lf)* did not enhance the CEM survival caused by any of several weak *ced-3* loss-of-function mutants (Tables 1A, 4; data not shown).

The *ced-9* Bcl-2-independent inhibition of CEM neuron death by *ceh-30* is unlikely to be mediated by any well-established death regulatory mechanism. In general, the regulation of caspase-mediated cell death involves members of the Bcl-2 superfamily (for review, see Kuwana and Newmeyer 2003). The principal exception is the regulation of caspases by the IAP (inhibitor of apoptosis) proteins, which directly inhibit caspase function and are themselves regulated by IAP inhibitors such as the *Drosophila* proteins Hid, Grim, and Reaper and the mammalian proteins Smac/DIABLO and ARTS (Bergmann et al. 1998; Wang et al. 1999; Du et al. 2000; Goyal et al. 2000; Verhagen and Vaux 2002; Gottfried et al. 2004). Loss of function and overexpression of the two *C. elegans* IAP genes have no apparent effect on cell death (Speliotes 2000), and we found that animals lacking both *C. elegans* IAP genes showed no reduction in the ability of increased *ceh-30* function to prevent the apoptotic deaths of CEM neurons of hermaphrodites (Supplementary Table S9). Other activities shown to regulate apoptotic cell death independently of the Bcl-2 superfamily, including ligand-mediated activation of caspase 8 and calpain-mediated regulation of caspase 12 (for review, see Benn and Woolf 2004), have no obvious parallels in *C. elegans*. Another conceivable mechanism to account for *ced-9* Bcl-2-independent regulation of cell death is the cell cycle regulation of Apaf-1 transcription by the DP-E2F heterodimer (Moroni et al. 2001); however, we found that a loss-of-function mutation in the only DP homolog, *dpl-1*, did not affect CEM survival (data not shown), suggesting that this mechanism is not important for CEM survival. Maurer et al. (2007) recently reported that the programmed death of the tail spike cell in *C. elegans* is effected by transcriptional regulation of the caspase gene *ced-3*. This report, which includes effects of *ced-9* Bcl-2 mutations similar to those we found in the CEM neurons, suggests a mechanism by which *ceh-30* might control CEM survival. We performed experiments using a *ced-3::gfp* reporter similar to those of Maurer et al. (2007) and failed to observe up-regulation of *ced-3* in the surviving CEMs of *ced-3(n717)* hermaphrodites (data not shown). We could have missed sexually dimorphic *ced-3* expression in the CEMs in these experiments because of the developmental stages we examined or because elements necessary for the appropriate transcriptional control of *ced-3* were not included in the reporter construct.

The loss of *ced-9* causes an activation of programmed cell death similar to that caused by expression of the

BH3-only proapoptotic protein EGL-1. For example, the HSNs of *ced-9(n1653*lf*)* hermaphrodites and the *egl-1*-expressing HSNs of *egl-1(n1084*gf*)* hermaphrodites undergo programmed cell death (Desai et al. 1988; Conradt and Horvitz 1999). Similarly, *ced-9* loss and *egl-1* overexpression each causes relocalization of CED-4 from the mitochondria to the perinucleus, a proposed early step in programmed cell death (Chen et al. 2000). *ceh-30* therefore protects by rendering cells that have activated programmed cell death less sensitive to this activation. A schematic representation of such a desensitizing effect of CEH-30 is shown in Figure 3C.

A genetic pathway for the regulation of CEM neuron survival

A proposed genetic pathway for the control of CEM neuron death is shown in Figure 3D. First, CEM neuron identity is established; one aspect of this identity is the activation of programmed cell death. The activation of CEM programmed cell death includes expression of the proapoptotic BH3-only protein EGL-1, which is required for somatic programmed cell deaths in *C. elegans* (Conradt and Horvitz 1998), including the deaths of the CEMs of hermaphrodites (Table 1A). Sexual identity, established by *tra-1* activity, controls *ceh-30* activity. *ceh-30* then acts downstream from or in parallel to *ced-9 Bcl-2* to establish whether the CEM neurons are sensitive to the activation of programmed cell death. In other words, *ceh-30* activity defines the sensitivity of the CEM neurons to the initiation of programmed cell death. How *ceh-30* exerts a cell-specific anti-apoptotic function independently of *ced-9 Bcl-2* remains to be determined.

The deaths of the CEM neurons in hermaphrodites normally require *egl-1* function. As described above, we have shown that *ceh-30* protects the CEMs from programmed cell death by acting downstream from or in parallel to *egl-1* and *ced-9*. In addition to the regulation of CEM survival by *ceh-30* downstream from or parallel to *egl-1* and *ced-9*, it is possible that the sexually dimorphic deaths of the CEM neurons are also regulated at the level of *egl-1* transcription, as are other cell-type-specific cell deaths. We could not directly assess *egl-1* expression in the CEM neurons, as our *egl-1*::*gfp* reporters were not expressed in the CEM neurons of cell death-defective larvae or in early embryonic CEM neurons (see Supplemental Material). *egl-1* might be expressed equivalently in the CEM neurons of both sexes, in which case the sexual dimorphism of CEM survival is established entirely by the function of *ceh-30* to alter the sensitivity of the CEMs to the death-inducing effects of *egl-1*, as described above. Alternatively, the sex determination pathway might also regulate *egl-1* expression in the CEMs, so that in hermaphrodites CEMs express *egl-1*, but in males CEMs do not. Importantly, the CEM neurons of *ceh-30(*lf*)* males died in an *egl-1*-dependent fashion. Thus, *egl-1* is active in the CEMs of *ceh-30(*lf*)* males. If *egl-1* is normally off in male CEMs, repression of *egl-1* expression in male CEMs must be dependent on *ceh-30*. We conclude that the regulation of *egl-1* expression in the CEMs by sexual identity, if it occurs, is performed by *ceh-30*. We depict this possible regulation of *egl-1* in the CEMs by sexual identity by a dashed line from *ceh-30* to *egl-1* in Figure 3D.

The novel anti-apoptotic function of ceh-30 *is evolutionarily conserved*

Parallels exist between the specific apoptotic loss of the *C. elegans* CEM sensory neurons caused by a loss of *ceh-30* function and the reported consequence of deleting the homologous murine gene *Barhl1*: *Barhl1* deletion mice are born healthy and able to hear, but progressively lose both their hearing and their sensory cochlear inner ear hair cells (Li et al. 2002). Similar to the sensory hair cells of *Barhl1* mice, but atypically for *C. elegans* cells that undergo programmed cell death, hermaphrodite CEMs persist for hours and show signs of differentiation (the extension of ciliated processes) prior to their apoptotic deaths (Horvitz et al. 1982 and references therein). Mice deleted for the *Barhl1* gene also display defects similar to those seen in mice lacking the neurotrophin survival factor NT-3 (Li et al. 2004), as well as increased apoptosis of neurons of the superior colliculus (Li and Xiang 2006). It seems likely that the sensory hair cell neurons of *Barhl1* mutant mice, like the CEM neurons of *ceh-30* mutants, lack protection from apoptotic cell death. *Barhl2* has been reported to be a possible positive regulator of cell death in *Xenopus* (Offner et al. 2005), raising the possibility that in different contexts Bar homeodomain proteins might be able to regulate targets involved in apoptosis either to promote or to prevent cell death.

Barhl1 and *Barhl2* transgenes rescued the CEM survival defect of *ceh-30* mutant males. These genes therefore encode proteins that retain the functions and target specificity of CEH-30. We conclude that *C. elegans* and vertebrate Bar homeodomain proteins likely share a conserved biological role as cell-type-specific regulators of programmed cell death. We suggest that vertebrate Bar proteins act to prevent neurodegeneration of certain neuron types and that the ectopic expression or increased activity of vertebrate Bar proteins might inhibit apoptotic cell death and promote oncogenesis. The novel mechanism by which *ceh-30* acts to prevent CEM neuron death together with the apparent conservation of the role of *ceh-30* in regulating cell survival suggests that further investigation of *ceh-30* and its vertebrate homologs might reveal a new evolutionarily conserved mechanism for the regulation of apoptotic cell death.

Materials and methods

C. elegans *genetics*

C. elegans strains were derived from the wild-type strain N2 and cultured using standard conditions (Brenner 1974). The mutations used are listed in the Supplemental Material.

We performed two screens using EMS mutagenesis (Brenner 1974) to identify revertants of *n3714*gf. In the first, F_1 progeny of

mutagenized *nIs133*; *unc-2 ceh-30(n3714) lon-2* hermaphrodites were placed singly on Petri plates, and the progeny of 523 F_1s were examined for decreased penetrance of the *n3714* phenotype of CEM survival in hermaphrodites. Suppressors were tested for linkage to *n3714* by outcrossing. In the second screen, mutagenized *nIs133*; *unc-2 ceh-30(n3714) lon-2* hermaphrodites were mated with *nIs133*; *him-5* males, 1960 larval hermaphrodite F_1 cross-progeny were placed singly on Petri plates, and their progeny were assessed for CEM survival. The only suppressor closely linked to *n3714*, *n4111*, came from the second screen. Adding a wild-type copy of the locus with the chromosomal duplications *mnDp57* or *yDp14* did not complement *n4111* for suppression of *ceh-30(n3714*gf*)* (data not shown), indicating that *n4111* does not cause loss of function in a second locus required to support the *n3714*gf phenotype.

ceh-30(n3713, *n3714*, and *n3720)* were mapped to the left end of the *unc-2 lon-2* interval on LGX using standard methods. *ceh-30(n3714)* was mapped between nucleotide 3383 on C33D12 (all references to cosmid C33D12 sequence refer to nucleotides of accession no. U64600) and 801 on F52E4 (accession no. U56964) using 124 Lon recombinants recovered after crossing *nIs133*; *unc-2 ceh-30(n3714) lon-2* hermaphrodites with males containing LGX from the Hawaiian strain CB4856 (Wicks et al. 2001). *ceh-30(n4111 n3714)* was mapped to the right of 14788 on M02F4 (accession no. U41548) using 127 Lon and 86 Unc recombinants recovered after crossing *nIs133*; *unc-2 ceh-30(n4111 n3714) lon-2* hermaphrodites with males containing LGX from the Hawaiian strain and further genotyping those recombinants that had broken left of 22142 on F52E4. None of these recombinant chromosomes contained *n3714*gf in the absence of *n4111*.

Isolation of ceh-30(n4289)

A library of mutagenized *C. elegans* was screened for deletions in *ceh-30* as described previously (Jansen et al. 1997). The deletion *n4289*, which removes from 22227 to 22893 of cosmid C33D12, was recovered. We used PCR to determine that sequences present in the wild type are missing in *ceh-30(n4289*Δ*)* homozygotes. *ceh-30(n4289*Δ*)* was outcrossed three times for the X chromosome and five times for the autosomal genome prior to strain construction and analysis.

DNA and RNA manipulations and generation of transgenic animals

DNA sequence determination was performed using an ABI DNA Sequencer model 373 and an ABI Genetic Analyzer 3100, and by Gene Gateway. DNA constructs used are described in the Supplemental Material. All germline transformation experiments were performed using the coinjection marker P76-16B (Bloom and Horvitz 1997) at 50 ng/µL as described (Mello et al. 1991). Cosmids and genomic and cDNA constructs were injected at 20 ng/µL, and reporter constructs were injected at concentrations from 2.5 to 50 ng/µL. Embryonic *ceh-30*::*gfp* expression was examined using embryos masculinized by *tra-2(n1106)*.

Total RNA from N2 and *him-5* was isolated using Trizol (Invitrogen). 5′ RACE and 3′ RACE were performed using appropriate reagents (Invitrogen). RNA ligase-mediated 5′ RACE was performed essentially as described (Maruyama and Sugano 1994) using appropriate reagents (Invitrogen; Epicentre; New England Biolabs). The 5′ end of the *ceh-30* transcript was also isolated by PCR with *ceh-30*-specific and vector-specific primers from cDNA libraries provided by Shai Shaham and by Zheng Zhou (pers. comm.). The 5′ ends determined using each of these methods were essentially identical and did not contain a splice leader sequence. The vector BSK-*ceh-30*-Sn (see Supplemental Material) was used to express the *ceh-30* cDNA and to express mouse *Barhl1* and *Barhl2* cDNAs (gifts of Shengguo Li and Mengqing Xiang, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, NJ), and these constructs were used to obtain the data shown in Table 2C.

Gel mobility shifts and competition experiments

Gel mobility shift experiments were performed essentially as described (Zarkower and Hodgkin 1993; Conradt and Horvitz 1999). Probes were generated by PCR using the primers CGT CATCATCAAATTTTCACC and AATGATGTTTTTATGTC GCAACT for *ceh-30* and the primers CTGTTCCAGCTCAAA TTTCCA and AACAAGTATCAGGCGGCATC for *egl-1* controls (data not shown). TRA-1A protein was generated by in vitro transcription and translation of a full-length *tra-1A* cDNA (plasmid pDZ118, a gift from David Zarkower, University of Minnesota, Minneapolis, MN). Reticulocyte lysate (1.5 µL), 0.5–1 ng of ^{32}P-labeled probe, and 0, 10×, 100×, or 1000× unlabeled competitor were incubated for 1 h at room temperature before electrophoresis through 4% acrylamide gels in 0.5× TBE.

Analysis of C. elegans *phenotypes*

Animals were examined for gross developmental defects using dissecting and Nomarski microscopy. In determining cell autonomy of the CEM survival caused by the pBSK-*ceh-30(n3714*gf*)* transgene, we used animals rescued for the Unc-76 phenotype. Programmed cell death in the anterior pharynx was quantified using Nomarski microscopy as described (Hengartner et al. 1992). Survival of Pn.aap cells was quantified using *lin-11*::*gfp* as described (Reddien et al. 2001). Corpse number in the heads of L1 hermaphrodites was scored as described (Yuan and Horvitz 1992). The number of neuronal nuclei in a region of the ventral nerve cords of late L4 larvae and young adults was determined following staining with DAPI as described (Sulston and Horvitz 1981; Fixsen 1985). Programmed cell death in the CEM lineage was assessed using a fluorescence-equipped dissecting microscope (M^2BIO; Kramer Scientific) to detect *pkd-2*::*gfp* expression in the cell bodies of CEM neurons or by using Nomarski microscopy as described (Schwartz 2007). The ventral CEMs of 1.5-fold stage embryos were identified by reference to Figure 8C of Sulston et al. (1983); the positions of the dorsal CEMs are less distinctive, and these cells were not examined.

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