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eor-1 and *eor-2* are required for cell-specific apoptotic death in *C. elegans*

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Abstract

Programmed cell death occurs in every multicellular organism and in diverse cell types yet the genetic controls that define which cells will live and which will die remain poorly understood. During development of the nematode *Caenorhabditis elegans*, the coordinated activity of four gene products, EGL-1, CED-9, CED-4 and CED-3, results in the death of essentially all cells fated to die. To identify novel upstream components of the cell death pathway, we performed a genetic screen for mutations that abolish the death of the hermaphrodite-specific neurons (HSNs), a homologous pair of cells required for egg-laying in the hermaphrodite. We identified and cloned the genes, *eor-1* and *eor-2*, which are required to specify the fate of cell death in male HSNs. In addition to defects in HSN death, mutation of either gene leads to defects in coordinated movement, neuronal migration, male tail development, and viability; all consistent with abnormal neuronal differentiation. *eor-1* encodes a putative transcription factor related to the human oncogene PLZF. *eor-2* encodes a novel but conserved protein. We propose that *eor-1* and *eor-2* function together throughout the nervous system to promote terminal differentiation of neurons and function specifically in male HSNs to promote apoptotic death of the HSNs.

Keywords: Apoptosis; Hermaphrodite-specific neuron (HSN); *egl-1* suppressor; *eor*; Differentiation; BTB/POZ; Krüppel

Introduction

During development of the nematode *Caenorhabditis elegans*, an invariant program of cell divisions generates 1031 somatic cells of which 131 are eliminated by a highly regulated process known as programmed cell death or apoptosis (Sulston, 1983; Sulston and Horvitz, 1977; Sulston et al., 1983). The invariant nature of these cell fate decisions facilitated the isolation of mutations that cause deviation from this highly stereotypical pattern of cell fate. Genetic screens for mutations that disrupt the normal pattern or kinetics of programmed cell death have identified more than 16 genes [reviewed in (Hengartner, 1997; Metzstein et al., 1998)]. Mutations in single genes can result in strong phenotypes that

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uncouple discrete phases of the programmed cell death pathway from one another: mutations in *ces-1* or *ces-2* alter the specification of a small number of cells that normally should die; mutations in *egl-1*, *ced-9*, *ced-4*, or *ced-3*, prevent the proper execution of cells fated to die; mutations in *ced-1*, *ced-2*, *ced-5, ced-6*, *ced-7*, *ced-10*, or *ced-12*, impair the engulfment of dying cells; and mutations in *nuc-1* affect the degradation of cell corpses.

Much has been learned regarding the biochemical nature of the orderly destruction of a cell. Once a cell has been specified to die by apoptosis, the killing machinery becomes engaged through a series of protein–protein interactions comprising at least four proteins: the BH3-domain protein EGL-1, the Bcl-2 homologue CED-9, the APAF-1-like adapter protein CED-4, and the caspase CED-3. These protein interactions ultimately lead to the proteolytic activation of pro-CED-3 into its catalytic form. Cleavage by CED-3 of its substrates finally brings on the cell's demise. Mutations that disrupt the function of *egl-1*, *ced-9*, *ced-4*, or *ced-3* result in cell death defects throughout the animal. Thus, these genes are required for most, if not all, developmental cell deaths in *C. elegans*.

Although there is a partial understanding of how the cell is dismantled, little is known regarding the signals that specify a particular cell to die, nor about whether a cell must attain a particular state of differentiation before it is competent to respond to a "death" signal to achieve its final fate. Mutations in genes that are required for cell fate determination have been shown to alter the pattern of cell death. In some cases, these genes are likely to not be directly involved in the life vs. death decision of a specific cell; rather they are involved in causing cell fate decisions that alter cell lineage. For example, in *lin-32* mutants, several neuronal lineages are missing and therefore the deaths that would have occurred in the missing lineage never had a chance to occur (Chalfie and Au, 1989; Zhao and Emmons, 1995). Conversely, mutations in the Zn-finger transcription factor, *pag-3*, cause specific death-containing neuroblast lineages to be reiterated, and thus result in the production of supernumerary cell deaths (Cameron et al., 2002).

Another example of cell-type-specific death occurs within the hermaphrodite-specific neurons (HSNs), two serotonergic neurons that innervate the vulval muscles and drive egg laying in hermaphrodites. These cells are not needed in males and die during development. Dominant gain-of-function *(gf)* mutations in the pro-apoptotic *egl-1* gene (Desai and Horvitz, 1989; Desai et al., 1988; Trent et al., 1983) selectively induce death of the HSNs in hermaphrodites (resulting in an egg-laying defect for which the gene was named), without affecting the death of any other cell. The ectopic HSN deaths in *egl-1(gf)* mutants is a result of de-repression of *egl-1* transcription specifically in the hermaphrodite HSNs (Conradt and Horvitz, 1999).

egl-1 transcription is in part regulated by *tra-1*; a switch gene that controls somatic sexual phenotype and is the terminal genetic element in the sex-determination pathway of *C. elegans* (Hodgkin, 1987). TRA-1A functions as a hermaphrodite-specific negative regulator of HSN death by binding to an *egl-1* regulatory element and thereby represses *egl-1* expression. The various *egl-1(gf)* mutations all localize to this regulatory element and prevent TRA-1A binding (Conradt and Horvitz, 1999). Thus, in *egl-1(gf)* mutants, where the TRA-1A binding site is altered, or in males, where TRA-1A activity is low, the *egl-1* gene is expressed, leading to HSN apoptosis. However, *egl-1* activity most likely is also controlled by multiple layers of regulation, including unidentified positive-acting factors that activate *egl-1* expression in response to the appropriate sexually dimorphic cues in males (Conradt and Horvitz, 1999).

To identify novel genes required to specify HSN death, we undertook a genetic screen for mutations that promote HSN neuron survival, by screening for suppressors of the egg-laying defect in *egl-1(gf)* mutants. Here, we present the isolation and characterization of two *egl-1(gf)* suppressors, *eor-1* and *eor-2*. Loss-of-function mutations in *eor-1* or *eor-2* prevent HSN death as induced by at least two distinct signals but do not result in the detectable inappropriate survival of any other cells. In addition, *eor-1* and *eor-2* mutants exhibit a common set of pleiotropic defects throughout the nervous system, suggesting that these genes function together to promote the terminal differentiation of multiple neuronal cell types. These genes might reveal a step in a genetic pathway to neuronal differentiation through which presumptive neurons must pass to appropriately respond to death signals and suggest that competence to die and competence to function are genetically separate aspects of HSN neuronal cell fate determination.

Materials and methods

Animals were cultured using standard conditions (Brenner, 1974) at 20°C, unless otherwise stated. Microscopic analysis of cells was performed using standard live animal mounting techniques and a Zeiss Axioplan fluorescence microscope fitted with Nomarski optics. The standard wild-type N2 strain was used unless otherwise stated.

Isolation of eor-1 and eor-2 mutations

Strain MT2251: *egl-1(n1084)* parental animals were mutagenized with 47 mM methanesulfonic acid ethyl ester (EMS) (Sigma M 0880) (Brenner, 1974) or 30 mM N-nitroso-N-ethylurea (ENU) (Sigma N 8509) (De Stasio et al., 1997) in M9 as L4 larvae. Pools of 10 parents were passaged onto fresh media every 6 h to generate synchronized cohorts of F1 progeny, which were allowed to develop. F1 cohorts were screened for non-Egl revertants when the majority of animals were gravid adults, and then removed. F2 embryos already laid by the gravid F1 mothers were allowed to develop to adulthood, and then screened again as above. Using EMS, 6 suppressors were isolated from a

screen of 54,000 haploid genomes in the F1 generation and 28 were isolated from a screen of 39,000 haploid genomes in the F2 generation. Using ENU, 2 suppressors were isolated from a F2 screen of 17,000 haploid genomes. *mh21* was isolated in a genetic screen for mutants with altered phasmid dye-filling (Herman et al., 1999; Zhao et al., 2002).

Measuring hermaphrodite-specific neuron (HSN) survival, CEM survival, and egl-1::gfp expression in dying HSNs

One side of animals in the first or second larval stage was scored for HSN survival using Nomarski optics. The CAN neuron, normally found in the same focal plane as the HSN, is not affected by any previously identified cell death mutations. CAN was used as a landmark to control for approximate position of the HSNs. Animals were only scored for HSN survival if CAN could first be detected. To determine sex in L1 animals, the large size of the B and C nuclei were used to indicate the male sex. To measure CEM survival, the integrated *pkd-2::gfp* transgene (Shai Shaham) was scored in *eor-1* and *eor-2* backgrounds. All integrated *pkd-2::gfp* and *ced-3* hermaphrodites express the transgene in the CEMs. *egl-1::gfp* reporter activity was measured in dying HSNs using *ced-3(n717)*; *him-5 (e1490)*; *bcIs1[egl-1::gfp]* animals. HSNs were identified as above then scored for gfp fluorescence. There is no ambiguity in *egl-1::gfp* expression in this strain background or derivatives reported here, all scores were clearly positive or negative.

Dye uptake assay

Animals were soaked in a 10 μg/ml solution of DiO (3,3′-dioctadecyloxacarbocyanine perchlorate; Molecular Probes) to asses amphid and phasmid dye-filling as previously described (Herman and Horvitz, 1994). Amphid neurons stain normally with DiO in *eor-1* and *eor-2* mutants. In some cases, DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine 4-chlorobenzenesulfonate, Molecular Probes) was used to double-label phasmids expressing the green fluorescent protein.

Analysis of eor-1 and eor-2 transcripts

The yk47h3 clone encodes the full-length *eor-1* cDNA including 119 bp upstream of the predicted ATG. This cDNA contains the exon/intron junctions as predicted by GeneFinder. The yk257c2 clone contains the full-length *eor-2* cDNA starting 16 nucleotides upstream of the predicted ATG. The 5′ ends of the *eor-1* and *eor-2* transcripts were determined by sequencing PCR products generated from N2 cDNA using internal gene-specific primers and a "universal" primer provided in the 5′ RACE kit (GIBCO). Reverse transcription was performed using 5′ gta gca gat ttc aca a 3′ for *eor-1* and 5′ gtt ggg tag ttt tta g 3′ for *eor-2*. Second strand synthesis was performed using 5′ gat aag caa ttg gcc gcg tc 3′ for *eor-1* and 5′ gtc cta gtt tgt ttt cga gc 3′ for *eor-2*. Amino acid sequence alignment was performed using the Vector NTI suite for EOR-1. EOR-2 amino acid alignment was performed as in Neuwald et al. (2003).

Construction of Peor-1::GFP and Peor-2::GFP expression constructs

A ~390 bp product from the intergenic region between R11E3.7 and R11E3.6 was generated by PCR using primers R11 int 5′ (5′ cgcggccgcgttggcgcgcctgatg 3′; including de novo *Not*I and *Asc*I restriction sites, underlined) and R11 int 3′ (5′ cgagcggccgcaagacgtc 3′; including a de novo *Not*I restriction site) and was cloned into pCR2.1-TOPO (Invitrogen) (=pR11int1-4/TOPO). The resulting plasmid was restricted with *Asc*I and *Not*I and the insert ligated to similarly digested pPD117.01 (Gift from Andy Fire) to create p117R11int1. This fragment is sufficient to drive GFP expression. All reporter constructs were co-injected with the dominant *rol-6(su1006)* marker plasmid pRF4 using standard methods (Mello et al., 1991).

To assay for promoter activity of *eor-2*, a 4-kb genomic region 5′ to C44H4.7 was amplified using primers C44 prom 5′ (5′ gcatcccggcgcgccattttgggatcctagcc 3′; including a de novo *Asc*I site) and C44 prom 3′ (5′ cattagtgcggccgcagttagataatctac 3′; including a de novo *Not*I site) and cloned into pCR2.1-TOPO (Invitrogen). The *Asc*I–*Not*I fragment was further subcloned into pPD117.01 to create pPD117C44pr1. This fragment is sufficient to drive expression of GFP.

Results

Isolation of genes that prevent egl-1(gf) induced HSN death

To identify factors required to specify or to mediate death of the HSNs, we performed a suppression screen for mutations that could revert the egg-laying defect in *egl-1(gf)* mutant animals. The dominant gain-of function (gf) *egl-1(n1084)* allele results in the inappropriate death of the hermaphrodite-specific neurons (HSNs) (Desai and Horvitz, 1989; Desai et al., 1988; Trent et al., 1983). This inappropriate HSN death is a result of de-repression of *egl-1* transcription in *egl-1(gf)* mutants (Conradt and Horvitz, 1999). Because the HSNs are required for egg-laying, the result of HSN death is readily detectable under a low-power microscope as an egg-filled bloated adult (the Egl phenotype). After EMS or ENU mutagenesis of *egl-1(n1084)* animals, first and second generation broods were scored for non-Egl revertants.

Homozygous mutant lines were generated from all non-Egl isolates. Stable non-Egl lines were examined both for the presence of HSNs and for any indication of general cell death: extra cells in the anterior pharynx (Ellis and Horvitz, 1986; Hengartner et al., 1992). Using these criteria, the suppressors were placed into three phenotypic groups

(Supplementary Table 1): "bypass suppressors" (one allele), which appeared to suppress the *egl-1*-induced egg-laying defect by a mechanism other than blocking HSN cell death, "general cell death suppressors" (all new alleles of *ced-3* or *ced-4*), which are required for all cell deaths in the animal, and the "HSN-specific suppressors", which are the focus of this study. HSN-specific suppressors allow survival of the HSNs but do not allow survival of other cells fated to die during normal development, such as cells of the anterior pharynx (Fig. 1, Supplementary Table 1).

The mutations that specifically affect HSN survival can be ordered by strength (Supplementary Table 2). Three of the strongest suppressors, *op181*, *op166*, and *op232*, completely suppress *egl-1*-induced HSN death and were characterized in further detail. Genetic analysis (see Supplementary Material) revealed that these mutations identified two new genes, which we named *eor-1* and *eor-2*, for *e*gl-1 suppressor, Di*O* uptake defective, *r*af enhancer—the nomenclature agreed upon to standardize the gene class that has been presented previously as *egs*-, *sue*- and *eor*- in unpublished formats. *eor-1* and *eor-2* mutations have been independently isolated and characterized by Howard and Sundaram (2002) and Rocheleau et al. (2002) based on their involvement in *ras* and *wnt* pathway signaling.

To determine whether *eor-1* and *eor-2* are general *egl-1* suppressors, we determined the extent of developmental cell death in the respective double mutants. Mutations in general cell death genes that act downstream of *egl-1*, such as *ced-3* and *ced-4*, prevent all *egl-1*-induced cell death, including those that occur in the anterior pharynx of the animal (Ellis and Horvitz, 1986; Hengartner et al., 1992), (Fig. 1; Supplementary Table 1). Similarly, the loss-of-function allele *egl-1(n1084 n3082)* results in the lack of cell death in both the anterior pharynx and in the HSNs (Conradt and Horvitz, 1998), (Fig. 1C). If *eor-1* and *eor-2* mutants produce their mutant phenotypes by inactivating *egl-1* in all cells, we would predict that these mutants would have extra cells in the anterior pharynx, as does the *egl-1(n1084 n3082)* mutant. However, we found that there are no extra pharyngeal cells in *eor-1* and *eor-2* mutants (Fig. 1C). Note that *ces-1* and *ces-2* mutants allow inappropriate survival of the sisters of the NSM neurons (Ellis and Horvitz, 1991). *ces-1* gain-of-function alleles allow survival of the I2 neuron sisters. Both of these neuron sisters are scored as part of the pharyngeal population and are not affected in *eor-1* or *eor-2* mutant backgrounds. Thus, loss of *eor-1* or *eor-2* function is not sufficient to generally prevent *egl-1*-induced apoptosis nor to allow NSM or I2 sister survival.

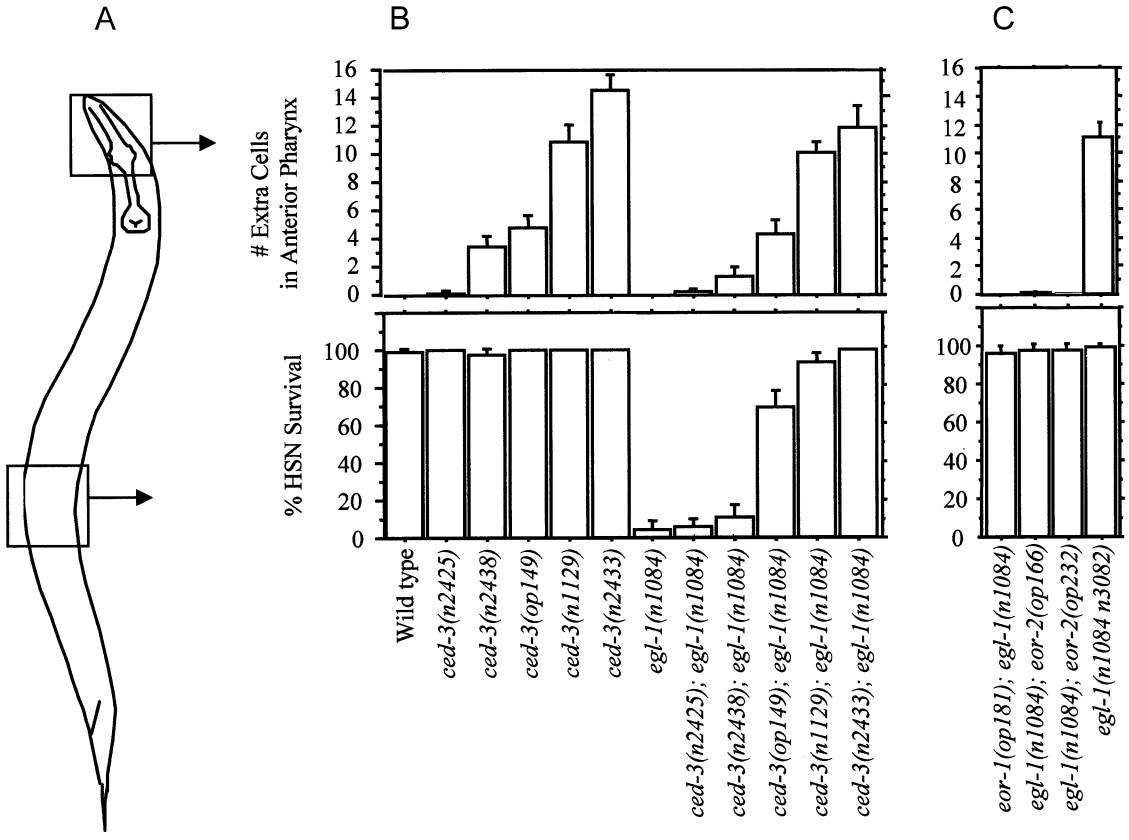

Fig. 1. General versus cell-type-specific cell death suppression. (A) Boxed regions indicate the positions of the two cell populations quantitated in B and C. (B) (Top) Mean number of extra surviving nuclei from the anterior pharynx. (Bottom) Mean percent survival of HSNs in animals of the indicated genotype, demonstrating general cell death blockage in both the anterior pharynx and HSNs. (C) *eor-1* and *eor-2* mutants are only defective for HSN death. Error bars represent 95% confidence limits. Top panels: $n = 20$ animals/genotype, bottom panels: $n = 90$ HSNs/genotype.

The results above do not exclude the possibility that *eor-1* and *eor-2* mutations are general, but rather poor *egl-1* suppressors, and that the inability to prevent pharyngeal cell death simply reflects a lower sensitivity of pharyngeal cells to weak pro-survival stimuli. To address this issue, we calibrated the penetrance of HSN survival in *eor-1* and *eor-2* mutants to known cell death mutants, by comparing the survival of HSNs and cells in the anterior pharynx in several *egl-1* and *ced-3* mutant backgrounds (Fig. 1B). The strongest *ced-3* alleles block nearly all HSN deaths while also preventing normal developmental death in the anterior pharynx; weaker alleles show progressively less activity in both assays. These data indicate that cells in the anterior pharynx respond to reductions in pro-death signals with the same dose-response as the HSNs. Together, our results suggest that mutations in *eor-1* and *eor-2* lead to HSN survival by antagonizing the killing activity of *egl-1* specifically in the HSNs.

eor-1 and eor-2 exhibit differentiation defects in several neurons

In addition to suppressing *egl-1*-induced HSN death, mutations in *eor-1* and *eor-2* cause a common set of pleiotropic defects. *eor-1* and *eor-2* mutants have an incompletely penetrant lethality defect that results in a terminal Rod-lethal phenotype (Fig. 2B, Table 1). Mutant animals arrest at all larval stages and as adults, but not as embryos. The growth arrest is accompanied by gradual paralysis and translucence, followed by a flaccid and shriveled body morphology.

In contrast to wild-type *C. elegans* that move through a lawn of *Escherichia coli* with a regular sine wave motion, *eor-1* and *eor-2* mutants produce tracks with an irregular pattern (Figs. 2C–E). The uncoordinated phenotype apparently involves a defect in relaxation in the posterior half of most animals after a complete turn, occasionally resulting in a weak Coiler phenotype. Although this movement defect appears similar to the movement defect in *unc-3(e151)*, which affects differentiation and organization of the ventral cord motor neurons, the ventral cord of *eor-1* and *eor-2* grossly appears normal (data not shown).

The *eor-1(mh21)* allele was isolated in an independent screen for phasmid-defective mutants. The phasmids are sensory structures in the tail composed of two socket cells, a sheath cell, and two phasmid neurons. The socket cells provide an opening to the environment for the phasmid neurons; this opening also facilitates the uptake by

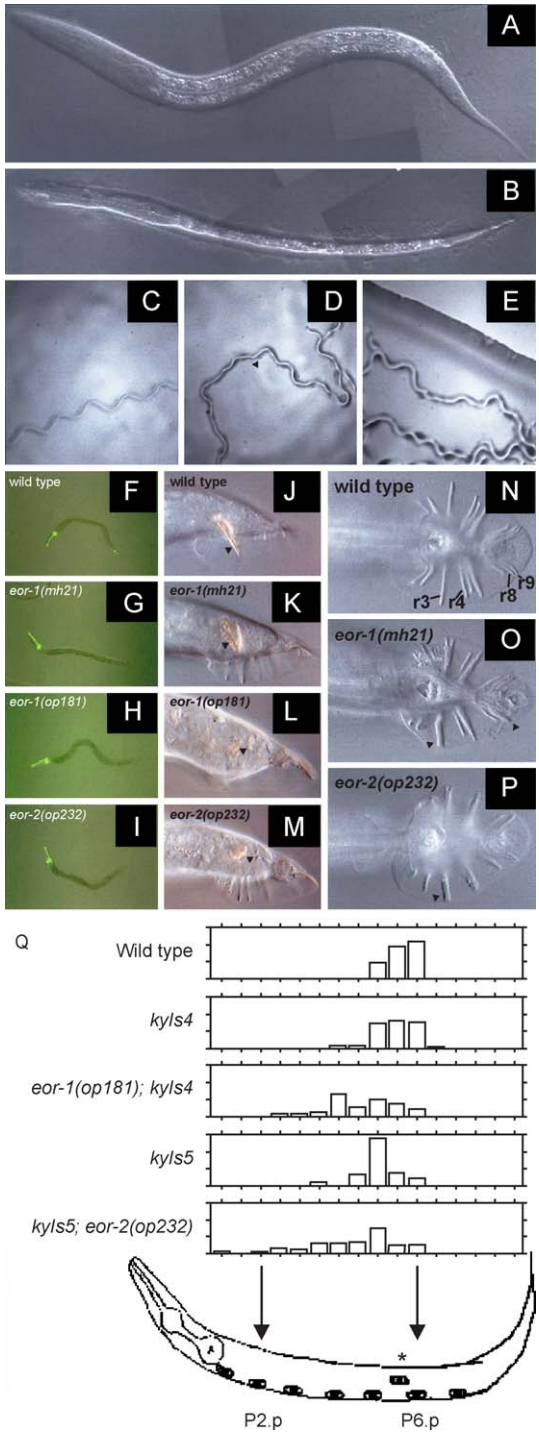

Fig. 2. *eor-1* and *eor-2* mutants have identical sets of pleiotropic defects throughout the nervous system. In all animal images, left is anterior. (A) Wild type and (B) *eor-1(op181)* L2 larvae. Note that animals can arrest at all stages from L1 to adult. The terminal phenotype in *eor-1* and *eor-2* mutants is indistinguishable. (C) Wild type, (D) *eor-1(op181)* and (E) *eor-2 (op166)* worm tracks demonstrating uncoordinated movement on a lawn of fresh *E. coli*. All animals are uncoordinated to varying degrees. (F–I) Dye uptake defects in *eor-1* and *eor-2* mutants. Note the absence of dye uptake in the posterior phasmids in G–I, whereas all worms show anterior amphid staining. (J–M) Spicule defects in *eor-1* and *eor-2* males. Arrowhead indicates the position of the left spicules. (N–P) Examples of male ray fusions in *eor-1* and *eor-2* mutants. Arrowheads indicate fused rays. (Q) CAN migration is defective in *eor-1* and *eor-2* mutant backgrounds. The frequency of finding CAN (*y*-axes, 0–60%) is plotted against the positions at which CAN is found (*x*-axes, P1.p–P8.p) in wild-type, transgenic, and mutant backgrounds. The positions of the Pn.p cells are indicated by the series of partially filled ovals at the bottom of the worm figure. The position of CAN in wild-type animals is indicated by an * on the worm figure.

Table 1
eor-1 and *eor-2* mutants exhibit incompletely penetrant lethality

Genotype	% Lethality		
	15°C	20°C	25°C
Wild type	<1	<1	<1
eor-1(op181)	6	12	10
eor-1(mh21)	25	36	45
eor-2(op166)	35	28	37
eor-2(op232)	31	28	38

Percent rod-larval lethality. $n > 250$ for each genotype at each temperature.

phasmid neurons of fluorescent dyes from the environment. *eor-1(op181), eor-2(op166)* and *eor-2(op232)* all also display phasmid dye-filling defects (Table 2 and Figs. 2F–I). Similar sensory structures in the head, the amphids, fill properly with dye in *eor-1* and *eor-2* mutant animals. To determine if phasmid neurons are present in *eor-1* mutants, we used the PHA/PHB phasmid neuron reporter, *srb-6*::*gfp* (Troemel et al., 1995). In *eor-1(mh21)* animals, the reporter was expressed normally in the neurons of all phasmids scored ($n = 50$), suggesting that the dye-filling defect of *eor-1* mutants is not caused by defects in phasmid neuron fate. However, there may be other aspects of phasmid neuron differentiation, such as the structure of the ciliated sensory endings that are defective in *eor-1* mutants.

We also observed several morphogenetic abnormalities in the male tail of both *eor-1* and *eor-2* mutants (Figs. 2J–P, Table 2). The male tail serves as a copulatory organ. Exterior male tail structures include nine bilaterally symmetric sensory rays embedded in a cuticular fan, two protracting spicules, and a sensory hook. The rays and hook are required for locating the hermaphrodite vulva and the spicules function to anchor the male to the vulva and thus facilitate sperm transfer (Sulston et al., 1980). We found that *eor-1* and *eor-2* mutants have deformed spicules that rarely protract and have morphologically abnormal or absent hooks. *eor-1* and *eor-2* male tails also have an incompletely penetrant, but fairly specific ray fusion phenotype, with rays eight and nine found to be fused most frequently. Occasionally, rays three and four fuse, giving rise to an abnormally thick ray that takes on the length property of that normally observed for ray three. We also found that the positions of rays five and six were sometimes transposed (data not shown).

In the process of scoring for the presence of HSNs, we noticed that the landmark CAN neurons had a variable position in *eor-1* and *eor-2* mutant animals. The bilaterally symmetric CANs are born in the head, migrate caudally to a defined position in the mid-body (Hedgecock et al., 1987; Sulston et al., 1983) and then extend one axon rostrally to the head and one caudally to the tail (Forrester and Garriga, 1997). At least two genes are expressed in and during differentiation of the CAN before its migration: *ceh-23* expression occurs in the CAN neurons after *ceh-10* has been expressed (Forrester et al., 1998). To investigate in more detail the CAN migration/terminal differentiation defect observed in *eor* mutants, we constructed strains that express the green fluorescent protein under the transcriptional control of the *ceh-23* promoter (*ceh-23*::*gfp*). Two integrated *ceh-23*::*gfp* transgenes, *kyIs4* and *kyIs5*, were used to build transgenic lines in *eor-1* and *eor-2* mutant backgrounds. On their own, *kyIs4* and *kyIs5* cause a slight rostral displacement of the CAN neurons. *eor-1* or *eor-2* mutations strongly enhance this CAN migration defect in isogenic backgrounds (Fig. 2Q). Thus, in *eor-1* and *eor-2* mutants, CAN differentiation proceeds at least through the *ceh-10*-dependent stage and through the stage that allows expression of *ceh-23*, but still results in incomplete CAN migration. These results suggest that a late aspect of CAN differentiation is defective in both *eor-1* and *eor-2* mutant backgrounds.

In summary, in addition to their role in specifying programmed cell death of the HSNs, *eor-1* and *eor-2* have diverse normal functions that are ostensibly unrelated to HSN death. The pleiotropic defects in *eor-1* and *eor-2* mutants include rod-larval lethality, neuronal migration defects, uncoordinated movement, male tail formation defects, and phasmid dye uptake defects. These pleiotropies are united by a common theme: incomplete neuronal differentiation.

Table 2
Phasmid and male tail defects of *eor-1* and *eor-2* mutants

Structure	% Abnormal (*n*)				
	Wild type	*eor-1(mh21)*	*eor-1(op181)*	*eor-2(op166)*	*eor-2(op232)*
Phasmid	0 (200)	96 (200)	92 (200)	92 (112)	90 (200)
Spicule	0 (86)	70 (134)	82 (76)	ND	81 (126)
Hook	0 (36)	29 (62)	33 (36)	ND	31 (61)
Rays					
3 fused to 4	0 (80)	11 (130)	12 (74)	ND	9 (124)
8 fused to 9	7.8 (77)	31 (126)	30 (71)	ND	27 (123)

Phasmids were assayed for defect by the inability of phasmid neurons to uptake DiO. There is one phasmid on each side of the animal. *n*, number of phasmids scored. Spicules, hooks and rays of *eor* mutants were scored in a *him-5(e1490)*, but otherwise wild type genetic background. *him-5* males are equivalent to wild-type males in this assay. Each male has two spicules. Spicule defects include morphological abnormalities, "crumpling," and shortened length; *n*, number of spicules scored. Hook defects include morphological abnormalities, mispositioning, and absence; *n*, number of hooks scored. Rays are bilaterally symmetric, nine on each side of the tail; *n*, number of sides scored. ND, not determined.

eor-1 and eor-2 are required for the death of HSNs in males but not for CEM death in hermaphrodites

One of the earliest sexually dimorphic events during development of *C. elegans* is cell death of the HSNs in males. This death requires a functional *egl-1* gene. To determine whether *eor-1* and *eor-2* mutations only suppress *egl-1* within a background where *egl-1* is overexpressed [*egl-1(n1084)*], or whether they can also suppress normal *egl-1* activity in HSN neurons, we asked if *eor-1* and *eor-2* are required for the death of HSNs in males. We found that *eor-1* and *eor-2* mutations allow survival of nearly half of the HSNs that normally die in males (Table 3A). This effect is not enhanced in an *eor* double mutant background (Table 3A). Thus, in addition to being required to kill hermaphrodite HSNs in response to ectopic expression of *egl-1*, *eor-1*, and *eor-2* are also required to transduce the normal physiological cell death signal from the sex-determination pathway in males. Indeed, *eor-1* and *eor-2* mutants also result in HSN survival in *egl-41(n1077)* hermaphrodites, which are partially transformed toward the male fate (Table 3B) (Desai and Horvitz, 1989; Desai et al., 1988), suggesting that the *eor-1* and *eor-2* genes function downstream of the sex determination pathway. We also tested whether *tra-1(lf)* could be suppressed by *eor-1* or *eor-2* mutations. HSN death in *tra-1(lf)* males is easily suppressed by *ced-3(n717)*. Consistent with *egl-41* suppression, *eor-2(op166)* suppresses HSN death in the strong *tra-1(e1099)* background, but not noticeably in the weaker *tra-1(e1076)* background (Table 3A). These data suggest that *eor-1* and *eor-2* act in an epistatic pathway downstream of sex determination.

Table 3
eor-1 and *eor-2* are required for HSN death downstream of sex determination, and upstream of or in parallel to *ced-9*

(A) Genotype	% HSN survival in males
him-5(e1490)	0 (50)
ced-3(n2433); him-5(e1490)	82 (50)
eor-1(op181); him-5(e1490)	36 (50)
eor-1(mh21); him-5(e1490)	62 (112)
him-5(e1490); eor-2(op166)	63 (80)
him-5(e1490); eor-2(op232)	52 (50)
eor-1(mh21); him-5(e1490); eor-2(op166)	60 (50)
tra-1(e1076)	75 (25)
tra-1(e1076); ced-3(n717)	95 (40)
tra-1(e1076); eor-1(op181)	80 (25)
tra-1(e1076); eor-2(op232)	72 (50)
tra-1(e1099)	7 (15)
tra-1(e1099); eor-2(op166)	75 (24)

(B) Genotype	% HSN survival in hermaphrodites
egl-41(n1077)	1 (91)
ced-3(n2433); egl-41(n1077)	99 (90)
eor-1(op181); egl-41(n1077)	61 (90)
egl-41(n1077); eor-2(op166)	74 (90)
egl-41(n1077); eor-2(op232)	86 (90)
ced-9(n1653)	16 (50)
ced-9(n1653); eor-1(mh21)	16 (50)
ced-9(n1653); eor-2(op232)	18 (50)

Percent HSN survival (*n*), number sides scores. *tra-1* data reflect HSN survival in transformed XX animals. All animals scored at 20°C.

We also asked whether *eor-1* or *eor-2* is required for another sexually dimorphic death: that of the CEMs. In wild-type hermaphrodites, CEM neurons normally die (0% survival, $n = 50$). These neurons also die in *eor-1* and *eor-2* mutant backgrounds (0% survival, $n = 50$ for each genotype). Since apoptotic germ cell death is well characterized in the hermaphrodite germline (Gumienny et al., 1999), we also examined germlines of *eor-1* and *eor-2* mutants and found that there is no gross alteration in germline cell death (Supplemental Table 3). Taken together, our data show that *eor-1* and *eor-2* are not general cell death genes in the cell types we examined.

eor-1 and eor-2 act upstream of or in parallel to ced-9

To test the hypothesis that *eor-1* and *eor-2* mutants allow inappropriate HSN survival by interfering with *egl-1* transcription in the HSNs, we tested whether *eor-1* or *eor-2* are required to activate transcription of the pro-apoptotic *egl-1* gene in HSNs fated to die. To monitor *egl-1* expression, we used *bcIs1*, an integrated *egl-1*::*gfp* transgene that contains all the regulatory elements necessary for normal *egl-1* repression in hermaphrodite HSNs and normal *egl-1* expression in male HSNs (Conradt and Horvitz, 1998; Thellmann et al., 2003). Normally, cells that express *egl-1* die. To block this death, the strong *ced-3(n717)* mutation was used to allow survival of HSNs even when a pro-apoptotic program is active. *him-5(e1490)* was used to increase the incidence of males in the cohort. We were able to score for *egl-1*::*GFP* expression in young larvae. As expected, gfp expression was not detectable in *ced-3; him-5; bcIs1 [egl-1*::*gfp]* hermaphrodite HSNs in the L1 to L2 stages ($n = 50$). In *ced-3; him-5; bcIs1 [egl-1*::*gfp]* males {83% ($n = 30$) of HSNs express the *egl-1*::*gfp* transgene}. In isogenic *eor-1(op181)* mutants, *egl-1* reporter expression was detectable in 93% ($n = 30$) of male HSNs. In isogenic *eor-2(op166)* mutants, 87% ($n = 30$) of male HSNs expressed gfp. However, given the caveats associated with transgene expression, these data do not exclude a role for *eor-1* and *eor-2* in regulating endogenous *egl-1* expression. It is possible that a high-copy transgenic array could bypass the requirement for *eor-1* and *eor-2* at the transgene promoter. It is also possible that *eor-1* and *eor-2* are required to specify the timing of *egl-1* expression, particularly in the embryo.

In cells that are not fated to undergo apoptosis, the pro-apoptotic protein CED-4 is kept in an inactive sequestered state by the anti-apoptotic protein CED-9. In cells doomed to die, the BH3 domain protein EGL-1 interacts with CED-9 (Conradt and Horvitz, 1998) forcing its dissociation from CED-4, resulting in a cascade of biochemical events that

lead to the demise of the cell. Strong loss-of-function *ced-9* alleles cause widespread ectopic engagement of the apoptotic machinery, resulting in embryonic lethality (Hengartner et al., 1992). The weak *ced-9(n1653ts)* allele results in a more restricted set of defects, including the death of the HSNs in hermaphrodites at 20°C, similar to the phenotype caused by *egl-1(gf)* (Hengartner et al., 1992). To determine whether *eor-1* or *eor-2* are required for HSN death induced by *ced-9(n1653ts)*, double mutants were constructed and raised at 20°C. As previously determined (Hengartner et al., 1992), only 16% of the HSNs scored were present in the parental *ced-9(n1653ts)* strain (Table 3B). Double mutants containing *ced-9(n1653ts)* and either *eor-1* or *eor-2* had the same HSN survival as the parental *ced-9(n1653ts)* strain (16% and 18%, respectively). Therefore, *eor-1* and *eor-2* mutations are sufficient to block HSN death induced by inappropriate *egl-1* expression, but are not able to block death once it is induced by inactivation of *ced-9* and consequent activation of CED-4/CED-3. Consistent with the failure to suppress *tra-1(lf)*, these data indicate that *eor-1* and *eor-2* function upstream of, or in parallel, to *ced-9*.

eor-1 encodes a putative transcription factor

eor-1(op181) was mapped to a 15-kb interval using single-nucleotide polymorphisms (SNPs) (Supplemental Figure 1). After sequencing predicted exons in the region, we identified mutations in the predicted gene R11E3.6 using genomic DNA from *eor-1(op181)* and from *eor-1(mh21)* mutant worms. One point mutation was found in each of the *eor-1* alleles (Fig. 3A). *eor-1(op181)* is a point mutation at the predicted splice acceptor site of the first coding exon and *eor-1(mh21)* is a nonsense mutation at amino acid 212. Using reverse-transcriptase-PCR (RT-PCR), we could not detect an *eor-1* product with either an SL1 or SL2 splice leader (data not shown). RT-PCR analysis confirmed that the *op181* mutation results in inappropriate retention of the first intron (data not shown).

Complete sequencing and conceptual translation of yk47h3, the longest available *eor-1* cDNA, generates a single open reading frame of 909 amino acids that encodes a multi-domain protein with an amino-terminal BTB/POZ domain, 9 C_2H_2-type zinc fingers and a carboxy-terminal Gly and Gln-rich domain (Figs. 3B–D). The *eor-1(mh21)* allele introduces a stop codon between the NH-terminal BTB/POZ domain and the central Zn-fingers (Trp212 > Opal).

The domains encoded by conceptual translation of the EOR-1 cDNA give important clues to its normal function. The BTB/POZ (Bric-A-Brack, Tramtrack, Broad Complex/Poxvirus and Zinc Finger) domain is most closely associated with protein–protein interactions, particularly homophilic interactions or heterophilic interactions between proteins containing BTB/POZ domains (Bardwell and Treisman, 1994). The nine C_2H_2-type zinc fingers of EOR-1 are most closely related to the nine C_2H_2-type zinc fingers of PLZF (Promyelocytic Leukemia Zinc Finger), a DNA binding transcriptional repressor protein that is the causative agent in a small proportion of Acute Promyelocytic Leukemia (APL) patients (Chen et al., 1993a,b; He et al., 1999). However, by analogy to the sea urchin stage-specific activator protein (SSAP), the Gly–Gln-rich region of EOR-1 (amino acids 785-883) may function as a transcription activation region (both contain 21% Gln at their C-termini) (Benuck et al., 1999).

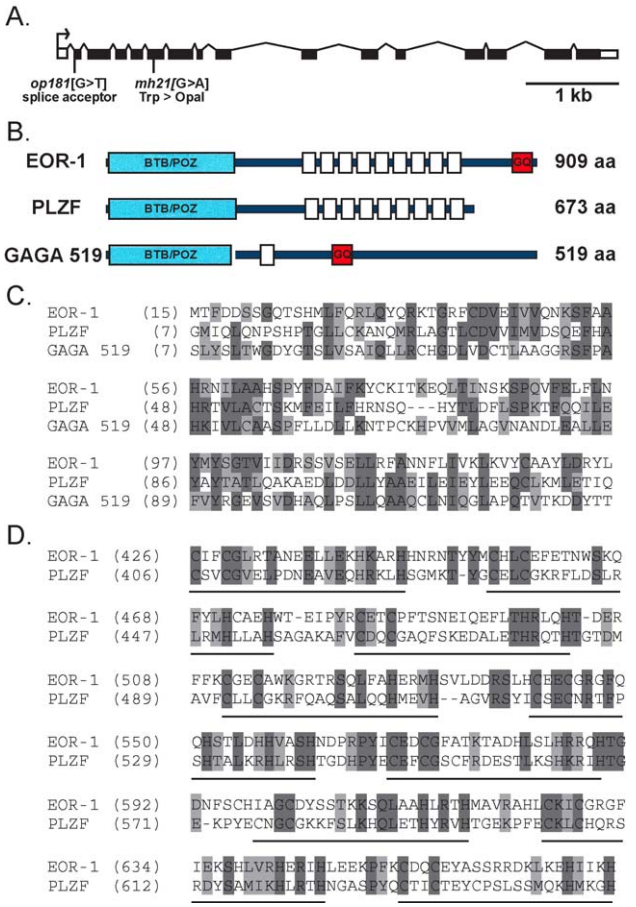

Fig. 3. *eor-1*. (A) Intron/exon structure of *eor-1*. Mutation positions are indicated. (B) Protein domains in conceptual translations of EOR-1, human PLZF and *Drosophila* GAGA 519. (C) BTB/POZ domain alignment of EOR-1 with *Drosophila* GAGA 519 and human PLZF. (D) C_2H_2 zinc finger alignment of EOR-1 with human PLZF. Dark shading indicates identity, light shading indicates similarity. Solid bars under alignment indicate positions of the 9 Zinc fingers. See supplementary materials for genetic, physical, and transcription map of the *eor-1* locus.

eor-2 encodes a conserved protein

The *eor-2* gene was cloned using the same methodology as *eor-1* (Supplemental Figure 2). SNP mapping of *eor-2(op232)* excluded all but two predicted genes. Single point mutations were found in the gene predicted by C44H4.7 by sequencing DNA from *op166* and *op232* mutants. Complete sequencing of the *eor-2* cDNA (yk257c2) leads to the conceptual translation of a 972

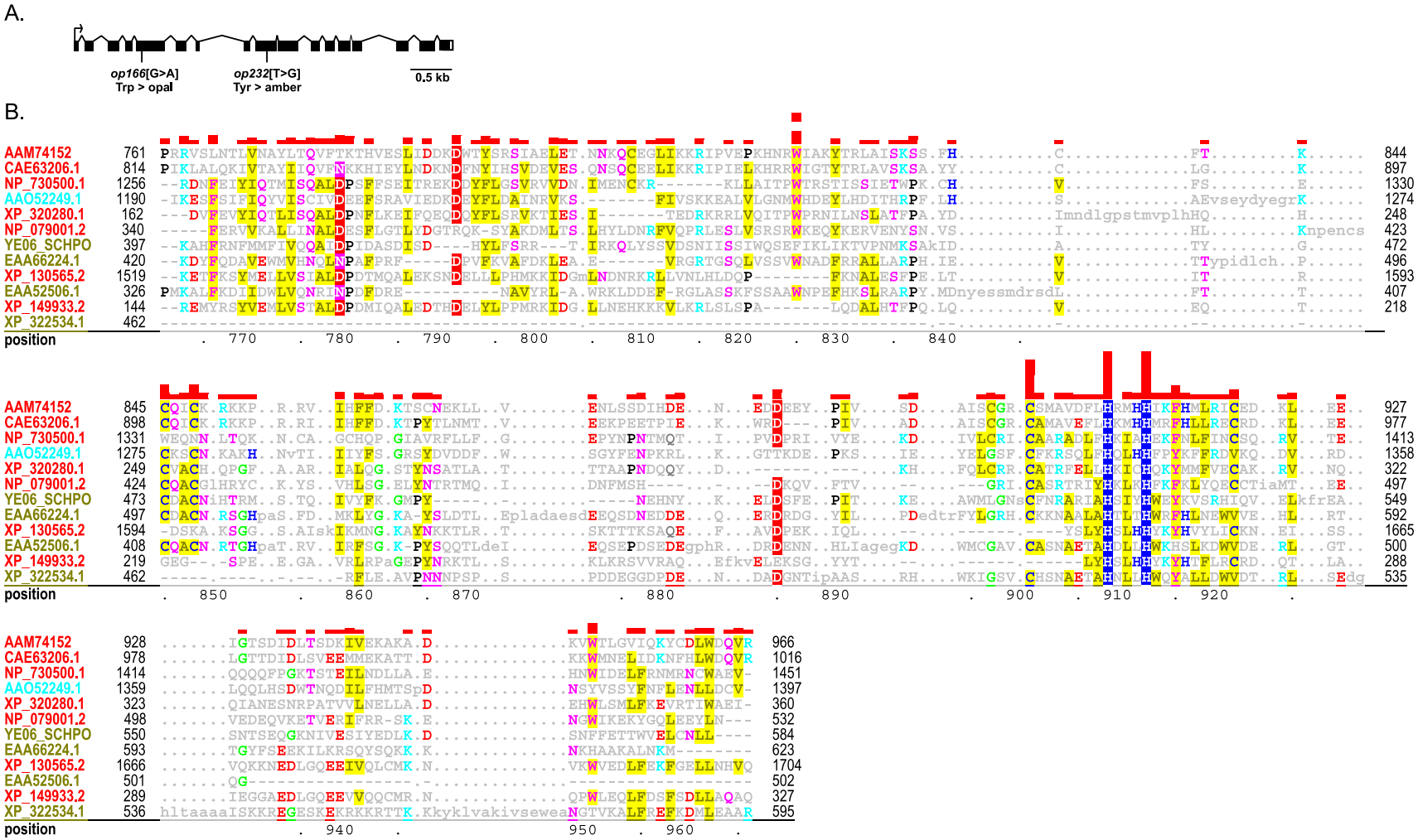

Fig. 4. *eor-2*. (A) Intron/exon structure of *eor-2*. Mutation positions are indicated. (B) Amino acid alignment of EOR-2 and related proteins. Alignment color code: red = metazoan, cyan = protozoan, dk yellow = fungi. See supplementary materials for genetic, physical, and transcription map of the *eor-2* locus.

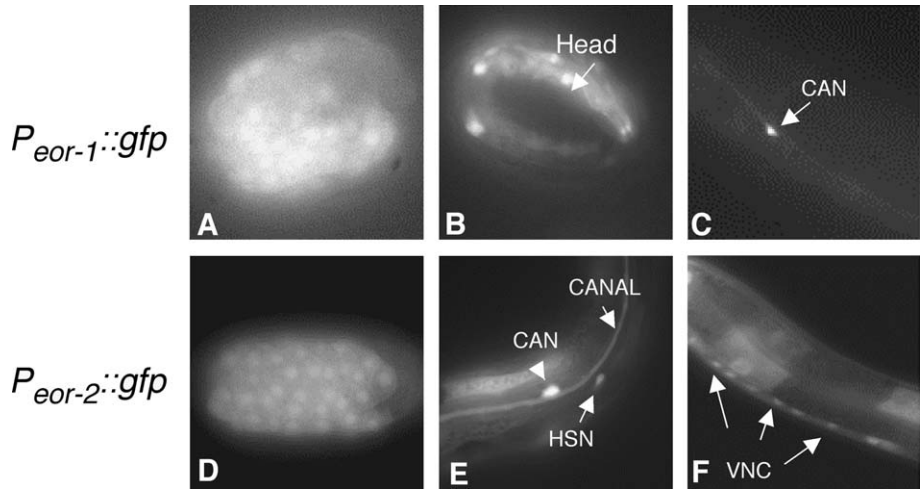

Fig. 5. Expression of P_{eor-1}::*gfp* and P_{eor-2}::*gfp* in wild type animals. (A–C) Transgenic worms expressing GFP under the transcriptional control of *eor-1* upstream sequences. (A) *eor-1* is expressed in most cells of the early embryo. (B) Expression becomes restricted in a 3-fold stage embryo. (C) *eor-1* is expressed in the CAN neuron of an L3 larva. (D–F) Transgenic worms expressing GFP under the transcriptional control of *eor-2* upstream sequences. (D) *eor-2*::*gfp* is expressed in most cells of the early embryo. (E) An L4 larva with expression in the HSN and CAN neurons and the excretory canal cell. (F) An L4 larva with expression in the ventral nerve cord (VNC). Anterior is left and ventral is down in larvae.

amino acid protein. Both *eor-2* alleles are nonsense mutations: *op166* results in a Trp177 to stop and op232 is a Tyr422 to stop mutation (Fig. 4A). The SL1 splice leader is spliced to the 5′ end of the transcript as detected by RT-PCR (data not shown). Curiously, there are several predicted short open-reading frames immediately downstream of the SL1 acceptor site, suggesting that EOR-2 expression may be regulated at the translational level.

EOR-2 protein has weak similarity to several proteins from protozoa through the fungi and metazoa (Fig. 4B). The carboxyl terminus of EOR-2 shares a potential Zn^{+} binding module with numerous related proteins (Neuwald et al., 2003). Although the overall degree of similarity is weak, this degree of conservation across distant organisms suggests an important conserved function. In addition, EOR-2 has a putative nuclear localization signal at amino acids 812–828 suggesting that this protein has a nuclear function perhaps to act in a complex with EOR-1. Our analyses of *eor-1* and *eor-2* sequence are consistent with the results of Howard and Sundaram (2002).

eor-1 and eor-2 are expressed in several neurons

We assessed the expression pattern of *eor-1* and *eor-2* using transcriptional reporter genes driving green fluorescent protein (GFP) expressed from extra-chromosomal arrays (Fig. 5). There is widespread expression of both genes in embryos that continues during elongation in some or all of these cells and then becomes restricted to several head neurons (Fig. 5B). This pattern persists into the adult stage (data not shown). Most importantly, *eor-2* is expressed in the HSN neurons (Fig. 5E), supporting a role for *eor-2* in suppressing *egl-1*-induced HSN apoptosis. *eor-2* is also localized in nuclei of the ventral nerve cord in L2 larvae suggesting a role for this gene in the normal function or development of these neurons, possibly explaining the uncoordinated phenotype observed.

Interestingly, expression of both *eor-1* and *eor-2* was detected in the CAN neurons, suggesting a cell-autonomous role for these genes in CAN differentiation. *eor-1* and *eor-2* expression in the CAN neurons is consistent with the CAN migration defect in *eor* mutants. We predicted to also see expression in the phasmid neurons. However, using DiD to label the phasmids in L3 larvae, we did not find co-localization with GFP expression (data not shown). One possible explanation for this is that *eor-1* and *eor-2* are expressed during embryogenesis, before it is possible to dye-fill the phasmids. In addition, we find that the pattern of expression of *Peor-2*::*gfp* is identical to the pattern in wild type and *eor-1(mh21)* worms suggesting that expression of *eor-2* does not require *eor-1* (data not shown). Similarly, *eor-2* is not required for normal expression of *Peor-1*::*gfp* (data not shown). Although these studies analyze reporter expression from a non-integrated extra-chromosomal array, this pattern of expression supports a role for these genes in the differentiation of multiple neuronal cell types and provides an explanation for the range of pleiotropic defects observed.

Discussion

During development and in adulthood, multicellular organisms eliminate many cells by a highly regulated process, known as apoptosis. Deviation from the strict regulation of this process in vertebrates leads to developmental defects, autoimmune diseases, cancer, and neurodegenerative diseases. Use of the nematode *C. elegans* as a

model genetic system provided major breakthroughs into the understanding of the molecular nature of this pathway (Horvitz, 2003). Most research in *C. elegans* apoptosis has focused on the machinery required to dismantle a cell. Surprisingly, few upstream regulators of the cell death pathway have been identified. Here, we undertook a genetic screen with the intention of identifying genes that are involved in the specification of cell death at single cell resolution. This screen revealed mutations in two genes, *eor-1* and *eor-2*, that allow the hermaphrodite-specific neurons to survive in response to conditions that normally lead to programmed cell death.

eor-1 and eor-2 are HSN-specific death regulators that function upstream of or in parallel to ced-9

Conradt and Horvitz (1998, 1999) (Hodgkin, 1999) conclusively demonstrated that the pro-apoptotic gene *egl-1* is transcriptionally repressed by the binding of the terminal sex-determination regulator TRA-1A in hermaphrodite HSNs. This repression prevents the activation of the downstream killing machinery. In males, the HSNs express *egl-1* and die. Therefore, *egl-1* likely responds to activating factors that promote HSN death in the absence of TRA-1A. In *eor-1* or *eor-2* mutant males, most HSNs fail to die, demonstrating that *eor-1* and *eor-2* are required to specify HSN death. Both *eor-1* and *eor-2* fail to suppress HSN death in *ced-9(lf)* hermaphrodites indicating that the cell death machinery downstream of *ced-9* (CED-4/CED-3) is intact in *eor-1* and *eor-2* mutants. Thus, the HSNs are competent to die in *eor-1* or *eor-2* mutant backgrounds but are not specified to do so.

Several neurons require eor-1 and eor-2 for terminal differentiation

The function of *eor-1* and *eor-2* extends beyond regulating cell death in the HSNs. The seemingly disparate pleiotropic defects observed with *eor-1* and *eor-2* mutants can be unified if one considers that *eor-1* and *eor-2* play a pivotal role during a late stage of differentiation of several neuronal precursors. The mutant neurons appear normal in fate choice, expression of differentiation markers, and in some cases, final migratory position. However, mutant neurons are each defective for a final stage of differentiation and do not function normally in *eor-1* and *eor-2* mutants. Examples described here include the phasmid neurons (PHA and PHB), which are present and appear morphologically normal, yet fail to fill with DiO; the spicules which are present but malformed; the rays which are present but often fused; the CAN neurons that are born and begin to migrate, but often do not reach their target; and the ventral nerve cord which is present and generally well organized, but often fails to coordinate normal movement, presumably due to a defect we did not detect morphologically, but is suggested by transgene expression. In addition, transgene expression analysis reveals that both *eor-1* and *eor-2* promoters are being actively expressed in the HSN and CAN neurons—cells which we identified here as having developmental defects. Finally, based on the observation that *eor-1* and *eor-2* exhibit identical pleiotropic defects and that they both function upstream of or in parallel to *ced-9*, it is tempting to speculate that they act at the same step of the genetic pathway, possibly in a physical complex, leading to terminal differentiation of a variety of neuronal precursors including CAN, PHA, PHB, HSN, and those of the V6, T, and B lineages (which generate the male rays). Taken together, these data suggest a model in which *eor-1* and *eor-2* are required for normal differentiation of many neuronal cell types in *C. elegans*.

eor-1 encodes a Krüppel-family protein

EOR-1 encodes a protein with three significant functional domains: a BTB/POZ domain, nine krüppel-type C_2H_2 zinc finger domains, and a Gly- and Gln-rich domain. Each of these domains in other proteins has a well-documented function, protein–protein interactions (Collins et al., 2001), DNA binding, and transcriptional activation (Mitchell and Tjian, 1989), respectively. Proteins containing these domains function in several aspects of transcriptional regulation including modifying chromatin structure, which can result in the activation or repression of transcription. EOR-1 is overall most closely related to the mammalian transcriptional repressor PLZF which contains a BTB/POZ domain and nine C_2H_2 zinc fingers. Domain mapping experiments with human PLZF indicates the presence of two transcriptional repression domains of different strengths (Li et al., 1997). The weaker repression domain maps to the BTB/POZ domain. The stronger repression domain maps to amino acids 200–300, which is not conserved in the predicted EOR-1 protein. In its role as a transcriptional repressor, PLZF binds DNA (Ball et al., 1999) and recruits histone deacetylase through its interaction with the mammalian co-repressors, SMRT and NcoR (Huynh and Bardwell, 1998). The critical residues required for transcriptional repression within the BTB domain of PLZF (D35 and R49) are conserved in EOR-1 (Melnick et al., 2002), suggesting that EOR-1 may have a function in transcriptional repression through homologous co-repressors in *C. elegans*. Indeed, EOR-1 was previously suggested to interact with LIN-36, a nuclear protein that participates in the Rb pathway and potentially plays a role in chromatin remodeling (Thomas and Horvitz, 1999; Walhout et al., 2000).

In patients with acute promyelocytic leukemia (APL), PLZF is translocated adjacent to the retinoic alpha receptor (RAR). The PLZF–RAR fusion protein functions as an oncogene by repressing retinoic acid-responsive genes, thus blocking progression to a more differentiated state (Slack, 1999). Mice that lack a functional PLZF gene show reduced

apoptosis in developing limb buds (Barna et al., 2000), consistent with a role for this protein in activating pro-apoptotic genes, such as mammalian puma and noxa (Villunger et al., 2003), perhaps indirectly by repressing an anti-apoptotic regulator.

Although EOR-1 contains the same number and type of zinc fingers as PLZF and shares homology over the BTB/POZ domain, there are notable differences between these two proteins. In addition to EOR-1 not containing the second (stronger) repression domain of PLZF, EOR-1 contains a GQ-rich COO-terminus that is not present in PLZF. However, the *Drosophila* protein GAGA (isoforms 519, or 581), encoded by the *ttl* gene, is also structurally related to EOR-1, in that it has a BTB/POZ domain, two Zn fingers and a large glutamine-rich domain at the COO-terminus (Gerber et al., 1994). The glutamine rich domain of GAGA functions as a transcriptional activation module (Vaquero et al., 2000). Further, the GAGA 519 (*melanogaster*) and GAGA A (*virilis*) isoforms both contain a glycine-rich domain in the "Intermediate Region", as does *eor-1*. No role has been ascribed to this region in GAGA, although its position is well integrated into the transcriptional activation domain.

Based on the fact that EOR-1 shares significant sequence similarity with the transcriptional regulator, PLZF, and has an acidic domain, we favor the hypothesis that EOR-1 normally functions as a DNA-binding transcriptional activator at promoters throughout the nervous system. Considering that the *eor-1* and *eor-2* mutant phenotypes were nearly indistinguishable in every context examined and *eor-1; eor-2* double mutants were as effective at suppressing male HSN death as either single mutant, we believe that EOR-1 functions in a complex with EOR-2 to promote expression of genes allowing normal differentiation of multiple cell types throughout the nervous system. Because the genetic pathway to cell death is conserved throughout evolution, both at the structural and functional levels, these data might lead to greater understanding of the transcriptional regulation of neuronal differentiation and cell death specification in vertebrates.

eors couple the ras signaling pathway with apoptosis

The search for upstream regulators and downstream targets involved in communicating cell death signals has led to the elucidation of a molecular pathway that couples *ras* signaling to cell death specification. Elegant studies in *Drosophila* demonstrated that the EGFR/RAS/MAPK signaling is required to suppress HID activity to prevent apoptosis in neurons (Bergmann et al., 1998, 2002; Kurada and White, 1998). *eor-1* and *eor-2* were isolated in an independent screen as enhancers of *raf* function (Howard and Sundaram, 2002; Rocheleau et al., 2002) and the authors show that *eor-1* and *eor-2* function downstream of—or in parallel to ERK. Whether *eor-1* and *eor-2* provide a similar link between *ras* signaling and the cellular execution machinery remains to be determined.

Some of the pleiotropic defects observed in *eor-1* or *eor-2* mutants are consistent with defects in the *ras* pathway. First, increased or decreased activation of the *ras* pathway alters the balance between anterior and posterior fate choice in B.a progeny, from which the male spicules are derived (Sulston et al., 1980), thereby influencing spicule fate specification (Chamberlin and Sternberg, 1994). Second, the Eor incompletely penetrant larval lethal phenotype, which includes a dramatic separation of gonad and intestine from the body wall, is highly reminiscent of the rod-like larval-lethal phenotype of loss-of-function mutations in the *let-60ras* pathway (Rogalski et al., 1982). Arrested *eor-1* and *eor-2* larvae are flaccid, non-Dumpy and often "kink" like a garden hose when they make a sharp turn.

As is the case with the core execution machinery, it is likely that the cell-type-specific apoptosis-regulatory functions of *C. elegans* genes are also conserved through evolution. For example, the CES-2-like protein HLF, when fused to E2A, is involved in regulating apoptosis (Chen et al., 2001), and the *ces-1*-related SLUG protein has anti-apoptotic activity (Inukai et al., 1999). Similarly, the EOR-1-like protein PLZF has been reported to play a role in apoptosis (Barna et al., 2000). Genetically, *ces-1* functions upstream of *egl-1*. The *egl-1* promoter/enhancer contains *ces-1* binding sites that compete with HLH2 and HLH3 to regulate expression of *egl-1* in the NSM sisters (Thellmann et al., 2003). A likely possibility is that the pattern of cell death specification throughout the worm is established by cell-type-specific combinations of activators and repressors bound to the promoter/enhancer of *egl-1* that integrate all signals through the apoptotic output of *egl-1* in combination with downstream factors that fine-tune the apoptotic signal in specific cells. Understanding the establishment of this pattern is critical to our ability to better understand cell fate decisions in the nervous system. The discovery that *ces* genes and *eor* genes target different subsets of neurons raises the possibility that selective interventions may be sought in the treatment of cell-type-specific neuronal diseases in man.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.06.022.

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Further reading

Accession Numbers:
eor-1 mRNA AF502568.1, protein AAM74151.1.
eor-2 mRNA AF502570.1, protein AAM74152.1.