Hox and a Newly Identified E2F Co-repress Cell Death in *Caenorhabditis elegans*

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ABSTRACT The development of an organism depends on individual cells receiving and executing their specific fates, although how this process is regulated remains largely unknown. Here, we identify a mechanism by which a specific cell fate, apoptosis, is determined through the cooperative efforts of Hox and E2F proteins. E2F transcription factors are critical, conserved regulators of the cell cycle and apoptosis. However, little is known about the two most recently discovered mammalian E2Fs—E2F7 and E2F8. In the nematode *Caenorhabditis elegans*, we identify a novel E2F7/8 homolog, EFL-3, and show that EFL-3 functions cooperatively with LIN-39, providing the first example in which these two major developmental pathways—E2F and Hox—are able to directly regulate the same target gene. Our studies demonstrate that LIN-39 and EFL-3 function in a cell type-specific context to regulate transcription of the *egl-1* BH3-only cell death gene and to determine cell fate during development.

THE generation of a complex, multicellular organism from a single cell is an immensely complicated process. Cell fate must be specified by factors such as contexts, mechanisms, and regulatory complexes for the proper number and types of cells to be generated. Here, we identified a mechanism governed by Hox and E2F genes in which cell fate is determined in a highly specific manner, according to cellular lineage and spatial positioning.

The genetics of development focus on how cells are directed to adopt specific fates. To this work *Caenorhabditis elegans* has brought the advantage of a known and essentially invariant lineage, making possible the discovery and analysis of developmental pathways that direct—with single-cell resolution—the precise patterns of cell fates, a feat that cannot be achieved in mammalian systems. The genetic pathway that underlies programmed cell death in all animals was first identified in *C. elegans* and includes four genes—*egl-1*, *ced-9*, *ced-4*, and *ced-3* (Ellis and Horvitz 1986; Hengartner *et al.* 1992; Conradt and Horvitz 1998; Horvitz 1999).

In many cells, apoptosis is initiated by transcriptional regulation of the BH3 domain-encoding gene *egl-1* (Conradt and Horvitz 1998). The EGL-1 protein binds the BCL-2 family member CED-9, releasing the APAF-1 homolog CED-4 to activate the caspase CED-3, killing the cell (Hengartner *et al.* 1992; Yuan *et al.* 1993; Chinnaiyan *et al.* 1997a,b; Seshagiri and Miller 1997; Wu *et al.* 1997a,b; Zou *et al.* 1997; Conradt and Horvitz 1998). The central cell death machinery is conserved across species, including *Drosophila*, mice, and humans (Danial and Korsmeyer 2004). How the apoptotic pathway is controlled in individual cells is not well understood, although it is of great importance to human disease, as the pathway is frequently abnormally regulated in cancer (see review by Chonghaile and Letai 2008). Here, we have characterized how an E2F transcription factor directly regulates the cell death pathway, providing insight into a mechanism of apoptosis regulation that is likely similar across species, given the conservation of the cell death pathway and E2F family functions.

E2Fs have been extensively studied *in vitro* and in cell culture models as regulators of the cell cycle and apoptosis (see review by Chen *et al.* 2009). The mammalian E2F family is composed of eight members, termed E2F1–8, with each member categorized according to structure and

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function—as an activator and/or repressor of transcription. It is thought that E2F7 and E2F8 are repressor E2Fs (De Bruin *et al.* 2003; Di Stefano *et al.* 2003; Logan *et al.* 2005) mostly on the basis of *in vitro* assays. Structurally, E2F7 and E2F8 are distinct from other family members, specifically lacking domains for binding E2Fs' dimerization partner (DP) and pocket proteins, including the retinoblastoma (RB) protein (De Bruin *et al.* 2003; Di Stefano *et al.* 2003; Logan *et al.* 2005). E2F7/8 are the most recently identified E2F family members. Thus far, only one murine study has examined the function of E2F7 and E2F8 *in vivo*. In that study, E2F7 and E2F8 double-knockout mice display widespread TUNEL staining in embryos, suggesting that E2F7 and E2F8 prevent apoptosis (Li *et al.* 2008), although currently the mechanisms and contexts by which they regulate target genes are unknown.

C. elegans has previously been employed to study how the E2F family functions *in vivo*. Like mammals, *C. elegans* possess a DP homolog, *dpl-1* ("*DP-*like"); an RB homolog, *lin-35* (Reddien *et al.* 2007); and E2Fs: *efl-1*, *efl-2* (Ceol and Horvitz 2001; Reddien *et al.* 2007), and *efl-3* ("*E2F-*like, *3*rd family member," introduced here). In a study by Reddien and colleagues (2007), *efl-1* was identified as a promoter of cell death in the anterior pharynx, functioning in the same pathway as *dpl-1* and *lin-35*. In the hermaphrodite germline, *lin-35*, *dpl-1*, *efl-1*, and *efl-2* possess context-specific pro-apoptotic functions (Schertel and Conradt 2007). *EFL-1* can be considered an activator E2F and shares a proposed structure similar to mammalian E2F4 and E2F5, while *EFL-2* is more similar to mammalian E2F3 and E2F6 (Ceol and Horvitz 2001).

C. elegans has also been used to study the Hox family of transcription factors, particularly in the context of the ventral nerve cord. The ventral nerve cord is a model for studying how cells differentiate and determine cell death based on varying developmental cues. The ventral nerve cord is generated from 12 P cell and 1 W blast cell lineages (Figure 1, A and B), each of which produces up to five types of motor neurons—VA, VB, VC, VD, and AS—and one hypodermal cell (Sulston 1976). All ventral nerve cord lineages are similar in terms of the number of progeny that they create and what types of neurons are generated, but because the lineages are spread across the length of the body, each is under the control of different spatial cues, leading to variations in cell fate based on developmental context. In the midbody, the survival of one neuron type, the VC neuron, allows for the innervation of egg-laying muscles, but in the anterior and posterior lineages, where the VC-lineal equivalents are unnecessary, the neurons undergo apoptosis (Clark *et al.* 1993). The Hox gene *lin-39* provides the spatial information to determine VC neuron survival *vs.* death; without *lin-39* to tell the midbody VC neurons where within the animal they are located, the neurons undertake the fate (death) of their lineal equivalents in the anterior or posterior of the animal (Clark *et al.* 1993; Potts *et al.* 2009) (Figure 1).

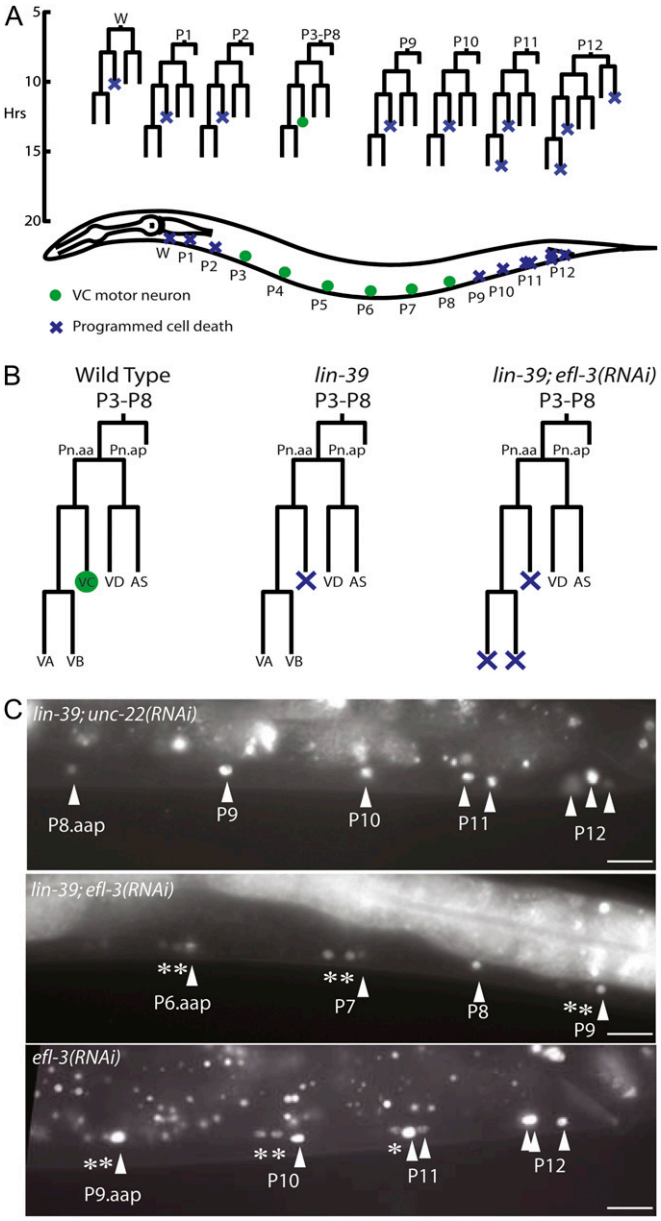

Figure 1 LIN-39 and EFL-3 regulate programmed cell death in the ventral nerve cord. (A) The ventral nerve cord is derived from 12 P cell and 1 W cell lineages across the length of the animal. In the anterior (W, P1–P2) and posterior (P9–P12) lineages, some neurons undergo programmed cell death. (B) Each midbody (P3–P8) lineage is composed of five neuron types, which all survive in wild-type animals. Loss of *lin-39* induces one neuron type, the VC, to express the cell death gene *egl-1* and die in each midbody lineage, in addition to the seven that typically die in the posterior. In our screen, we identified that *lin-39; efl-3(RNAi)* animals have two additional neurons per midbody lineage expressing *egl-1*. (C) On *unc-22* control RNAi, *lin-39* animals express *Pegl-1gfp* in cells that undergo programmed cell death. In *lin-39; efl-3(RNAi)* animals, there is an increase in the number of neurons in the midbody and posterior expressing *Pegl-1gfp*. In *efl-3(RNAi)* animals, there is an increase in posterior neurons expressing *Pegl-1gfp*. Arrowheads indicate neurons expressing *Pegl-1gfp* in a *lin-39* mutant background, and asterisks indicate additional neurons expressing *Pegl-1gfp*. All animals shown are also homozygous for *ced-1; ced-3; Pegl-1gfp*. Scale bars, 10 μm.

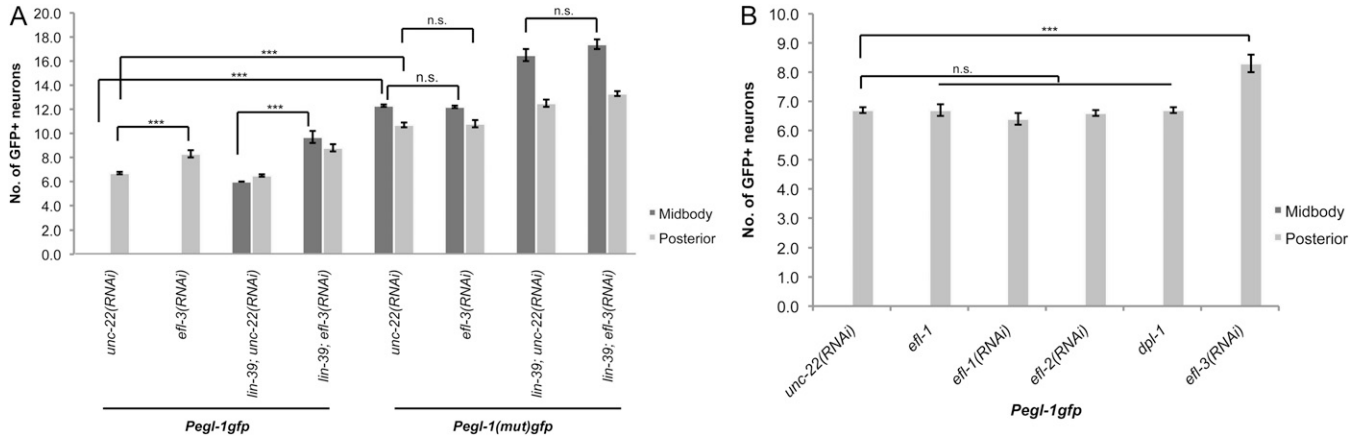

Figure 2 *egl-1* is repressed by *efl-3* and *lin-39* in a context-specific manner in the ventral nerve cord. (A) A repressive E2F, likely EFL-3, directly binds *Pegl-1*. (B) Loss of *efl-3* induces an increase in the number of neurons expressing *egl-1*. All strains are homozygous for *ced-3*. Standard errors are shown. ***$P < 0.0001$; "n.s." indicates $P > 0.05$.

Materials and Methods

Worm strains and scoring

"Wild type" refers to the Bristol N2 strain. All worms were maintained under standard conditions (Brenner 1974) at 20°, unless otherwise noted. The RNA interference (RNAi) screen background was *ced-1(e1735) I; lin-39(n709ts) III; ced-3(n717) IV; mxIs14 X*. *mxIs14* is an integrated $P_{egl\text{-}1}$ *histone:gfp* transgene (Liu *et al.* 2006). The strains used to identify VA, VB, and VC neurons included *wdIs4*[$P_{unc\text{-}4}$*gfp*] *II* (Pflugrad *et al.* 1997), *wdIs6*[$P_{del\text{-}1}$*gfp*] *II* (Winnier *et al.* 1999), *nIs106*[$P_{lin\text{-}11}$*gfp*] *X* (Reddien *et al.* 2001), *lin-39 (n709ts)*, and *ced-3(n717)*. *wdIs4* and *wdIs6* were generously provided by David Miller. Strains used to study how cell death is affected by E2Fs include *efl-1(se1) V* that was raised at 26°, *dpl-1(n2994) II*, and *efl-3(gk835)/mIn1[mIs14 dpy-10(e128)] II*. From strains used for scoring—including $P_{egl\text{-}1}$*gfp*, $P_{unc\text{-}4}$*gfp*, $P_{del\text{-}1}$*gfp*, and $P_{lin\text{-}11}$*gfp* reporter strains—between 17 and 31 individuals were scored for each experiment. Raw *P*-values from the scoring results were used to calculate significance in Figure 2 and Figure 4.

RNAi analyses and imaging

dsRNA-expressing bacteria from the Ahringer RNAi library were fed to worms according to the procedure described by Kamath *et al.* (2001). L4 animals were fed on RNAi bacteria and their L2 progeny were scored 2–3 days later. For RNAi clones used after the initial screen, the validity of gene targets was confirmed by sequencing. *dpl-1* RNAi was constructed by PCR, amplifying a 1.1-kb segment of *dpl-1* ORF from N2 genomic DNA. The *dpl-1* gene fragment was then dropped into the L4440 RNAi backbone, using the following primers to introduce *Nco*I and *Pst*I sites for cloning: 5′-TAGCCATGGACAAACTACGATCCCCGTATC-3′ and 5′-CTACTGCAGCTTACTGGCAATGATTTCGTC-3′. For RNAi screening and for all images, we used a Zeiss Axiophot microscope.

Conservation across species

To identify putative E2F-binding sites in $P_{egl\text{-}1}$, the 7.67-kb *Bam*HI-*Stu*I promoter of *egl-1* (Liu *et al.* 2006) was first searched using MatInspector (http://www.genomatix.de) for general core promoter binding sites, with a matrix similarity of 5, indicating similarity across many species. All sequences identified as possible E2F- or E2F/DP-binding sites were then examined for conservation with *Caenorhabditis briggsae* and *Caenorhabditis remanei* using Family Relations II and Cartwheel (Caltech; http://cartwheel.caltech.edu/).

To search for domains conserved between human and *C. elegans* E2Fs, human E2F proteins were examined in Uniprot (http://www.uniprot.org) for their respective domains. Each E2F domain was aligned with *C. elegans* E2F protein sequences (Wormbase http://www.wormbase.org) using MacVector Pustell Protein Matrix software.

Reporter constructs

$P_{egl\text{-}1}$*gfp* is $P_{egl\text{-}1}$*histone:gfp* (Liu *et al.* 2006). To create $P_{egl\text{-}1(mut)}$*gfp*, the $P_{egl\text{-}1}$*gfp* construct was first split into two parts. A 3.5-kb *Pst*I fragment was dropped out, creating pJW001, and the 3.5-kb *Pst*I fragment was cloned into pBluescript II KS, creating pMP024. Mutations were made using Phusion (Finnzymes) site-directed mutagenesis. In pJW001, two mutations were made by changing 5′-TTTCCCGCATGAA-3′ to 5′-TTTAGGCCTTGAA-3′ and 5′-AGTTCCCGCGTTT-3′ to 5′-AGTACTAGTGTTT-3′. In pMP024, three mutations were made, changing 5′-TTTCGCGCATT-3′ to 5′-TTTCGATCATT-3′, 5′-TTTCGCGCATTTC-3′ to 5′-TTTAGGCCTTTTC-3′, and 5′-ATTGCGCGAGACC-3′ to 5′-ATTACTAGTGACC-3′. Mutations were confirmed by sequencing. The pMP024 *Pst*I fragment was then excised and recombined with pJW001 to create the final $P_{egl\text{-}1(mut)}$*gfp* construct. $P_{egl\text{-}1(mut)}$*gfp* (5 ng/μL) was injected into *ced-1(e1735) I; ced-3(n717) IV; lin-15(n765) X* with the *lin-15* rescuing construct pL15EK (50 ng/μl). Non-Muv transgenic progeny were

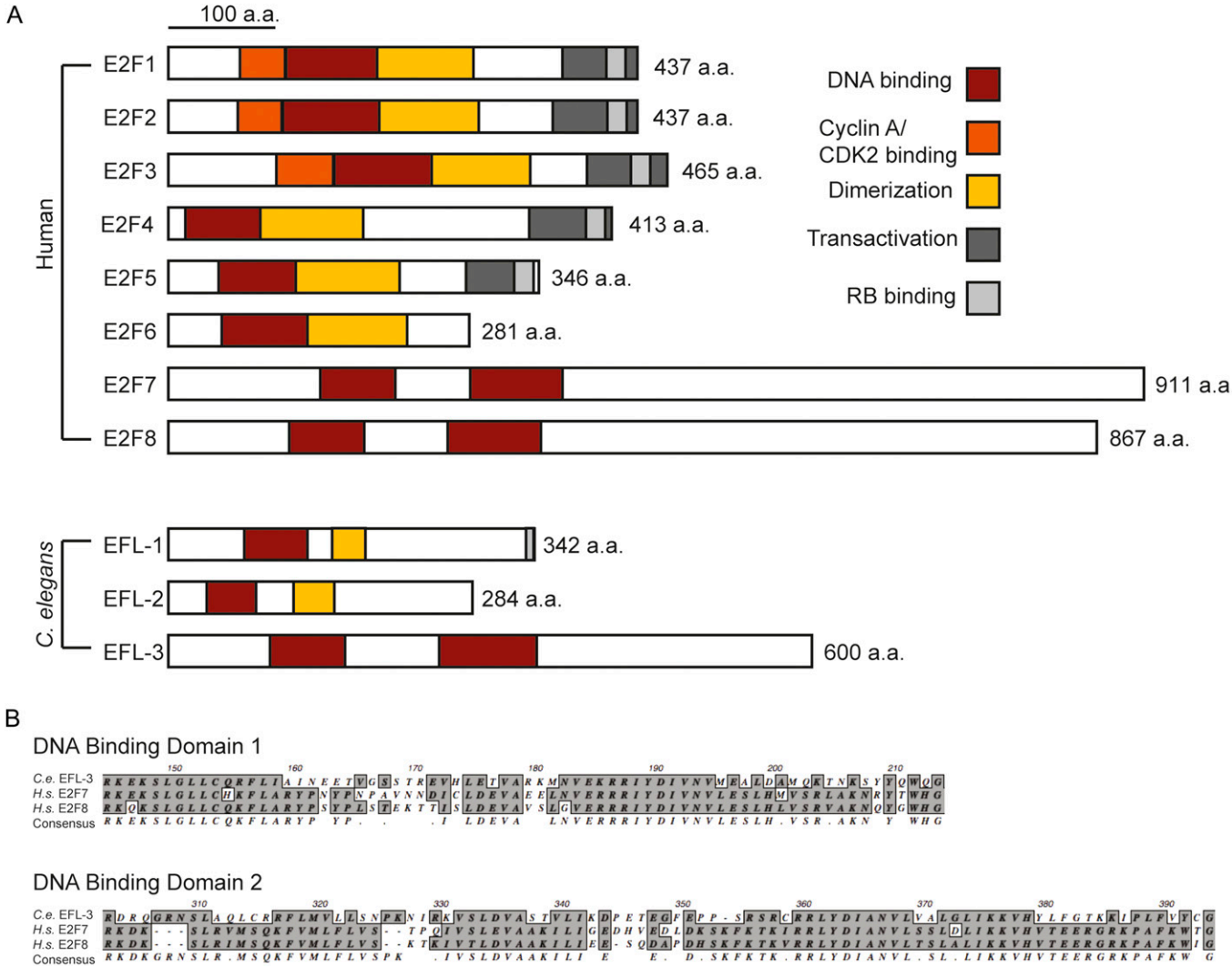

Figure 3 EFL-3 is a homolog of mammalian E2F7 and E2F8. (A) The mammalian and *C. elegans* E2F families of transcription factors. (B) Alignment of the DNA-binding domains of *C. elegans* EFL-3 and *Homo sapiens* E2F7 and E2F8.

maintained. Three stable transgenic lines gave similar *gfp* expression. Mutations of single sites induced no change in *egl-1* expression, indicating that a combination of multiple binding sites is required for E2F regulation of *egl-1*. One line was crossed with *lin-39(n1760) III* to give *lin-39; $P_{egl\text{-}1\ (mut)}$gfp*.

$P_{efl\text{-}3}$mCherry was created by first amplifying a 3.2-kb fragment upstream of *efl-3*'s ATG start. To this fragment, *Sph*I and *Xma*I sites were added by PCR using the following primers: 5′-TAGTAGGCATGCCCAGCAGTGTGACTGTACATG TTC-3′ and 5′-CTACTACCCGGGATTTGTTGAGCTCAATTA CCAGATG-3′. *$P_{efl\text{-}3}$* was cloned into a pPD95.70 construct in which the *gfp* coding sequence was replaced with *mCherry*. The final *$P_{efl\text{-}3}$mCherry* construct was confirmed by sequencing. *$P_{efl\text{-}3}$mCherry* (50 ng/μl) was injected with a *$P_{myo\text{-}2}$gfp* (30 ng/μl) co-injection marker into N2 worms. Three stable transgenic lines gave similar *mCherry* expression.

Results

EFL-3 is identified in a screen for repressors of cell death redundant with LIN-39

Although the Hox transcription factor LIN-39 is expressed in all five neuron types generated by each midbody P3-8 lineage, only the VC neurons express *egl-1* and subsequently undergo apoptosis in a *lin-39* mutant (Maloof and Kenyon 1998) (Figure 1). With loss of LIN-39, what causes the VC neurons to die and the other four neuron types to remain alive? One possibility is that there is a repressor of *egl-1* that functions redundantly with *lin-39* in the VA, VB, VD, and/or AS neurons.

Using an RNAi screen of 387 transcription factors and 263 chromatin-remodeling factors (Kamath and Ahringer 2003), we sought repressors of *egl-1* that are redundant with Hox function in the non-VC motor neurons. *egl-1* expression was determined by a *$P_{egl\text{-}1}$gfp* reporter in a cell

death-defective (*ced-3*) background so cells that initiate the cell death cascade would express $P_{egl\text{-}1}gfp$, but the execution of death would be blocked by a downstream mutation in the caspase *ced-3*, allowing the cells to remain alive for scoring by fluorescence microscopy. In the screening background of *lin-39*; *ced-3;* $P_{egl\text{-}1}gfp$, we discovered that the gene *F49E12.6*, which we refer to as *efl-3*, represses *egl-1* expression in a subset of ventral nerve cord neurons in a manner that is at least partially redundant with *lin-39* (Figure 1).

EFL-3 and LIN-39 repress egl-1 in the ventral nerve cord in a partially redundant manner

Additional neurons expressed *egl-1* on *efl-3(RNAi)* in a *lin-39* background (Figure 2A). In the midbody, loss of LIN-39 was sufficient to derepress *egl-1* in the VC neurons. Additional loss of EFL-3 led to derepression of *egl-1* in the VA and VB neurons. Loss of EFL-3 alone was not sufficient to induce any change in *egl-1* expression in the midbody. In the posterior, where LIN-39 is not expressed, loss of EFL-3 alone was sufficient to result in ectopic *egl-1* expression. These findings suggest that, in the midbody, EFL-3 and LIN-39 act partially redundantly to repress *egl-1* transcription in the VA and VB motor neurons.

EFL-3 is the C. elegans *homolog of mammalian E2F7 and E2F8*

The predicted protein structure of EFL-3 includes two DNA-binding domains characteristic of the E2F family of transcription factors (Figure 3). Like E2F7 and E2F8, EFL-3 lacks a transactivation domain and binding domains for RB and cyclin A. EFL-3 is the only predicted protein in the completely sequenced *C. elegans* genome that shares all these characteristics with E2F7 and E2F8.

EFL-3 induces cell death without altering VA and VB neuron differentiation

To examine whether a differentiation defect might explain the additional *egl-1*-expressing neurons that we observed in the screen, we examined the expression of cell type-specific *gfp* markers after treatment with *efl-3(RNAi)* (Figure 4). We found that the VA, VB, and VC cell-type differentiations were apparently unaltered with *efl-3(RNAi)*, supporting the hypothesis that *efl-3* represses *egl-1* expression without altering the identity of the VA, VB, and VC neurons.

EFL-3 directly represses egl-1 in the VA and VB motor neurons

In the sole murine E2F7/8 study (Li *et al.* 2008), widespread apoptosis was observed in embryos with loss of E2F7/8, but it is unclear whether the apoptosis occurred indiscriminately or in a cell type-specific manner. Furthermore, whether E2F7/8 regulation of cell death is direct (E2Fs binding to the promoters of cell death genes to repress transcription) or indirect (possibly as the result of cell cycle deregulation) is unclear. In *C. elegans*, we sought to investigate these two

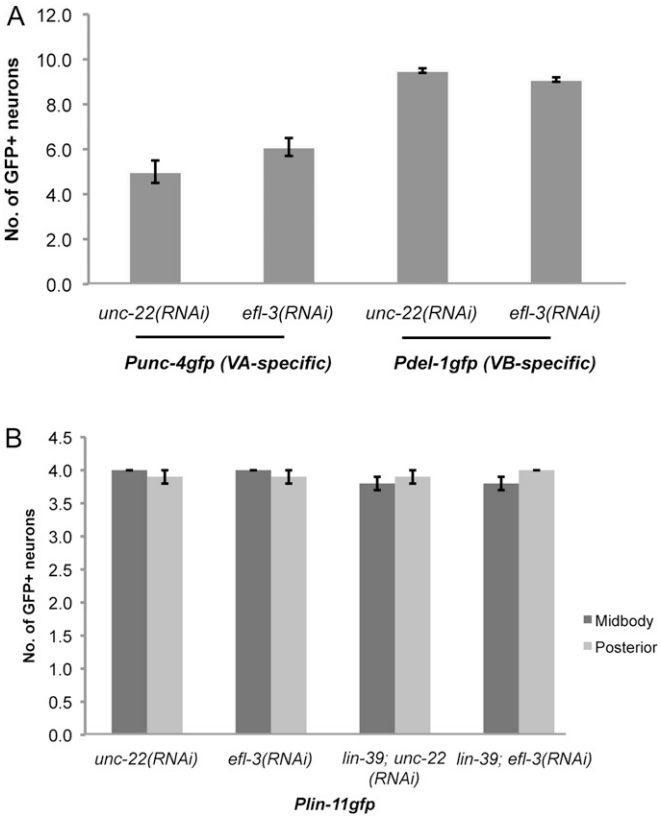

Figure 4 *efl-3(RNAi)* does not alter the identity of the VA, VB, and VC neurons. (A) Differentiation of the VA and VB neurons is unaffected with *efl-3(RNAi)*. (B) Differentiation of the VC neurons is unaffected with *efl-3 (RNAi)*. The *Plin-11gfp* strain is homozygous for *ced-3*. Standard errors are shown.

questions. Our hypothesis was that EFL-3 may function directly at the site of a cell death gene, *egl-1*, to repress its transcription in the VA and VB neurons.

To determine whether EFL-3 is able to directly bind $P_{egl\text{-}1}$ *in vivo*, we mutagenized two to five nucleotides at each of the five consensus E2F-binding sites across the 7.6-kb *egl-1* promoter to give $P_{egl\text{-}1(mut)}gfp$. In *ced-3;* $P_{egl\text{-}1(mut)}gfp$ transgenic animals, we observed an increase in the number of ventral nerve cord neurons expressing *egl-1* (Figure 5) when compared with *ced-3;* $P_{egl\text{-}1}gfp$ animals (Figure 1 and Figure 2A). Thus, an E2F likely binds *egl-1* to directly regulate its transcription.

It is thought that all E2Fs are capable of binding a similar E2F consensus sequence (Zheng *et al.* 1999; De Bruin *et al.* 2003; Di Stefano *et al.* 2003; Logan *et al.* 2005), so it is possible that *efl-1* or *efl-2* is capable of repressing *egl-1*. However, loss of *efl-1* and/or *efl-2* or their putative obligate dimerization partner *dpl-1* did not affect cell death in the ventral nerve cord, although *efl-3* did (Figure 2B). Furthermore, *ced-3;* $P_{egl\text{-}1(mut)}gfp$ animals expressed GFP in two neurons per midbody lineage (Figure 5), similarly to the two additional neurons per midbody lineage that expressed *egl-1* in *lin-39; ced-3;* $P_{egl\text{-}1}gfp$; *efl-3(RNAi)* animals, as compared to control RNAi (Figure 1). In addition, although *efl-3(RNAi)*

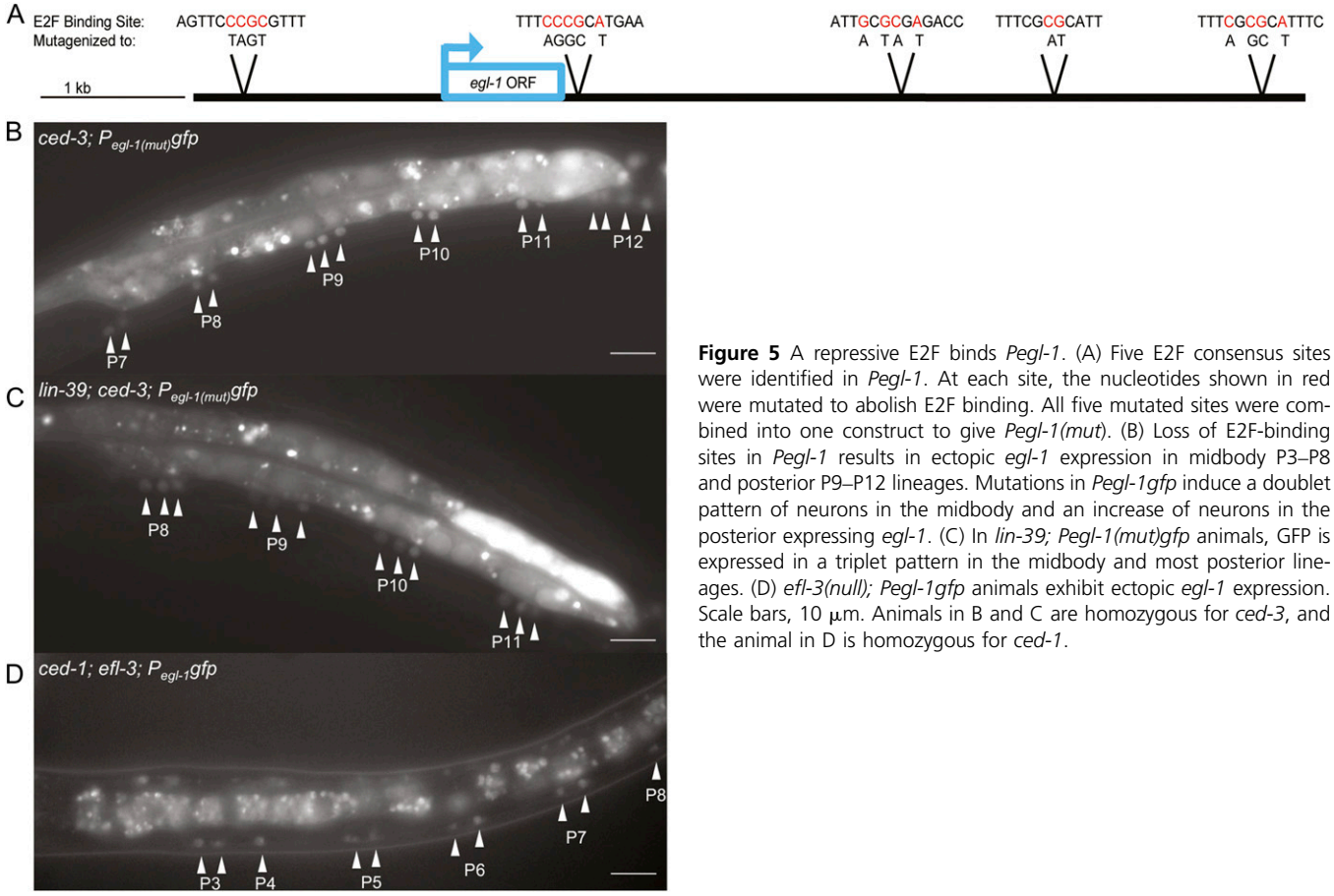

Figure 5 A repressive E2F binds *Pegl-1*. (A) Five E2F consensus sites were identified in *Pegl-1*. At each site, the nucleotides shown in red were mutated to abolish E2F binding. All five mutated sites were combined into one construct to give *Pegl-1(mut)*. (B) Loss of E2F-binding sites in *Pegl-1* results in ectopic *egl-1* expression in midbody P3–P8 and posterior P9–P12 lineages. Mutations in *Pegl-1gfp* induce a doublet pattern of neurons in the midbody and an increase of neurons in the posterior expressing *egl-1*. (C) In *lin-39; Pegl-1(mut)gfp* animals, GFP is expressed in a triplet pattern in the midbody and most posterior lineages. (D) *efl-3(null); Pegl-1gfp* animals exhibit ectopic *egl-1* expression. Scale bars, 10 μm. Animals in B and C are homozygous for *ced-3*, and the animal in D is homozygous for *ced-1*.

induced additional neurons to express *egl-1* in *ced-3;* $P_{egl\text{-}1}$*gfp* animals, *efl-3(RNAi)* induced no change in expression in *ced-3;* $P_{egl\text{-}1(mut)}$*gfp* animals, suggesting that *efl-3* repression had been lost by removing $P_{egl\text{-}1}$'s E2F-binding sites (Figure 2A).

EFL-3 is a stronger repressor of egl-1 than LIN-39 in the midbody VA and VB neurons

In the midbody of $P_{egl\text{-}1}$*gfp* transgenic animals, no neurons express *egl-1*, but in the midbody of $P_{egl\text{-}1(mut)}$*gfp* animals, there are two neurons per lineage that expressed *egl-1* (Figure 5). We knew these to be VA and VB neurons on basis of differential interference contrast analysis. Furthermore, in *lin-39; ced-3;* $P_{egl\text{-}1(mut)}$*gfp* animals, we observed three cells per midbody lineage expressing *egl-1*—the VA, VB, and VC neurons (Figure 5). The pattern of *egl-1* expression in *lin-39; ced-3;* $P_{egl\text{-}1(mut)}$*gfp* animals was similar to the pattern observed in *lin-39; ced-3;* $P_{egl\text{-}1}$*gfp; efl-3(RNAi)* animals, but more consistent (see ranges of *gfp*-expressing neuons in supporting information, Table S1). One likely reason for the inconsistency of *egl-1* expression in *lin-39; ced-3;* $P_{egl\text{-}1}$*gfp; efl-3(RNAi)* animals is that *efl-3(RNAi)* provides incomplete knockdown of *efl-3*, as supported by the observation that *efl-3 (RNAi)* worms are viable, but deletion of alleles *efl-3—gk835* and *gk896*—are larval lethal.

ced-3; $P_{egl\text{-}1(mut)}$*gfp* animals (compared with *ced-3;* $P_{egl\text{-}1}$*gfp* animals) exhibited an increase of midbody neurons expressing *egl-1* independently of *lin-39*. Previously, we had shown that *lin-39* loss was required for *efl-3(RNAi)* to have an effect on *egl-1* expression in the midbody (Figure 2A). However, with E2F-binding sites removed, we created an essentially null condition in terms of EFL-3 binding $P_{egl\text{-}1}$*gfp*, indicating that, in the midbody VA and VB neurons, LIN-39 functions as a weak repressor and EFL-3 functions as a strong repressor of *egl-1*. That is, complete loss of LIN-39 (via a null allele) and partial loss of EFL-3 (via RNAi) is sufficient to derepress *egl-1*, while complete loss of EFL-3 alone (via $P_{egl\text{-}1}$ mutagenesis) is sufficient to derepress *egl-1* (Figure 2A).

One possible mechanism to explain the partial redundancy of LIN-39 and EFL-3 in the midbody VA and VB neurons is that, by abolishing E2F sites, we inadvertently disrupted one or more Hox sites as well, leading to derepression of *egl-1*. However, this hypothesis seems unlikely, as a previous study has shown that, of the 116 putative $P_{egl\text{-}1}$ Hox/Pbx-binding sites, LIN-39 is predicted to repress *egl-1* at multiple sites or indirectly by binding one or more $P_{egl\text{-}1}$-bound transcription factors (Potts *et al.* 2009).

A second hypothesis is that LIN-39 represses *egl-1* by forming a complex with EFL-3 bound to $P_{egl\text{-}1}$. Partial knockdown of EFL-3 (via RNAi) would lead to variable expression

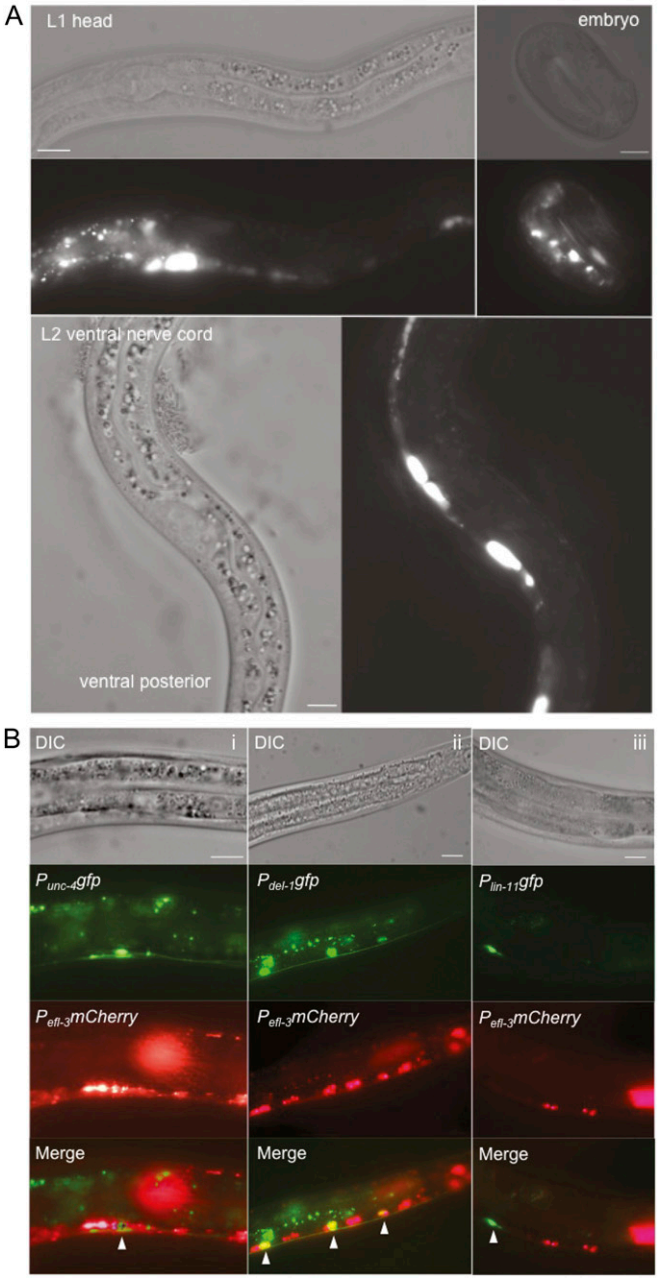

Figure 6 *efl-3* is expressed in a subset of cells during development. (A) *Pefl-3mCherry* is expressed throughout development in embryos, L1 animals (with highest expression in the head), and L2 animals (with highest expression in the head and ventral nerve cord). Three independent transgenic lines exhibit the same expression pattern. (B) *Pefl-3mCherry* is expressed in the (i) VA neurons and (ii) VB neurons. (iii) EFL-3 is not expressed in the VC neurons. Images are false-colored epifluorescence of L2–L3 animals. Scale bars, 10 μm.

of *egl-1* due to a minimal amount of EFL-3 being present to allow for inconsistent LIN-39/EFL-3 repression of *egl-1*, and elimination of E2F-binding sites would lead to a more predictable pattern of *egl-1* expression, which is what we observed (Figure 2 and Table S1).

To directly test whether complete loss of *efl-3* function is sufficient to cause ectopic expression of *egl-1*, we attempted to look at *efl-3(gk835); ced-3;* P_{egl-1}*gfp* animals. This experiment failed because the majority of these worms die as larvae before completing cell deaths in the ventral nerve cord. We found, however, that a rare few *ced-1*; *efl-3*; P_{egl-1}*gfp* animals are able to survive long enough to display a GFP pattern in the ventral nerve cord matching that of *ced-3;* $P_{egl-1(mut)}$*gfp* animals (Figure 5). (*ced-1* is necessary for the normal engulfment of cell corpses.) In *ced-1; efl-3;* P_{egl-1}*gfp* animals, similar to *ced-3;* $P_{egl-1(mut)}$*gfp* animals, two neurons per midbody lineage expressed *egl-1*, supporting the hypothesis that *efl-3* is a repressor of *egl-1* in the VA and VB neurons.

EFL-3 is expressed in the VA and VB motor neurons

EFL-3 is required in the VA and VB neurons to repress *egl-1* in a manner partially redundant with LIN-39, but loss of LIN-39 alone is sufficient to derepress *egl-1* in the VC neurons. Why is EFL-3 not required in the VC neurons to repress *egl-1*? One possibility is that EFL-3 is not present in the VC neurons. To determine the pattern of *efl-3* expression, we created a P_{efl-3}*mCherry* reporter. P_{efl-3}*mCherry* transgenic worms displayed expression beginning in multiple cells in embryos (Figure 6A). The expression continued throughout the life span of the worm, with maximal expression appearing in the L1–L2 larval stages. In addition, EFL-3 expression was observed consistently in head neurons from L1 to adult stage (Figure 6A).

In the ventral nerve cord, EFL-3 was most clearly expressed during the L2 stage. By crossing P_{efl-3}*mCherry* transgenic animals with integrated lines of VA (P_{unc-4})-, VB (P_{del-1})-, and VC (P_{lin-11})-specific GFP markers, we determined that EFL-3 is expressed in the VA and VB neurons but not in the VC neurons (Figure 6B). This finding provides insight as to why EFL-3 represses *egl-1* in the VA and VB but not in the VC neurons.

Discussion

Here we have examined how a newly identified E2F, *efl-3*, is able to regulate transcription of the *egl-1* cell death gene. We found that *efl-3* functions in the ventral nerve cord VA and VB cells to repress *egl-1* in a manner partially redundant with a Hox, *lin-39*, but that *efl-3* function is not required to repress *egl-1* in a third cell type, the VC neuron (Figure 7). In the posterior, where *lin-39* is not expressed, *efl-3* alone is sufficient to repress *egl-1*. Across the ventral nerve cord, *lin-39* provides information to developing lineages that tells them where they are located, and within each lineage, *efl-3* provides information to the developing VA and VB neurons that tells them how their fate should be different from that of the VC neurons. Thus, spatial information from *lin-39* Hox is integrated with lineage-specific information from *efl-3* at the site of the *egl-1* cell death gene to determine cell fate (Figure 7).

Our findings demonstrate a context in which two major developmental pathways—Hox and E2F—cooperatively regulate a target gene, *egl-1*. This interaction is dependent on context, with the transcription of *egl-1* being determined in a spatial- and cell type-specific manner. Although our studies

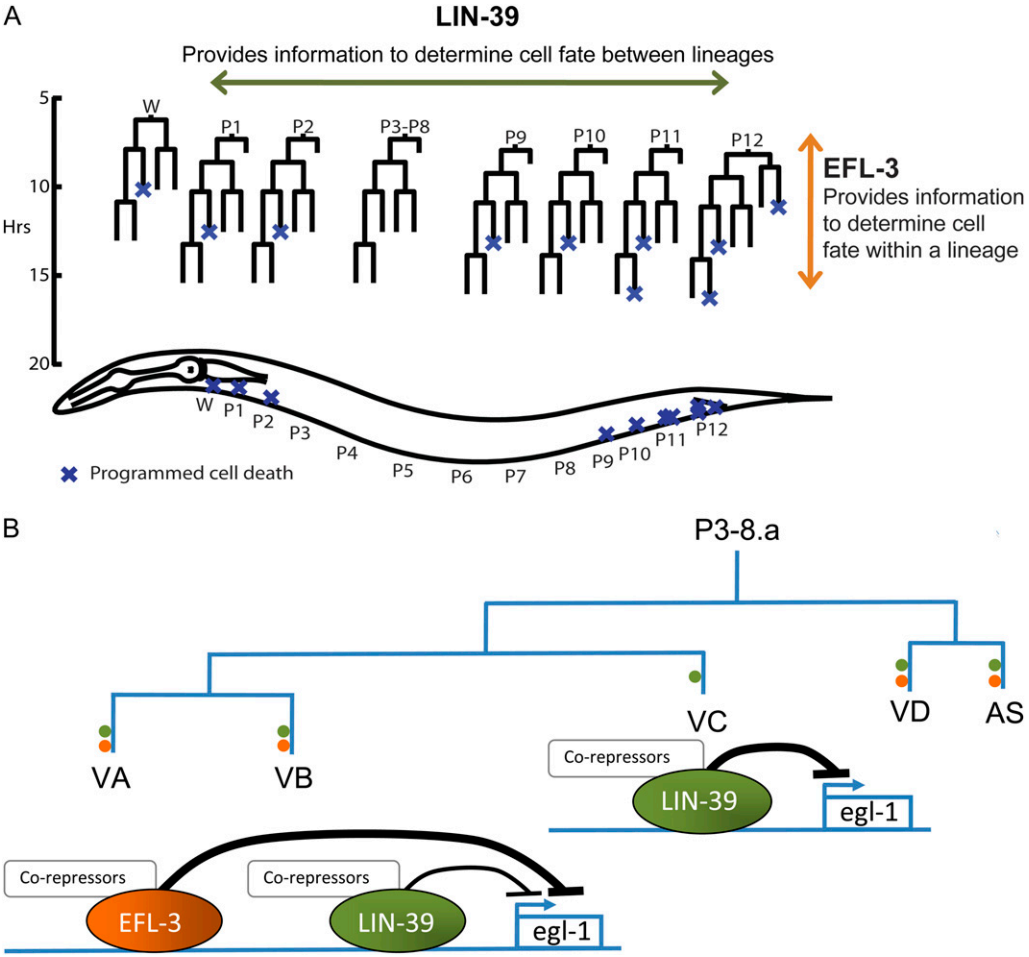

Figure 7 EFL-3 and LIN-39 interact to specify ventral nerve cord development. (A) LIN-39 provides spatial-specific information to developing lineages. EFL-3 provides information within each lineage to specify cell fate between the different cell types. (B) In the midbody P3–P8 lineages, EFL-3 and LIN-39 integrate their lineage and spatial-specific cues on the *egl-1* promoter. EFL-3 and LIN-39 co-repress *egl-1* in the VA and VB neurons, with LIN-39 functioning as a weaker repressor. In the VC neurons, LIN-39 is necessary to repress *egl-1*. EFL-3 is not expressed in the VC neurons and has no apparent affect on *egl-1* expression in the VCs. Green dots represent neurons that express *lin-39*; orange dots represent neurons that express *efl-3*.

examined how Hox and E2F interact within the limited setting of the *C. elegans* ventral nerve cord, it is possible that the mechanism of cell fate regulation by Hox and E2F is relevant across species. Hox proteins have previously demonstrated broad functional conservation, with *Drosophila* Hox proteins able to function in *C. elegans* (Hunter and Kenyon 1995) and vertebrate Hox proteins able to function in *Drosophila* (Malicki *et al.* 1990; McGinnis *et al.* 1990; Zhao *et al.* 1993; Lutz *et al.* 1996). In the E2F family, mechanistic similarities can also be drawn between species, particularly between mouse, *Drosophila*, and *C. elegans*, although precise functional comparisons are more difficult given the variety and redundancy of E2F family proteins and their extensive range of functions (Van Den Heuvel and Dyson 2008). For future studies, it would be interesting to determine how the E2F and Hox families may interact in different species and whether the cooperative action of the two families is limited to cell fate regulation, or if their interaction affects other developmental processes as well.

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Hox and a Newly Identified E2F Co-repress Cell Death in *Caenorhabditis elegans*

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Table S1 ***efl-3*** **directly represses** ***egl-1*** **in a context-specific manner with** ***lin-39***

	Midbody				Posterior				
Genotype	Average			Range	Average			Range	n
ced-3; $P_{egl\text{-}1}$gfp; unc-22(RNAi)	0.0	±	0.0	0	6.7	±	0.1	4-7	30
ced-3; $P_{egl\text{-}1}$gfp; efl-3(RNAi)	0.0	±	0.0	0	8.3	±	0.3	7-12	30
lin-39; ced-3; $P_{egl\text{-}1}$gfp; unc-22(RNAi)	6.0	±	0.0	6	6.5	±	0.1	5-7	31
lin-39; ced-3; $P_{egl\text{-}1}$gfp; efl-3(RNAi)	9.7	±	0.5	6-15	8.8	±	0.3	7-13	30
ced-3; $P_{egl\text{-}1(mut)}$gfp; unc-22(RNAi)	12.3	±	0.1	12-14	10.7	±	0.2	9-13	26
ced-3; $P_{egl\text{-}1(mut)}$gfp; efl-3(RNAi)	12.2	±	0.1	10-13	10.8	±	0.3	8-14	27
lin-39; ced-3; $P_{egl\text{-}1(mut)}$gfp; unc-22(RNAi)	16.5	±	0.5	13-19	12.5	±	0.3	11-15	17
lin-39; ced-3; $P_{egl\text{-}1(mut)}$gfp; efl-3(RNAi)	17.4	±	0.4	13-19	13.3	±	0.2	12-15	21
ced-3; $P_{egl\text{-}1}$gfp; efl-1	0.0	±	0.0	0	6.7	±	0.2	6-7	30
ced-3; $P_{egl\text{-}1}$gfp; efl-1(RNAi)	0.0	±	0.0	0	6.4	±	0.2	5-7	20
ced-3; $P_{egl\text{-}1}$gfp; efl-2(RNAi)	0.0	±	0.0	0	6.6	±	0.1	6-7	20
dpl-1; ced-3; $P_{egl\text{-}1}$gfp	0.0	±	0.0	0	6.7	±	0.1	6-7	30