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EMB-4: A Predicted ATPase That Facilitates *lin-12* Activity in *Caenorhabditis elegans*

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ABSTRACT

The *sel-6* gene was previously identified in a screen for suppressors of the egg-laying defect associated with hypermorphic alleles of *lin-12* (Tax *et al.* 1997). Here we show that *sel-6* and two other previously defined genes, *mal-2* and *emb-4*, are the same gene, now called "*emb-4*." We perform a genetic and molecular characterization of *emb-4* and show that it functions cell autonomously as a positive regulator of *lin-12* activity. Viable alleles identified as suppressors of *lin-12* are partial loss-of-function mutations, whereas the null phenotype encompasses a range of lethal terminal phenotypes that apparently are not related to loss of *lin-12/Notch* signaling. *emb-4* encodes a large nuclearly localized protein containing a predicted ATPase domain and has apparent orthologs in fission yeast, plants, and animals.

RECEPTORS of the LIN-12/Notch family mediate cell–cell interactions that specify cell fate during animal development and have an unusual mechanism of signal transduction (Greenwald 2005). LIN-12/Notch proteins are type I transmembrane proteins that undergo modifications upon their transit to the cell surface. Binding of a protein of the Delta-Serrate-LAG-2 family triggers proteolytic cleavage in the extracellular domain, resulting in ectodomain shedding. This extracellular cleavage is mediated by a protease of the ADAM family and creates a truncated form with a short extracellular domain. This truncated form serves as the substrate for a protease complex that includes presenilin, which cleaves within the transmembrane domain. Transmembrane cleavage releases the intracellular domain, which translocates to the nucleus and forms a transcriptional activation complex with the sequence-specific DNA-binding protein LAG-1 (Suppressor of Hairless in Drosophila and CBF1/RBP-J in mammals) and other cofactors. Thus, LIN-12/Notch proteins are essentially membrane-tethered transcription factors that are released upon ligand binding.

The identification of *trans*-acting factors that constitute the core machinery of signal transduction or modulate the activity of the signaling pathway has been fruitfully approached using genetics in *Caenorhabditis elegans*. The main approach has been to isolate extragenic suppressors of phenotypes caused by elevated or reduced *lin-12* activity. Genes thereby defined—many named *sel* genes, for ***s**uppressor/**e**nhancer of **l**in-12*—include those that encode proteases involved in cleaving LIN-12/Notch, components of the transcription activation complex, and factors that affect LIN-12 stability or trafficking. Many of these proteins are conserved and play similar roles in other animals (reviewed in Greenwald 2005).

The *sel-6* gene was first identified in a screen for suppressors of a constitutively active allele of *lin-12* (Tax *et al.* 1997). Our additional genetic analysis indicates that *sel-6* functions as a positive regulator of *lin-12* activity. The alleles of *sel-6* that were recovered in screens for *sel* genes appear to be hypomorphic. During the course of investigating *sel-6,* we found that alleles had been recovered in two independent screens for maternal-effect essential genes and had been assigned to two genes, *emb-4* (Miwa *et al.* 1980; Schierenberg *et al.* 1980) and *mal-2* (Hekimi *et al.* 1995). As *emb-4* was the first published name, it has become the official name of this gene. We show that *emb-4* encodes a protein with a putative ATPase domain with apparent orthologs in higher eukaryotes and that it affects *lin-12* activity in a cell-autonomous manner. We discuss possible mechanisms by which *emb-4* may influence *lin-12* activity. In an accompanying article in this issue, Checchi and Kelly (2006) discuss the role of *emb-4* in a mechanism that keeps germ cell precursors transcriptionally quiescent.

MATERIALS AND METHODS

Strains: Experiments were performed at 20° unless indicated otherwise. The wild-type parent for all strains was *C. elegans* var. Bristol N2 (Brenner 1974), except for mapping experiments using GS3063, a hybrid strain in which *lin-12(n302)* was placed into the Hawaiian strain CB4856 background by six

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backcrosses (data not shown). GS3063 [*lin-12(n302); him-5(e1467)*] has all the single-nucleotide polymorphisms (SNPs) that we tested on LG V.

The main alleles used in this study are the following: LG III, *lin-12(n302), lin-12(n950), lin-12(n379), lin-12(n941)* (Greenwald *et al.* 1983; Greenwald and Seydoux 1990), *lin-12(ar170)* (Hubbard *et al.* 1996), and *glp-1(ar202)* (Pepper *et al.* 2003); and LG V, *sel-6(sa44, n1256*) (Tax *et al.* 1997), *mal-2(qm31)* (Hekimi *et al.* 1995), and *emb-4(hc60)* (Miwa *et al.* 1980). Additional information about these alleles and markers used for mapping and for facilitating genetic analysis can be found via Wormbase at http://www.wormbase.org.

arIs53[lin-12(ΔE)::gfp] expresses a GFP-tagged version of LIN-12 lacking most of its extracellular domain, a constitutively active form of the receptor (Shaye and Greenwald 2005).

We used transgene *arIs51[cdh-3::gfp]* to facilitate visualization of anchor cells (Karp and Greenwald 2003).

Mutant analysis and scoring: For scoring the egg-laying defective (Egl) phenotype, L4 larvae were picked onto separate plates and scored 2 days later. An animal was scored as non-Egl if it laid eggs and as Egl if it did not lay eggs or if it formed a bag of worms. The egg-laying potential of sterile animals could not be assessed.

To assess the number of anchor cells (ACs), L3 larvae were scored by Nomarski optics and ACs were identified by morphology and GFP expression using the marker *arIs51[cdh-3::gfp]*. For the Multivulva (Muv) phenotype, L4 larvae were generally picked onto separate plates and checked for the number of pseudovulvae the next day.

For germline proliferation, L1 larvae of hermaphrodites carrying *glp-1(ar202)* were transferred to individual plates at 25° and scored for progeny production.

Fine-scale genetic mapping of *sel-6(sa44)*: We used GS3063, a *lin-12(n302)*-containing derivative of the Hawaiian strain CB4856, for further mapping to facilitate recombinant analysis.

lin-12(n302); sel-6(sa44) unc-51(e369) rol-9(sc148) hermaphrodites were mated with GS3063 males and non-Unc, non-Rol F_2's were recovered. Homozygosity for *sel-6* was determined by cloning 10 F_3 progeny from each recombinant candidate and checking that all segregated egg-laying-competent animals. A total of 78 Sel non-Unc non-Rol recombinants were obtained. Of the 78 recombinants, 4 placed *sel-6* to the left of a newly identified C-to-T SNP, ik1, between genes Y80D3A.10 and Y80D3A.5 (using the primer pair ik1F: 5′-CACAGGTGC TCAAAATCACAA-3′ and ik1R:5′-TGGCGCAATTCTCGATG TTGC-3′). This SNP introduces an *Xba*I restriction site.

GS3063 males were mated with *lin-12(n302); dpy-21(e428) sel-6(sa44)* hermaphrodites. Non-Dpy F_2's were picked from F_1 cross-progeny. We assessed the presence and homozygosity of *sel-6(sa44)* as above. A total of 79 Sel non-Dpy recombinants were obtained. Of the 79 recombinants, 1 placed *sel-6* to the right of a newly identified C-to-A SNP, ik2, between genes Y17D7A.2 and Y80D3A.1 (using the primer pair ik2F: 5′-AAA CCAGGAAAGGGGCGGAGC-3′ and ik2R: 5′-AGAGCATCGC GAAAAAGGTGG-3′). This SNP introduces a *Dra*I restriction site.

Combined SNP mapping data delineate the region of *sel-6* between the predicted genes Y17D7A.2 and Y80D3A.5, containing five predicted genes.

Phenocopy of *lin-12(n302)* suppression by RNA-mediated interference against predicted genes in the *sel-6* interval and sequencing: We constructed plasmids expressing double-stranded RNA (dsRNA) in the vector pPD129.36 (Timmons *et al.* 2001), corresponding to four of the five genes in the *sel-6* mapping interval (for construct details, see below). These constructs were transformed into bacterial strain HT115 (Timmons and Fire 1998; Timmons *et al.* 2001). *lin-12(n302)* adults were placed on lawns of such bacteria and their progeny were scored for egg-laying ability. Some eggs were observed upon feeding with pIK21 (corresponding to Y80D3A.2) and pIK24 (corresponding to Y80D3A.3). Y80D3A.3 is contained within intron 12 of Y80D3A.2. These two predicted genes were sequenced using DNA from *sel-6(sa44)* and *sel-6(n1256)* backgrounds. Single-base-pair changes were found within the coding region of Y80D3A.2 in both cases. The PCR product obtained using one pair of primers, Y80D3A.2(7F) (5′-TAGTCCTAAGCTCTCCAGGTG-3′) and Y80D3A.2(7R) (5′-AAAATCCTCAATTTTCCGGGG-3′), contains a G-to-A mutation in *sel-6(sa44)* (G884R). The PCR product obtained using a different pair of primers, Y80D3A.2(10F) (5′-AAATTTTAGAGTTTTTCGACG-3′) and Y80D3A.2(10R) (5′-CCAAATTTCAGCAAATTCAGC-3′), contains a C-to-T mutation in *sel-6(n1256)* (T1040I). *sa44* is in exon 10, and *n1256* is in exon 13 of Y80D3A.2. Y80D3A.4 and Y80D3A.10 were eventually also sequenced to confirm that they did not contain mutations in *sel-6* mutant strains. Two additional noncomplementing mutations have been sequenced and found to be in exons of Y80D3A.2 (P. Checchi, personal communication).

cDNA analysis: yk1132d03 was the only cDNA available that appeared to be full-length. It meets the following criteria for a full-length cDNA: (1) it starts from the consensus 3′ splice site for *cis*- and *trans*-splicing; (2) a partial spliced-leader sequence is present, but it is not possible to determine whether it is SL1, SL2, or another variant; (3) the ATG translation start site is 8 bp downstream from the 3′ splice site and is preceded by two A residues; and (4) the cDNA ends in a poly(A) sequence. All exon junctions were examined in terms of their genomic context and none appear to result from amplification mistakes. Our analysis does not exclude the possibility that there are alternative splice isoforms, but we found no evidence of it in the available cDNA clones. The full-length SEL-6 protein has 1467 amino acids encoded by 19 exons.

RNA interference assays: We assessed whether the null phenotype of *sel-6* was indeed embryonic and larval lethality as reported by Kamath *et al.* (2003). We used the two protocols below; only protocol II yielded a high percentage of lethality.

Protocol I: Either three to five young N2 adults or ~100 bleached eggs were placed on lawns of bacteria expressing the desired RNA interference (RNAi) constructs against *sel-6* and controls at 20°, room temperature, or 25°. The progeny of these worms were observed for occurrence of any embryonic or larval lethality.

Protocol II: Between 10 and 15 L3–L4 N2 worms were placed on lawns of bacteria expressing the desired RNAi constructs against *sel-6* and controls and left at 15° for 72 hr. Three animals, now adults, were removed and placed onto individual plates and allowed to lay eggs for 24 hr at 20°, room temperature, or 25°. The three worms were then removed. The phenotypes of worms that hatched from these eggs (F_1) as well as their progeny (F_2) were then examined for embryonic and larval lethality (Kamath *et al.* 2003).

Complementation tests with *emb-4(hc60)* and *mal-2(qm31)* and sequencing the *mal-2(qm31)* allele: The *emb-4* or *mal-2* mutant phenotypes include lethality and ectopic growths reminiscent of those observed upon *sel-6* RNAi (Cassada *et al.* 1981; Hekimi *et al.* 1995). We performed complementation tests as follows: *emb-4(hc60)* progeny of *emb-4(hc60)* segregate 100% dead eggs at 25°. *emb-4(hc60)/sel-6(sa44) unc-51(e369)* were allowed to lay eggs at 15°. A total of 60 of their non-Unc progeny were picked to individual plates as L4 larvae, switched to 25°, and scored for segregation of live progeny. About one-third of these worms segregated only dead eggs; the rest segregated both Unc and non-Unc live worms and occasional dead eggs. *emb-4(hc60)/unc-51(e369)* were scored in parallel as a negative control and showed the same segregation patterns. *sel-6(sa44)* thus complements the maternal-effect lethality of *emb-4(hc60).*

mal-2(qm31) self-progeny of *mal-2(qm31)* mothers segregate a high percentage of embryonic and larval lethals at 20°. However, we observed no difference in lethality between progeny of *mal-2(qm31)/sel-6(sa44)* and *mal-2(qm31)/+* animals (4/231 eggs laid failed to hatch *vs.* 2/420). This indicates complementation between *sel-6(sa44)* and *mal-2(qm31)* for maternal-effect embryonic lethality.

We sequenced the predicted coding region of Y80D3A.2 in the *mal-2(qm31)* background. PCR product using the primer pair Y80D3A.2(10F) (5′-AAATTTTAGAGTTTTTCGACG-3′) and Y80D3A.2(10R) (5′-CCAAATTTCAGCAAATTCAGC-3′) contains a C-to-T mutation in *mal-2(qm31)* (R1137STOP).

Plasmid construction: For RNAi constructs corresponding to four genes in the *sel-6* interval, a PCR product was amplified from N2 genomic DNA within each gene, TOPO-cloned, and then subcloned between *Xho*I and *Sac*I sites (for pIK21–pIK23) or *Eco*RV sites (for pIK24) of pPD129.36. The following PCR primers were used: for Y80D3A.2, pIK21F (5′-AGTACTTGG AGGCCTATTTATGG-3′) and pIK21R (5′-GCTCCAAACTGT TCAAAACTCG-3′); for Y80D3A.4, pIK22F (5′-GATCACCCC ATTCTCTTCTTC-3′) and pIK22R (5′-TTATAGCACCGTTC CAGACG-3′); for Y80D3A.10, pIK23F (5′-GAGAGTGCAAGT AGTCACAC-3′) and pIK23R (5′-CAATTTTGGGTCTCACCA CG-3′); and for Y80D3A3.3, pIK24F (5′-GCAATTAACAGCTC CAAGAAG-3′) and pIK24R (5′-AACATGCTTCTGGTCCTCC TC-3′). The RNAi construct containing the full-length cDNA of Y80D3A.2 (pIK25) was obtained by digesting the yk1132d03 cDNA plasmid with *Eco*0109I and *Xba*I and ligating the insert into the corresponding sites in the vector pPD129.36 (Timmons *et al.* 2001).

The vector PIN2 (D. Levitan and I. Greenwald, unpublished observations) drives inserted sequences under the control of *sel-12* regulatory sequences. It contains a unique *Not*I site inserted at the second amino acid of a *sel-12*-rescuing genomic fragment. To make pIK33, PIN2::*emb-4*cDNA, we first inserted a *Not*I site in front of the ATG of the cDNA by amplifying a PCR product from the cDNA using the following pair of primers: NruF (5′-CAACAAGCGGCCGCT**ATG**GTGACTAAA CGTCATCAAG-3′, where the *Not*I site is underlined and the ATG of *emb-4* is in boldface type) and NruR (5′-CAATCTAGA CGCTCTTCCTGCAACGAAAGCTGC-3′, with the added *Xba*I site underlined). This piece was cloned into the *Not*I and *Xba*I sites of pPD129.36. The resulting plasmid was then cut with *Nru*I and a *Nru*I fragment from pIK25 was subcloned into it, yielding pIK30. pIK32 was constructed by ligating the *Rsr*II/*Xba*I fragment of pIK25 into pIK30. This *Xba*I site is 3′ to the second *Not*I site in IK31 that follows the *emb-4* cDNA poly(A) sequence. pIK33 was finally obtained by cloning the *Not*I insert containing the entire *emb-4* cDNA into the *Not*I site of PIN2. All joints and correct orientation of the cDNA were confirmed by sequencing.

To construct pIK56, *lin-31p::gfp::emb-4*cDNA, we first used the PCR fusion approach to generate the N-terminally tagged EMB-4 (Hobert 2002) using a KSGFPS65T vector and a vector containing the *emb-4* cDNA subcloned into *Not*I sites. The relevant primers were A*big (5′-CAACAAGCGGCCGCAAAAATG AGTAAAGGAGAAGAAC-3′), Bbig (5′-CTTGATGACGTTTAG TCACCATTTTGTATAGTTCATCCATGC-3′), and D*big (5′-TACTTCATTGGGCTCACAAGCGGC-3′), where the *Not*I site is underlined. The PCR product was TOPO cloned and subsequently subcloned into the *Not*I sites of the plasmid pB253 (Tan *et al.* 1998).

Worm transformation and rescue experiments: DNA was micro-injected into the germline of *C. elegans*. pIK33, PIN2::*emb-4*cDNA, was injected at different concentrations together with the co-injection marker *ceh-22::gfp*. Many different combinations of micro-injection mixes and recipient strains yielded only eight lines. Lines were obtained only when the concentration of pIK33 was no >2.5 ng/μl. For rescue experiments, the transgenes were placed into appropriate genetic backgrounds. Egg laying was scored in the *lin-12(n302); emb-4(sa44)* background and the number of pseudovulvae was scored in the *arIs53[lin-12(ΔE)::gfp]; emb-4(sa44)* background. No rescue was observed in either background with any of the transgenic arrays. All of the arrays were transmitted at a very low frequency, suggesting that overexpression of *emb-4* may be toxic to animals.

Subcellular localization: pIK56, *lin-31*p*::gfp::emb-4*cDNA, was micro-injected into the germline of *C. elegans* at 20 ng/μl together with the co-injection marker *ceh-22::gfp*. Three lines were obtained, but no GFP expression was observed. Two lines were then analyzed by immunofluorescence. For immunofluorescence, transgenic animals were fixed according to the modified Finney–Ruvkun procedure and stained with a polyclonal rabbit anti-GFP antibody (Molecular Probes, Eugene, OR) at a 1:300 dilution and a mouse anti-MH27 (Priess and Hirsh 1986) at a 1:10 dilution in antibody buffer A. Secondary antibodies used were goat-anti-rabbit conjugated to Cy2, and goat-anti-mouse conjugated to Cy3, both at 1:300 (Jackson ImmunoResearch, West Grove, PA, nos. 111225144 and 115165146, respectively). The stained animals were mounted onto an agarose pad with a drop of SlowFade (Molecular Probes, no. S-7461) and visualized with a Zeiss Axio Imager Z1 microscope.

Laser microsurgery: The nucleus of Z4 was ablated with a laser microbeam in newly hatched L1 larvae. Success of ablations was confirmed and the presence of an AC was scored during the late L3 stage.

RESULTS

Molecular identification of *sel-6*: *sel-6* had been mapped previously to the right arm of LG V (Tax *et al.* 1997). We mapped the reference allele *sel-6(sa44)* to a region containing five predicted genes (Figure 1; see materials and methods). We provisionally identified *Y80D3A.2* as *sel-6* by feeding RNAi (Timmons *et al.* 2001) using protocol I (see materials and methods) on the basis of the presumption that loss of gene activity would confer a Sel phenotype, *i.e.*, suppression of the 0 AC–Egl defect of *lin-12(n302)*.

We did not perform a conventional rescue experiment, as the genomic region is incompletely covered by cosmids and *Y80D3A.2* is the second gene in the predicted three-gene operon that spans 45 kb. However, when we sequenced the exons of *Y80D3A.2* from *sel-6(sa44)* and *sel-6(n1256)* mutants, we observed single-base-pair changes within the coding region of this gene, corroborating the RNAi evidence that *Y80D3A.2* is indeed *sel-6* (Figure 1). The sequence of putative null, noncomplementing mutations provides further corroboration. We will describe features of the predicted protein product below.

***sel-6*, *mal-2*, and *emb-4* are a single gene:** When N2 hermaphrodites were fed using protocol II with bacteria expressing double-stranded RNA corresponding to the full-length *Y80D3A.2* cDNA, only 8% (8/96) hatching was observed, as compared to 98% (138/141) observed

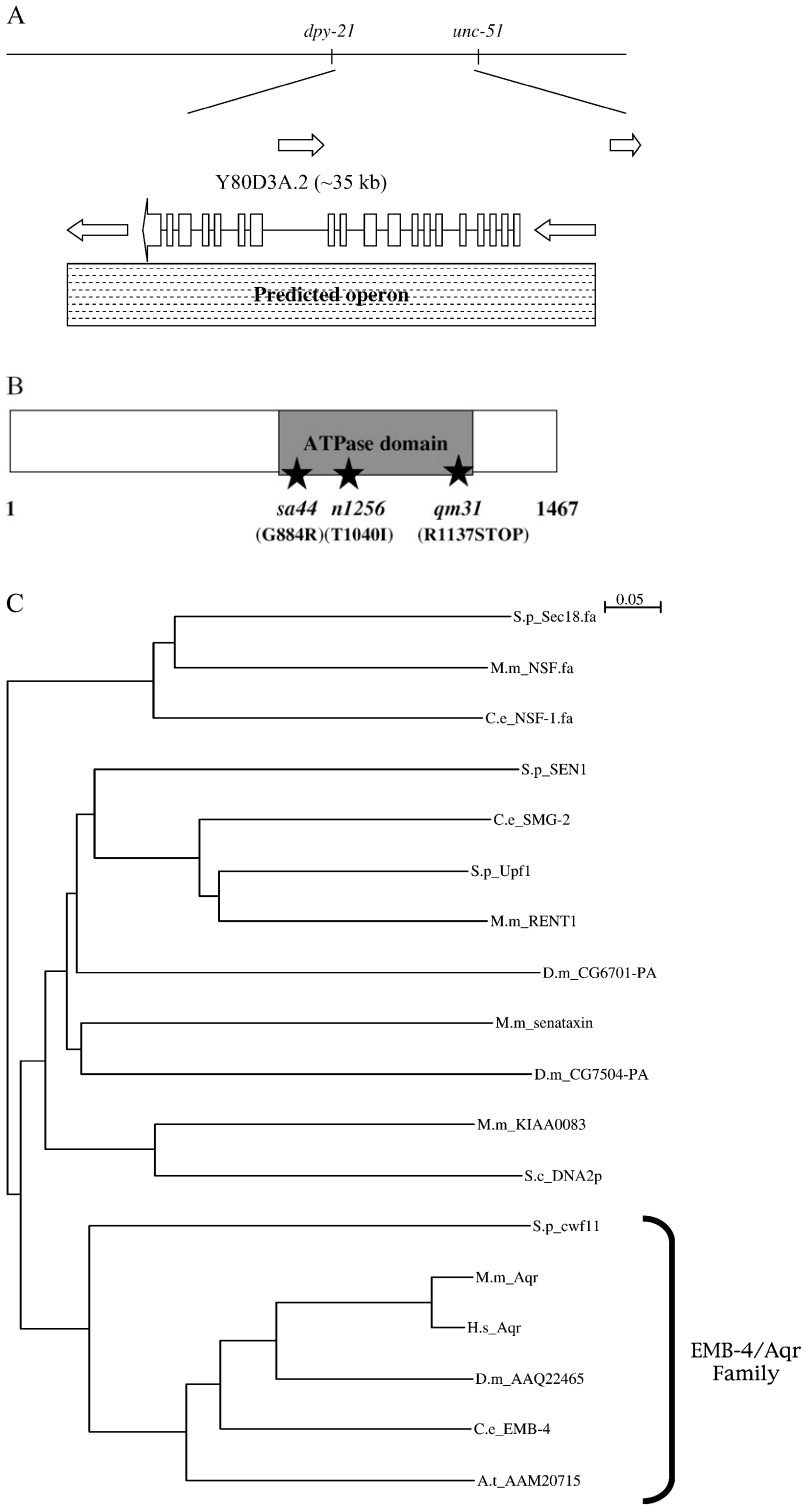

FIGURE 1.—Molecular cloning and DNA sequence analysis of *emb-4*. (A) Mapping and cloning of *emb-4*. *emb-4* was genetically mapped to the region including the five genes depicted schematically. Y80D3A.2, the second gene in the predicted operon, is *emb-4*. (B) Schematic of the EMB-4 protein. The ATPase domain has similarity to DEAD-box helicases and AAA ATPases. (C) Phylogenetic analysis. The predicted *C. elegans* EMB-4 sequence was compared to predicted proteins in *Saccharomyces cerevisiae*, *S. pombe*, *A. thaliana*, *C. elegans*, *Mus musculus* and *Homo sapiens* using NCBI BLAST (http://www.ncbi.nih.gov/BLAST/) (ALTSCHUL *et al.* 1997; SCHAEFFER *et al.* 2001). Sequences showing significant alignment were downloaded in FastA format, and the labels for each protein were modified to include a code for species and sequence identifier. Many duplicate entries were eliminated by identifying identical sequences. ClustalX was then used to align the sequences. The orthologs of EMB-4 shown are *S. pombe* cwf11p (SPBC646), *A. thaliana* AAM20715, *Drosophila melanogaster* AAQ22465, *M. musculus* Aquarius (BAC 65592), and *H. sapiens* Aquarius (BAA25486). *N*-ethylmaleimide-sensitive factor family proteins were included as outliers because of their distant similarity to the apparent EMB-4 orthologs.

with an empty vector construct. Embryonic lethality was also observed upon dsRNA injection (data not shown). *Y80D3A.2(RNAi)* viable escapers often displayed unusual outgrowths on the head or the tail of the larvae. The lethality and the presence of ectopic bumps in escapers were reminiscent of mutants in a previously described but molecularly unidentified gene in the region, *mal-2*, which was recovered in a screen for *m*aternal-effect morphologically *a*bnorma*l* mutants (HEKIMI *et al.* 1995). When we sequenced the *Y80D3A.2* coding region of the *mal-2* canonical allele *qm31*, we observed a single-base-pair change in exon 13 coding for a stop within the protein, establishing *sel-6* and *mal-2* as the same gene. Subsequently, Paula Checchi and William Kelly identified *emb-4(hc60)* as having a stop mutation in exon 5 of *Y80D3A.2*, demonstrating that *emb-4* also corresponds to

this gene (see Checchi and Kelly 2006, accompanying article in this issue).

Alleles of *mal-2* and *emb-4* were identified in different screens (Miwa *et al.* 1980; Hekimi *et al.* 1995), but the three alleles [*mal-2(qm31, qm35)* and *emb-4(hc60)*] exhibit completely penetrant maternal-effect embryonic lethality at 25°, as well as maternal-effect embryonic and larval lethality and morphological abnormalities at 20° (Miwa *et al.* 1980; Hekimi *et al.* 1995). As three alleles are associated with stop codons in Y80D3A.2 and have completely penetrant and similar phenotypes, we infer that they are all null alleles. We will refer to Y80D3A.2 as *emb-4*, the first published name of this gene, for the remainder of this article.

The alleles isolated as suppressors of *lin-12(n302)* are associated with missense mutations and are fully viable. In addition, the suppressor mutation *emb-4(sa44)* complements the maternal-effect lethality of the null alleles *qm31* and *hc60* (data not shown; materials and methods). In genetic studies described below, we refer to the viable allele *emb-4(sa44)* as *emb-4(sel)* and to the two null alleles *emb-4(qm31)* and *emb-4(hc60)* as *emb-4(0)*.

***emb-4* interactions with *lin-12* and *glp-1*:** We examined genetic interactions between *emb-4* and *lin-12* or the *lin-12* paralog *glp-1*, as well as the phenotype of viable *emb-4(0)* escapers. Our analysis, together with previous studies by Tax *et al.* (1997), suggests that *emb-4* is a positive regulator, but is not absolutely required for *lin-12/Notch* activity in *C. elegans*.

The AC/ventral uterine precursor cell decision: In wild-type hermaphrodites, the gonadal cells Z1.ppp and Z4.aaa interact so that one becomes the AC and the other becomes a ventral uterine (VU) precursor cell (reviewed in Greenwald 2005). In mutants with elevated *lin-12* activity, both cells become VUs, whereas loss of *lin-12* activity causes both cells to become ACs. When *lin-12* activity is elevated, the absence of an AC prevents the formation of a vulva, causing an Egl phenotype that is easy to score in the dissecting microscope. The *emb-4(sel)* alleles were identified as suppressors of the 0 AC–Egl phenotype (Tax *et al.* 1997).

The AC/VU decision proceeds normally in *emb-4* null mutant animals: 33/33 *emb-4(qm31)* hermaphrodites raised at 20° have 1 AC, and 15/15 eggs shifted to 25° 1 hr postlaying gave rise to animals that have 1 AC. However, *emb-4* appears to be a positive regulator of *lin-12* activity in the AC/VU decision, as *emb-4(0)* alleles can suppress the 0 AC–Egl phenotype associated with *lin-12(n302)* (Table 1). An *emb-4(0)* allele does not suppress the 0 AC–Egl phenotype caused by two copies of a stronger *lin-12(d)* allele, *lin-12(n950)*, but suppresses the 0 AC–Egl phenotype caused by one copy, in *lin-12(n950)/lin-12(0)* animals (Table 1). In addition, *emb-4(sel)* enhances the 2AC defect of *lin-12(ar170)*, a hypomorphic allele: 17/70 (25%) *lin-12(ar170)* hermaphrodites form two anchor cells, but the penetrance of that defect rises to 65% (22/34) in *lin-12(ar170); emb-4(sel)* animals. These results indicate that suppression is not allele specific, but depends on the level of *lin-12* activity.

Vulval precursor cell specification: LIN-12, together with other signaling pathways, specifies the fates of P3.p–P8.p, the six vulval precursor cells (VPCs) (reviewed in Sternberg 2005). In wild-type hermaphrodites, only P5.p and P7.p adopt the vulval 2° fate. Strong hypermorphic *lin-12* alleles such as *lin-12(n950)* cause all six VPCs adopt the 2° fate, resulting in a Muv phenotype. *emb-4(sel)* and *emb-4(0)* do not appear to suppress the Muv phenotype caused by *lin-12(n950)* (Table 2; Tax *et al.* 1997), but are able to suppress *lin-12(n950)/lin-12(0)* (Table 2). *emb-4(sel)* and *emb-4(0)* alleles are also able to suppress the Muv phenotype associated with *arIs53* [*lin-12*(ΔE)::gfp], a constitutively active form of LIN-12 (Shaye and Greenwald 2005) (materials and methods; data not shown). These observations suggest that *emb-4* also acts as a positive regulator of *lin-12* activity in VPC specification.

It is notable that the *emb-4(0)* alleles suppress both the 0 AC–Egl and multivulva defects of *lin-12(d)* alleles to a lesser degree than the original *sel* mutations (Tables 1 and 3). The molecular lesions and the fact that *emb-4(sel)* alleles lack, and complement, the embryonic phenotypes characteristic of *emb-4(0)* alleles are consistent with these alleles being non-null and affecting only a subset of the activities of the protein. As *emb-4(sel)* alleles *sa44* and *n1256* are stronger suppressors than *emb-4(0)* alleles *qm31* and *hc60* and are weakly semidominant, they may be dominant negative in action.

Embryonic cell fate specification: Animals that lack zygotic activity of both *lin-12* and its paralog, *glp-1*, arrest development in the L1 stage with a Lag phenotype, one hallmark of which is the absence of a rectum (Lambie and Kimble 1991). All *emb-4(0)* mutant larvae that we examined have a rectum [122/122 *emb-4(hc60)* larvae], indicating that, while playing a positive role, *emb-4* is not essential for *lin-12/Notch* activity.

Development of the germline: Loss of *glp-1* activity causes germline precursors to cease mitotic division and to enter meiosis prematurely, resulting in sterility. *emb-4(sel)* alleles enhance sterility caused by *glp-1* loss of function (Tax *et al.* 1997), suggesting that *emb-4* facilitates *glp-1* activity in the germline. Constitutive *glp-1* activity, as caused by the *glp-1(ar202)* missense mutation at 25° (Pepper *et al.* 2003), also causes sterility, caused by excessive proliferation at the expense of meiosis. However, the *emb-4(sel)* allele *sa44* does not suppress the sterility caused by *glp-1(ar202)*: 100% (82/82) of *glp-1(ar202); emb-4(sel)* hermaphrodites are sterile at 25°, similar to control animals (80/80 sterile). We could not assess suppression of *glp-1(ar202)* by *emb-4(0)* because of its lethal null phenotype at 25°.

Cell autonomy of *emb-4* functions as a suppressor of *lin-12(d)*: In wild-type hermaphrodites, when a laser microbeam is used to ablate Z4 (the precursor of Z4.aaa), Z1.ppp always becomes an AC, because the cellular

TABLE 1

***emb-4* suppresses the 0AC-Egl defect caused by *lin-12(d)* alleles**

Genotype	Egg-laying competent (non-Egl)/total (%)
A. *emb-4* mutations reduce the activity of *lin-12(d)* alleles in the AC/VU decision	
N2	>1000 scored (100)
lin-12(n302)	>1000 scored (0)
lin-12(n302); emb-4(sel)	88/104 (84)
lin-12(n302); emb-4(qm31)[a]	6/41 (15)
lin-12(n302); emb-4(hc60)	6/118 (5)
lin-12(n950)	>1000 scored (0)
lin-12(n950); emb-4(sel)	92/218 (42)
lin-12(n950); emb-4(qm31)	0/65 (0)
lin-12(n950)/lin-12(0)[b]	44/143 (31)
lin-12(n950)/lin-12(0); emb-4(sel)[b]	77/77 (100)
lin-12(n950)/lin-12(0); emb-4(qm31)[b]	37/63 (59)
B. *emb-4(0)* alleles fail to complement *emb-4(sel)* for suppression of *lin-12(n302)* 0AC-Egl defect	
lin-12(n302)	>1000 scored (0)
lin-12(n302); emb-4(sel)/+	1/39 (3)
lin-12(n302); emb-4(qm31)/+	0/17 (0)
lin-12(n302); emb-4(sel)/emb-4(qm31)	34/42 (81)
lin-12(n302); emb-4(hc60)/+	0/18 (0)
lin-12(n302); emb-4(sel)/emb-4(hc60)	10/13 (77)

[a] Of 107 animals, 41 had living progeny; the rest were sterile.
[b] Actual genotype on chromosome III is *lin-12(n950)/unc-36(e251) lin-12(n941)*.

source of the ligand for LIN-12, Z4.aaa, has been removed (Kimble 1981; Seydoux and Greenwald 1989). However, when Z4 is ablated in *lin-12(n302)* hermaphrodites, Z1.ppp adopts the VU fate, suggesting that LIN-12(n302) is constitutively active in the absence of ligand (Table 3; Greenwald and Seydoux 1990). We have found that when Z4 is ablated in *lin-12(n302); emb-4(sel)* hermaphrodites, Z1.ppp becomes an AC (Table 3), suggesting that LIN-12(n302) constitutive activity has been lowered. We infer that *emb-4* acts cell autonomously to facilitate *lin-12* activity in the presumptive VU.

EMB-4 is highly conserved and contains a predicted ATPase domain: The predicted EMB-4 protein, based on the apparent full-length clone yk1132, contains 1467 amino acids. We analyzed the predicted protein using two programs, SMART (http://smart.embl-heidelberg.de/) (Schultz *et al.* 1998; Letunic *et al.* 2006) and BLAST (http://www.ncbi.nih.gov/BLAST/) (Altschul *et al.* 1997; Schaeffer *et al.* 2001), and found that EMB-4 shows similarity to ATPases, particularly to helicases of the DEAD-box family (Rocak and Linder 2004). These helicases are characterized by the presence of Walker A- and B-type motifs used for ATP binding, as well as other, family-specific regions of conservation. It is unclear whether EMB-4 is a *bona fide* member of this family, as it shows homology to only the N-terminal 57% of the

TABLE 2

***emb-4* affects *lin-12* activity in VPC specification**

Genotype	Average no. of pseudovulvae (*n*)	
	non-Egl animals	Egl animals
lin-12(n950)		4.7 ± 0.1 (50)
lin-12(n950); emb-4(sel)[a]		4.5 ± 0.2 (28)
lin-12(n950); emb-4(qm31)[a]		4.3 ± 0.1 (65)
lin-12(n950)/lin-12(0)[b]	2.6 ± 0.1 (44)	4.2 ± 0.1 (99)
lin-12(n950)/lin-12(0); emb-4(sel)[b]	0 ± 0.0 (77)	NA[c]
lin-12(n950)/lin-12(0); emb-4(qm31)[b]	0.7 ± 0.2 (37)	2.2 ± 0.2 (26)

The strains compared were grown and scored in parallel.
[a] Only 0 AC-Egl animals were scored to account only for the role of *emb-4* during the VPC specification, and not as a secondary consequence of the AC/VU decision (the presence of an AC decreases the number of pseudovulvae).
[b] Actual genotype on chromosome III was *lin-12(n950)/unc-36(e251) lin-12(n941)*.
[c] All *lin-12(n950)/lin-12(0); emb-4(sel)* animals were able to lay eggs.

TABLE 3

Cell autonomy of EMB-4 function in the AC/VU decision

Genotype	*n*[a]	Fate of Z1.ppp
lin-12(n302)	4/4 + 11/11[b]	VU
lin-12(n302); emb-4(sel)	5/5	AC

The nucleus of Z4 was ablated with a laser microbeam in newly hatched L1 larvae of *lin-12(n302)* and *lin-12(n302); emb-4(sa44)* genotypes. Success of ablations was confirmed and the presence of an AC was scored during the late L3 stage.

[a] Number of animals that exhibited the cell fate indicated divided by the total number of animals examined.

[b] Four *lin-12(n302)* animals were scored in this work; previously, 11 animals were handled identically by Wen *et al.* (1997).

consensus DEAD-box helicase domain, including the Walker A motif. The C-terminal part of the ATPase domain shows significant alignment to the consensus sequence of the superfamily I DNA and RNA helicases.

EMB-4 is remarkably conserved in higher eukaryotes, with the BLAST *E*-value of 0.0 for the human, mouse, fruit fly, and *Arabidopsis thaliana* orthologs (Figure 1 and see below). There are no other *C. elegans* proteins highly homologous to it, and other organisms with EMB-4 homologs have only a single copy of the gene encoding this protein. The mouse EMB-4 ortholog, named *Aquarius*, was recovered in a screen for retinoic-acid-responsive genes (Sam *et al.* 1998), but functional analysis of Aquarius has not been reported. *Schizosaccharomyces pombe* has a single EMB-4 ortholog, Cwf11p, which was isolated as a component of a multiprotein complex copurifying with Cdc5p (Ohi *et al.* 2002). Cdc5p is an essential Myb-related protein implicated in pre-mRNA splicing (Ohi *et al.* 2002), and the majority of the proteins that copurified with Cdc5p were known pre-mRNA splicing factors. cwf11 also exhibits synergistic genetic interactions with alleles of cdc5 itself (Ohi *et al.* 2002). We assessed whether feeding animals RNAi constructs targeting the *C. elegans* orthologs of other Cdc5-complex related proteins (Ohi *et al.* 2002) could suppress the 0 AC phenotype of *lin-12(n302)* and *lin-12(n379)* animals. However, as RNAi for most of these components was highly lethal before the completion of the AC/VU decision (data not shown), we could not determine whether or not the Cdc5p complex proteins positively regulate *lin-12* activity in the AC/VU decision.

EMB-4 shows similarity to SMG-2, a helicase required in the process of nonsense-mediated mRNA decay (Figure 1; Page *et al.* 1999; Grimson *et al.* 2004). *unc-54(r293)* mutant animals are uncoordinated and egg laying deficient due to a premature stop codon in the *unc-54* mRNA. These phenotypes are suppressible by mutations in all known *smg* genes (Hodgkin *et al.* 1989). However, as the loss of *emb-4* activity does not suppress either phenotype [120/120 *unc-54(r293); emb-4(hc60)* animals are Unc-54], there is no evidence for the involvement of EMB-4 in nonsense-mediated decay.

Subcellular localization of EMB-4: Expression of EMB-4 under the control of a heterologous promoter that is generally expressed appears to be toxic (see materials and methods). Therefore we expressed a GFP-tagged EMB-4 protein under the control of the VPC-expressed *lin-31* promoter (Tan *et al.* 1998) to analyze its subcellular localization. Nuclear staining is observed when transgenic animals are stained with an anti-GFP antibody (Figure 2), consistent with a role for EMB-4 in a nuclear process such as transcription or RNA processing.

DISCUSSION

Genetic analysis demonstrates that *emb-4* is a positive regulator of *lin-12* activity, as alleles that reduce or eliminate the activity of *emb-4* suppress phenotypes associated with constitutively activated forms of LIN-12. Suppression is cell autonomous, consistent with an influence on LIN-12 signal transduction or events that occur downstream of *lin-12* signaling. However, viable "escaper" *emb-4(0)* hermaphrodites do not display the hallmark cell fate transformations associated with loss of *lin-12* activity, and arrested larvae do not display defects associated with concomitant loss of *lin-12* and *glp-1* activity. This observation suggests that *emb-4* influences, but is not required for, *lin-12/Notch* activity in *C. elegans* or that another gene provides a required but redundant function. However, *emb-4(0)* mutants arrest with many developmental defects, and the profound phenotypes caused by the absence of *emb-4* activity suggest that, in some processes, *emb-4* plays a unique or required role. The mechanism(s) by which germ cell precursors are kept transcriptionally quiescent appears to be one such process (Checchi and Kelly 2006, accompanying article in this issue).

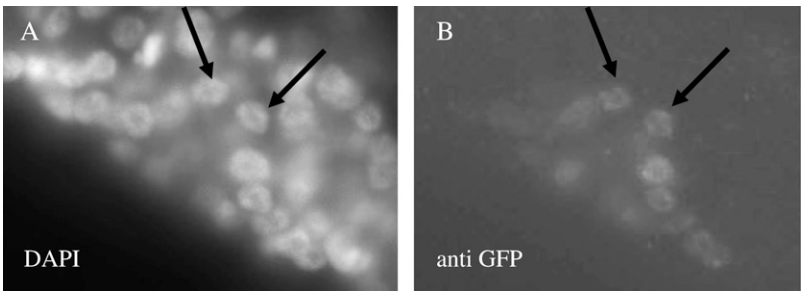

Figure 2.—EMB-4 is localized to the nucleus. Photomicrographs of VPC descendants in an L3 hermaphrodite expressing a GFP::EMB-4 protein under the control of *lin-31* regulatory sequences stained with DAPI (A) or with an antibody against GFP (B). Arrows point to two of the stained vulval nuclei. Anterior is to the left and ventral is down.

EMB-4 has orthologs in fission yeast (but not budding yeast), animals, and plants. The *S. pombe* ortholog of EMB-4, Cwf11p, copurifies with the Cdc5p complex; many components of this complex are known splicing factors. However, cwf11 differs from most known splicing factors in *S. pombe* in that it is not an essential gene and therefore was proposed to play only an ancillary role in splicing (Ohi *et al.* 2002); furthermore, no biochemical evidence for a role in splicing exists at this time. RNAi for most of the *C. elegans* orthologs of these components was highly lethal, precluding a definitive assessment of their roles in the AC/VU decision. The Senataxin family of proteins, whose ATPase domain is similar to the ATPase domain of EMB-4, has been shown to have both RNA and DNA helicase activities (Kim *et al.* 1999; Moreira *et al.* 2004). Whether Cwf11p/EMB-4 has helicase activity is unknown, but the nuclear localization of EMB-4::GFP suggests a role in a nuclear process.

Recent work from the Kelly lab (H. Furahashi, P. Checchi and W. Kelly, unpublished results) shows that RNAi depletion of *ama-1*, the gene coding for the large subunit of RNA polymerase II, phenocopies a histone modification defect associated with *emb-4(0)*, suggesting a link between *emb-4* and transcription. The link to transcription may be direct, as in participation of EMB-4 in a transcription activation complex or chromatin-modifying complex. Alternatively, it may be indirect, through an effect on RNA metabolism, such as the coupling of transcription with capping, splicing, or 3′-end formation (reviewed in Reed 2003; Zorio and Bentley 2004; Bentley 2005) or in an RNA-dependent chromatin modification process (Bernstein and Allis 2005). A role for EMB-4 in efficient expression of *lin-12* itself, a limiting component of the *lin-12*-signaling system, or key target genes, by any of the above mechanisms, could account for its function as a positive regulator of *lin-12* activity.

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