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Cell fates and fusion in the *C. elegans* vulval primordium are regulated by the EGL-18 and ELT-6 GATA factors – apparent direct targets of the LIN-39 Hox protein

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SUMMARY

Development of the vulva in *C. elegans* is mediated by the combinatorial action of several convergent regulatory inputs, three of which, the Ras, Wnt and Rb-related pathways, act by regulating expression of the *lin-39* Hox gene. LIN-39 specifies cell fates and regulates cell fusion in the mid-body region, leading to formation of the vulva. In the lateral seam epidermis, differentiation and cell fusion have been shown to be regulated by two GATA-type transcription factors, ELT-5 and -6. We report that ELT-5 is encoded by the *egl-18* gene, which was previously shown to promote formation of a functional vulva. Furthermore, we find that EGL-18 (ELT-5), and its paralogue ELT-6, are redundantly required to regulate cell fates and fusion in the vulval primordium and are essential to form a vulva. Elimination of *egl-18* and *elt-6* activity results in arrest by the first larval stage; however, in animals rescued for this larval lethality by expression of ELT-6 in non-vulval cells, the post-embryonic cells (P3.p-P8.p) that normally become vulval precursor cells often fuse with the surrounding epidermal syncytium or undergo fewer than normal cell divisions, reminiscent of *lin-39* mutants. Moreover, *egl-18/elt-6* reporter gene expression in the developing vulva is attenuated in *lin-39(rf)* mutants, and overexpression of *egl-18* can partially rescue the vulval defects caused by reduced *lin-39* activity. LIN-39/CEH-20 heterodimers bind two consensus HOX/PBC sites in a vulval enhancer region of *egl-18/elt-6*, one of which is essential for vulval expression of *egl-18/elt-6* reporter constructs. These findings demonstrate that the EGL-18 and ELT-6 GATA factors are essential, genetically redundant regulators of cell fates and fusion in the developing vulva and are apparent direct transcriptional targets of the LIN-39 Hox protein.

Key words: Cell fusion, Vulva, GATA factor, Hox, *C. elegans*

INTRODUCTION

A relatively small number of signal transduction pathways are used repeatedly during development of multicellular organisms to orchestrate diverse cell fates in many tissues. For example, in the fly, Notch signaling regulates cell fates many times during development of sensory systems such as the eye and bristle (for a review, see Artavanis-Tsakonas et al., 1995). In the nematode *Caenorhabditis elegans*, Wnt/Wingless pathway components are involved in many asymmetric cell divisions throughout embryonic and larval development (for a review, see Thorpe et al., 2000), and a Ras signaling pathway regulates cell fates in the hermaphrodite vulva and male tail, as well as several other tissues (for a review, see Sternberg and Han, 1998). Although extensive genetic and molecular studies have identified many conserved components of these signaling pathways that operate in multiple tissue types, relatively little is known about their target genes in specific tissues. Recent studies have demonstrated that Hox genes, which are best known for their role in pattern formation along the anterior-posterior body axis (for reviews, see Kenyon et al., 1997; Krumlauf, 1994), are important targets of several signaling pathways. In this context, they integrate multiple regulatory inputs and provide specific responses to common signaling inputs with diverse developmental outcomes (Clandinin et al., 1997; Eisenmann et al., 1998; Jiang and Sternberg, 1998; Maloof and Kenyon, 1998; Maloof et al., 1999). We have investigated the role of two GATA transcription factors, which appear to be targets of the *C. elegans* Hox gene, *lin-39*, in the developing vulva.

Development of the vulva in *C. elegans* hermaphrodites is controlled by the intersection of several conserved signaling pathways, including the Ras, Wnt, Notch and Rb-related pathways; it has therefore served as a useful model system with which to study the function of these pathways (for reviews, see Greenwald, 1997; Kornfeld, 1997; Wang and Sternberg, 2000). During the L1 larval stage, 12 cells (P1.p-P12.p) are born along the ventral midline. While Pn.p cells in the anterior

and posterior body regions (P1.p-P2.p and P9.p-P11.p, respectively) fuse with the surrounding syncytial epidermis shortly after their birth, the central Pn.p cells (P3.p-P8.p), or vulval precursor cells (VPCs), remain unfused and are competent to generate cells of the vulva. In response to a Ras-mediated inductive signal from the gonadal anchor cell during the L3 stage, three central VPCs, P5.p, P6.p, and P7.p, undergo three rounds of cell division, adopting the secondary, primary and secondary vulval fates, respectively. P4.p and P8.p divide once and then fuse with the surrounding syncytium, adopting the tertiary fate, and P3.p either fuses without dividing (adopting the 'F' fate) or adopts the tertiary fate.

The *C. elegans* Hox gene, *lin-39*, plays a pivotal role in the development of the mid-body region, and controls several aspects of vulval development (Clark et al., 1993; Clandinin et al., 1997; Maloof and Kenyon, 1998; Wang et al., 1993). The LIN-39 protein is expressed in mid-body cells, including the VPCs, and is required to prevent the VPCs from fusing with the surrounding syncytium (Clark et al., 1993; Wang et al., 1993). LIN-39 performs an additional function in induction of vulval cell fates by the anchor cell-activated Ras signaling pathway. Recent studies have demonstrated that several regulatory inputs control *lin-39* expression in the developing vulva. The Ras, Wnt, and Rb-related pathways coordinately regulate *lin-39* in the VPCs (Chen and Han, 2001; Eisenmann et al., 1998). Moreover, the SEM-4 transcription factor also regulates *lin-39* in the VPCs during the L2 and L3 stages (Grant et al., 2000) and *lin-39* itself is required to upregulate *lin-39* expression in response to Ras signaling (Maloof and Kenyon, 1998). Although the regulatory inputs into *lin-39* expression have been characterized, the downstream targets of this Hox gene and how it executes its regulatory functions in the vulva are unknown.

We report that a pair of GATA-type transcription factors, ELT-5 and ELT-6, previously shown to be essential for regulation of epidermal seam cell fusion and differentiation (Koh and Rothman, 2001), are essential regulators of cell fates and fusion during vulval development. Our results indicate that ELT-5 is encoded by the *egl-18* gene, previously identified in screens for mutants with vulval and egg-laying defects (Eisenmann and Kim, 2000; Trent et al., 1983), and that EGL-18 (ELT-5) and ELT-6 are likely to be direct targets of *lin-39* in the developing vulva.

MATERIALS AND METHODS

Strains and alleles

C. elegans Bristol N2 was used as the wild-type strain. Maintenance of strains was as described (Brenner, 1974). Experiments were conducted at 20°C unless otherwise noted. The genes and alleles used are as follows:

LGIII, *lin-39(n709)*;

LGIV, *egl-18(ga97)*, *egl-18(n162)*, *egl-18(n474)*, *egl-18(n475)* and *egl-18(ok290)*.

Molecular identification of *egl-18*

Rescue of an *egl-18* mutation by the cloned *elt-5* gene (Koh and Rothman, 2001) was tested using a GFP-tagged *elt-5* transgene containing ~3.4 kb of upstream sequence and all intronic sequences (pKK52) (Koh and Rothman, 2001). Although 100% (n>200) of *egl-18(n162)* mutant animals exhibited Lumpy and Uncoordinated phenotypes at hatching, most (73%, n=66) of the *egl-18(n162)* animals carrying the *elt-5* transgene (as determined by the presence of GFP) had a wild-type morphology and movement. The *elt-5* transgene also partially rescued the vulval defects, or abnormal vulval invaginations, at the L4 stage (30% wild type, n=79, without the *elt-5* transgene versus 60% wild type, n=109 with the *elt-5* transgene; $\chi^2(1)=16.7$; $P<0.0001$).

We sequenced the entire *elt-5*-coding region and intron-exon boundaries of the four previously identified *egl-18* alleles (*ga97*, *n162*, *n474* and *n475*), as well as a deletion allele (*ok290*), recently isolated by the Genome Knockout Consortium, from two independent PCR reactions of genomic DNA each. For the latter, we identified an 816 bp deletion that removes sequences from a region of exon 2 through to a region of exon 4 (corresponding to base pairs 698-1513 relative to the *egl-18* ATG).

RNA-mediated interference (RNAi)

egl-18 or *elt-6* dsRNA (~2 mg/ml), prepared as described (Koh and Rothman, 2001), was injected into young hermaphrodites (Fire et al., 1998) and progeny laid at least 12 hours after injection were analyzed. Injection of *egl-18* dsRNA into N2 adults resulted in fully penetrant lethality of their progeny. When *egl-18* dsRNA was injected into *wEx1070* animals, in which ELT-6 is driven by a partial (~3.4 kb) promoter of *egl-18* (pKK47) (Koh and Rothman, 2001), the progeny developed to fertile adults but exhibited variable vulval defects. We report data pooled from multiple injected animals whose progeny were affected to varying degrees.

Characterization of lethal and vulval phenotypes

To determine the penetrance of the lethal phenotypes, embryos from individual hermaphrodites were collected over sequential 12-24-hour periods and the embryos and larvae counted. Three days after the initial count, the live adults were counted. To characterize vulval phenotypes of *egl-18(RNAi)* animals rescued for lethality, *wEx1070* hermaphrodites injected with *egl-18* dsRNA were allowed to lay eggs and develop at 15, 20 or 25°C. F_1 animals were scored at the L4 stage by Nomarski microscopy to determine the number of VPCs that had adopted induced cell fates and contributed to the vulva. In addition, F_1 progeny of injected animals were allowed to develop into adults and their vulval phenotypes examined. Lineage analysis was performed as described (Eisenmann and Kim, 2000).

To determine the time of VPC fusion, *egl-18* RNAi was performed on worms carrying both *wEx1070* and *jcIs1* (*ajm-1*::GFP) (Koppen et al., 2001; Mohler et al., 1998). The presence of the adherens junction GFP expression surrounding a VPC indicated that it had not yet fused or divided, while the absence of expression was taken as evidence of fusion.

Reporter constructs and germ-line transformation

DNA constructs were made according to standard methods (Sambrook et al., 1989). Two transcriptional constructs, pKK62 and pKK63, were used as the basic *egl-18/elt-6*::GFP reporters. Each contains the 792 bp fragment surrounding intron 2 of the *egl-18* gene (positions 622-1413 relative to the *egl-18* ATG) and 200 bp of the *egl-18* basal promoter immediately upstream of the ATG. pKK62 contains the *gfp*-coding region (derived from pPD95.67; all pPD vectors are gifts of A. Fire) fused in frame shortly after the *egl-18* ATG, and pKK63 contains the *gfp* and β-galactosidase coding regions (derived from pPD96.04) fused at the same site. Transgenic animals carrying either construct showed GFP expression in the VPCs, their descendants and VC neurons. Some lines also showed GFP expression in the intestinal cells. pKK63 was used to characterize the normal *egl-18/elt-6* expression pattern, and pKK62 for generating mutant versions. Several pKK62 derivatives were made as follows (mutated bases are in lower case).

pKK73: site 1 mutation (TGATATAT to TctcgagT)

pKK74: site 2 mutation (TGATTGAT to aGcTcGAg)

pKK68: 248 bp deletion (removes base positions 1166 – 1413, including Site 2)

pKK70: site 1 mutation as in pKK73 and 248-bp deletion as in pKK68

Site-directed mutagenesis for pKK70, pKK73 and pKK74 was performed using the QuickChange kit (Stratagene) according to the manufacturer's protocol. Constructs were sequenced to confirm the targeted mutations.

Each construct (~100 μg/ml) was co-injected with *unc-119*(+) (pDP#MM016B, ~100 μg/ml) into *unc-119(ed4)* hermaphrodites. We observed qualitatively similar, albeit weak, expression at lower levels (~50 μg/ml) of injected DNA. Some lines showed weak, widespread neuronal expression, apparently an artifact attributable to the *unc-119(+)*maker. To confirm expression patterns, some constructs were also co-injected into N2 worms with *ceh-22*::GFP and *gcy-5*::GFP constructs (each at ~50 μg/ml, gifts of P. Okkema and D. Garbers, respectively) as co-injection markers.

Effects of reducing *lin-39* activity on *egl-18/elt-6*::GFP

To examine *egl-18/elt-6*::GFP expression in animals with reduced *lin-39* activity, we performed two experiments. First, we used *n709*, a temperature-sensitive allele of *lin-39*, to construct a strain, JR2195, containing the *n709* mutation as well as an integrated array with *egl-18/elt-6*::GFP (*wIs129*) and an integrated array with *ajm-1*::GFP (*jcIs1*). *ajm-1*::GFP was used to identify unfused P5.p-P7.p cells during the late L2 through mid L3 stages, and the percentage of cells expressing *egl-18/elt-6*::GFP in JR2195 animals was compared with the percentage in a *lin-39(+)* strain (JR2193) carrying *wIs129* and *jcIs1*. Both *lin-39(n709)* and *wIs129* were temperature-sensitive: at higher temperature, more VPCs fused in *n709* animals and more VPCs expressed GFP in *wIs129* animals. The experiment was performed at 20°C, which allowed some VPCs to remain unfused in *n709* animals and GFP expression was detectable in the majority of P5.p-P7.p cells in *wIs129* animals.

Second, we compared JR2193 worms (containing *egl-18/elt-6*::GFP and *ajm-1*::GFP) soaked in *lin-39* dsRNA with worms soaked in either H_2O or control dsRNA. L1 larvae were soaked in concentrated (~2 mg/ml) dsRNA or H_2O in the presence of food for ~16 hours. The larvae were transferred to plates and allowed to continue to develop before examination by fluorescence microscopy. Soaking in *lin-39* dsRNA caused the majority of VPCs to fuse, but some remained unfused, and only the unfused P5.p-P7.p cells during the late L2 to mid-L3 stages were scored. Data from animals soaked in H_2O were combined with those from animals soaked in control dsRNA, as they were comparable. The worms were grown at 25°C throughout the experiment.

Overexpression of EGL-18 in *lin-39(RNAi)* animals

Two *egl-18* heat-shock constructs, pKK8 and pKK9, were made by cloning the entire *egl-18*-coding region into vectors pPD49.78 (*hsp-16.2*) and pPD49.83 (*hsp-16.41*). Both constructs were co-injected with *ceh-22*::GFP and *gcy-5*::GFP markers into N2 hermaphrodites to obtain JR2268. *lin-39* RNAi by feeding was carried out as described (Gleason et al., 2002). The timing of this RNAi protocol does not interfere with the early function of *lin-39* (VPC generation), but does affect the later function of *lin-39* (VPC fate specification). Control (N2) and experimental (JR2268) animals were given five heat shocks (37°C for 15 minutes) during the L2 and L3 stages. The first heat shock was administered at the midpoint of the L2 stage, followed by a heat shock 1 hour later, and then three more heat shocks every 2.25 hours. These animals were allowed to continue development at 20°C and were then scored by Nomarski microscopy for the number of VPCs adopting induced vulval fates as described above.

LIN-39 and CEH-20 protein purification

6His-tagged versions of LIN-39 and CEH-20 were produced using the pRSET vectors pJKL430 and pRL434, respectively (gifts from J. Liu and A. Fire.) BL21-Codon Plus cells (Stratagene) carrying these vectors were grown to an O.D of 0.6-0.8 and induced with 1 mM IPTG for 3-4 hours. Cells were lysed in buffer A [8 M urea/10 mM Tris HCl (pH 8.0)/100 mM NaH_2PO_4/20 mM BME/30 mM imidazole]. Proteins were purified on a Superflow Ni^{2+}-NTA column (Qiagen) via FPLC (BioRad BioLogic HR Workstation). Proteins were renatured on the column using a linear gradient of buffer B (500 mM NaCl/20 mM Tris HCl pH 8.0/20% glycerol) and eluted with buffer E [250 mM imidazole/300 mM NaCl/50mM NaH_2PO_4 (pH 8.0) plus protease inhibitors]. Fractions (1 ml) were collected and those with LIN-39 or CEH-20 were pooled and glycerol was added to 50% for storage. Proteins are greater than 80% pure based on Coomassie staining.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were performed as described (Chang et al., 1995) with modifications. DNA binding was carried out at 4°C in a volume of 15 μl with 250 ng of LIN-39 and/or 2.5 μg of CEH-20 and final buffer conditions of 2 μg poly dI-dC/75 mM NaCl/1 mM EDTA/1 mM DTT/10 mM Tris HCl (pH 8.0)/2 μg BSA/25% glycerol. ^{32}P labeled oligonucleotide (5000 cpm) was added per reaction. After 30 minutes, samples were loaded onto a 6% polyacrylamide gel run in 0.5×TBE buffer at 100 V. To make labeled oligonucleotides, 5 pmole of one oligonucleotide was incubated with 10 μCi of ^{32}P (300 Ci/mmol, NEN) and T4 polynucleotide kinase (New England Biolabs) at 37°C for 10 minutes and then at 80°C for 5 minutes. Complementary strand oligonucleotide (10 pmole) was then added, incubated an additional 5 minutes at 80°C and then slow cooled to room temperature. The labeled, double-stranded oligonucleotide was purified over a Centri-Spin-20 column (Princeton Separation). The oligonucleotides used (one strand only) are as follows (lower case indicates mutated bases).

Antp/Exd: ACCGCGTTGATTAATGACCAGACCGGAT
S1: CGAACAAAGGAAAGATATATCACCCCGGGAGGCGGC
S1M: CGAACAAAGGAAAGActcgagACCCCGGGAGGCGGC
S2: GTGAATGTATTTATTGATTGATTGAAGGAGCTGCTG
S2M: GTGAATGTATTTATaGcTcGAgTGAAGGAGCTGCTG

RESULTS

egl-18 encodes the ELT-5 GATA factor

The *elt-5* and *elt-6* genes encode single-finger GATA transcription factors that function continuously in epidermal seam cell development (Koh and Rothman, 2001). However, analysis of reporter genes suggests that they are also expressed in other cell types, including in the post-embryonic vulval primordium. While elimination of *elt-5* and *elt-6* function results in lethality, viability can be restored by expressing ELT-6 under control of a partial promoter lacking transcriptional regulatory elements for the vulval primordium but containing elements that drive transcription in the seam epidermis and certain embryonic cell lineages (as expressed from the extrachromosomal array *wEx1070*). However, we found that the animals rescued for lethality show vulvaless (Vul), protruding vulva (Pvl), and egg-laying defective (Egl) phenotypes (see below), implying that expression of the genes in the vulval primordium is essential for normal vulval development. These phenotypes are similar to those of *egl-18* mutants (Eisenmann and Kim, 2000; Trent et al., 1983), which maps close to *elt-5*, and we therefore sought to determine whether *egl-18*, defined only by mutation, is identical to *elt-5*.

egl-18 chromosomal mutants are lumpy and uncoordinated

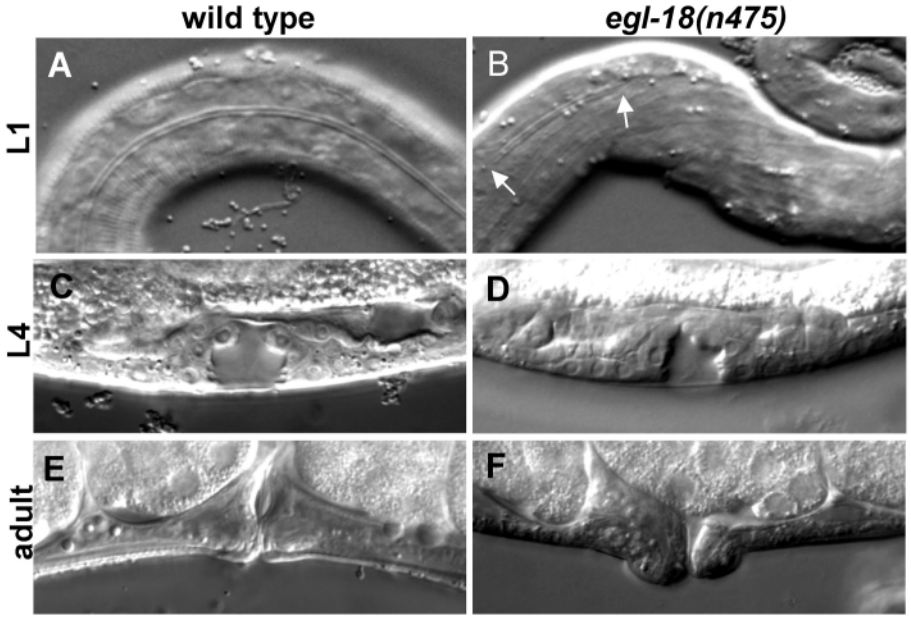


Fig. 1. Phenotypes of *egl-18* chromosomal mutants. (A) Surface view of a wild-type L1 larva. Alae are clearly visible as two unbroken lines along the length of the body. (B) Surface view of an *egl-18(n475)* L1 larva, showing breaks in alae (arrows) and a twisted body (the basis for the Rol phenotype). (C) Wild-type vulval opening at the 'Christmas tree' (L4) stage larva. (D) Defective vulval opening of an *egl-18(n475)* larva at the L4 stage. (E,F) Adult hermaphrodites, showing the wild-type vulva (E) and the protruding vulva (Pvl) phenotype (F). In these and subsequent photos, anterior is towards the left and dorsal is towards the top.

at hatching and show partially penetrant embryonic or larval lethality (see Table 1), defects in alae (seam-specific cuticular structures) and the vulva, and an occasional roller (Rol) phenotype (Fig. 1) (Eisenmann and Kim, 2000). We found that these *egl-18* phenotypes can be rescued with a transgenic *elt-5* gene (see Materials and Methods). Moreover, we identified molecular legions in the *elt-5*-coding region in all four previously identified *egl-18* alleles (Fig. 2). Three alleles, *n475*, *ga97* and *n162*, contained nonsense mutations; the fourth, *n474*, carried a deletion of a single base pair, causing a frame-shift and introduction of a premature stop codon. All four alleles are predicted to encode polypeptides that are truncated before the DNA-binding domain. In addition, we found that *ok290* mutants, recently isolated by the *C. elegans* Genome Knockout Consortium, show similar phenotypes to the previously described *egl-18* mutants. This latter mutation is a deletion of an 816 bp fragment of *elt-5* that removes the zinc-finger region. We conclude that *egl-18* encodes the ELT-5 GATA factor.

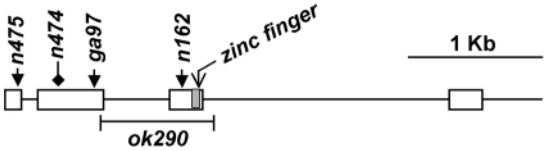
Table 1. Functional redundancy between *egl-18* and *elt-6*

Genotype	Inferred *egl-18* activity	Inferred *elt-6* activity	% survival (*n*)
egl-18(RNAi)	–	–	0 (many)
egl-18(ga97)	–	+	18 (148)
egl-18(n162)	–	+	43 (302)
egl-18(n474)	–	+	31 (495)
egl-18(n475)	–	+	46 (516)
egl-18(ok290)	–	+	59 (362)
elt-6(RNAi)	+	–	>99 (many)
egl-18(ga97) elt-6(RNAi)	–	–	0 (66)
egl-18(n162) elt-6(RNAi)	–	–	0 (205)
egl-18(n474) elt-6(RNAi)	–	–	1* (371)
egl-18(n475) elt-6(RNAi)	–	–	2.6* (268)
egl-18(ok290) elt-6(RNAi)	–	–	0 (78)

*The survivors tended to be the earliest progeny, which may have been born before *elt-6* dsRNA became fully effective.

egl-18 is functionally redundant with *elt-6*

Whereas 100% of the progeny of adults injected with high doses of *egl-18* dsRNA arrest by the early L1 larva stage (Koh and Rothman, 2001), *egl-18* chromosomal mutants that are likely null alleles based on their molecular lesions (e.g. the *n475* mutation truncates over 90% of the protein), show only partially penetrant lethality (Table 1). The survival of *egl-18* mutants may be attributable to intact *elt-6* activity. Indeed, interfering with *elt-6* function by treatment with *elt-6* dsRNA in *egl-18* chromosomal mutants caused nearly fully penetrant late-embryonic/early-larval lethality (Table 1). These results indicate that *egl-18* and *elt-6* are functionally redundant during embryonic development and imply that *egl-18* mutations affect *egl-18* activity alone, *elt-6* dsRNA affects *elt-6* activity alone, and *egl-18* dsRNA affects both *egl-18* and *elt-6* activity. This is consistent with previous observations suggesting that *egl-18* and *elt-6* are both monocistronically and dicistronically transcribed and that *egl-18* dsRNA interferes with expression



allele	DNA change	AA change
n475	AAA→TAA (100)	K34stop
n474	1 bp deletion (272)	
ga97	CGA→TGA (622)	R193stop
n162	CAA→TAA (1297)	Q245stop
ok290	816 bp deletion (698-1513)	

Fig. 2. Mutations in the *egl-18* gene. Boxes represent exons and lines represent introns. The nature of each mutation is described in the lower part of the figure. The numbers in parentheses indicate the position of the changed bases in the genomic sequence relative to the *egl-18* ATG.

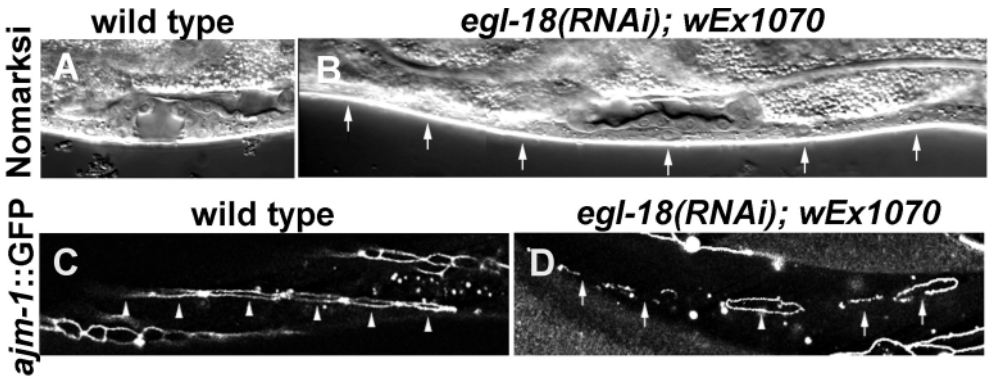


Fig. 3. Vulval phenotypes of *egl-18(RNAi)* animals rescued for lethality by *wEx1070.* (A) Nomarski image of a wild-type vulval opening at the 'Christmas tree' (L4) stage. (B) Nomarski image of an *egl-18(RNAi); wEx1070* animal, in which all of the six P3.p-P8.p cells (arrows) did not divide and appear to have fused. (C) *ajm-1*::GFP (adherens junction marker) expression in a wild-type animal at the early L3 stage. All six VPCs (arrowheads) are clearly demarcated by *ajm-1*::GFP. One of the VPCs is partially out of focus in this image. (D) *ajm-1*::GFP expression in an *egl-18(RNAi); wEx1070* animal at a similar stage to the animal shown in C. All but one VPC are in the process of fusion as indicated by fragmented *ajm-1*::GFP expression (arrows). One VPC remains unfused as shown by a complete ring (arrowhead).

of an *elt-6* reporter gene containing the *egl-18*-coding region (Koh and Rothman, 2001).

egl-18 and *elt-6* appear to function redundantly in vulval development as well as viability. Whereas approx. two-thirds of *egl-18(RNAi)* animals rescued for lethality by the extrachromosomal array *wEx1070* are Vulvaless (i.e., all VPCs adopted either the F or tertiary fate, Table 2 and Fig. 3), such strong vulval defects were rarely observed in *egl-18* chromosomal mutants (Eisenmann and Kim, 2000) (Fig. 1). This difference is unlikely to result from non-specific interference of genes other than *egl-18* and *elt-6*, as dsRNA made from two non-overlapping regions of *egl-18* yielded essentially the same results (not shown). Rather, these results imply that *egl-18(RNAi)* affects both *egl-18* and *elt-6* activity, which function redundantly. For simplicity, we will refer to animals subjected to *egl-18* dsRNA as *egl-18(RNAi)* mutants, though it is probable that both *egl-18* and *elt-6* activity is compromised in such animals.

Table 2. Vulval phenotypes of *egl-18(RNAi)* animals rescued for lethality by *wEx1070*

	L4 phenotype*				
	% Vul; all F	% Vul; some tertiary	% underinduced	% wild type	*n*
15°C	23	46	23	8	200
20°C	21	47	27	5	204
25°C	31	56	3	9	188

	Adult phenotype†			
	% Bag	% Egl	% wild type	*n*
15°C	84	9	6	420
20°C	68	26	5	580
25°C	79	15	6	331

*L4-stage larvae were examined by Nomarski microscopy for vulval phenotypes. Animals in which no VPCs are induced are scored as having a vulvaless (Vul) phenotype. Vulvaless animals are further categorized into those in which all VPCs adopted the F fate (Vul; all F) and those in which some VPCs adopted the tertiary fate (Vul; some tertiary). Animals in which only one or two VPCs were induced were categorized as underinduced.

†Adults were scored under a dissecting microscope for the bag-of-worms (Bag – animals bag without laying any eggs), egg-laying-defective (Egl – animals lay some eggs but accumulate late-stage embryos or L1-stage larvae) or wild-type phenotype.

Vulval defects in the absence of *egl-18* and *elt-6* function

To further characterize the vulval defects in *egl-18(RNAi); wEx1070* animals (in which lethality is rescued but *egl-18/elt-6* are not expressed in the vulval primordium) we followed the cell lineages of the VPCs in ten animals (Table 3). Almost all the VPCs followed adopted inappropriate cell fates. Many VPCs inappropriately adopted the F fate, and the P5.p-P7.p cells often stopped dividing after one or two cell divisions ('SS' or 'NNNN'). Previous lineage analysis of *egl-18* chromosomal mutants (Eisenmann and Kim, 2000) revealed similar, but milder, defects in VPC fusion and number of divisions. These results indicate that EGL-18 and ELT-6 are key regulators of vulval development.

To determine when VPCs fuse in *egl-18(RNAi); wEx1070* animals, we examined expression of *ajm-1*::GFP (a marker of epithelial adherens junctions) (Koppen et al., 2001; Mohler et al., 1998) at several times from late L1 through L3 stages. In wild-type animals, six Pn.p cells (P3.p-P8.p) remain unfused

Table 3. Vulval lineages of *egl-18(RNAi)* animals rescued for lethality by *wEx1070*

P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
F	F	F	F	F	F
F	F	F	F	F	F
F	F	F	SS	F	F
F	F	SS	SS	F	F
F	F	SS	SS	F	F
F	F	F	F	SS	SS
F	F	F	NNNN	F	F
F	F	F	NNNN	F	F
F	F	F	NNNN	F	F
F	F	F	TTTT	F	F

Each row corresponds to the lineage data from an individual animal. The last division patterns of the VPCs or their descendants are reported. F (fused) indicates cells that did not divide and appear to have fused. SS indicates that cells fused after dividing once. T indicates a transverse division and N indicates that the cell did not divide. TTTT is a wild-type primary fate and SS is a wild-type tertiary fate.

during the late L1 and early L2 stages. During the ensuing L2 and L3 stages, only P3.p fuses, and does so only 50% of the time; thus, there are five or six Pn.p cells surrounded by the *ajm-1*::GFP signal at this stage. In all *egl-18(RNAi); wEx1070* animals examined, six Pn.p cells expressed *ajm-1*::GFP through the early L2 stage. However, beginning around the mid-L2 stage, *egl-18(RNAi); wEx1070* animals often contained fewer than five Pn.p cells demarcated by *ajm-1*::GFP, indicating that some VPCs had inappropriately fused (Fig. 3 and data not shown). Fusion occurred between the mid L2 and L3 stages, which correspond to the time at which the V3.p cells fuse in wild-type animals. These results implicate *egl-18* and *elt-6* in maintaining VPCs in an unfused state during later stages of vulval development. However, it remains possible that these genes also function in the generation of the VPCs (see Discussion).

The vulval defects observed in *egl-18/elt-6* mutants resemble those in animals with reduced *lin-39* function (Clandinin et al., 1997; Clark et al., 1993; Maloof and Kenyon, 1998), suggesting a close relationship between *lin-39* and *egl-18/elt-6*.

egl-18/elt-6::GFP is expressed in the VPC lineages and VC neurons

To further investigate the role *egl-18* and *elt-6* play in vulval development, we examined their larval expression. Previous work demonstrated that these genes are expressed in many cell types, apparently under the control of separable enhancer elements for different cell types (Koh and Rothman, 2001). Using a series of partial promoter reporter constructs (Koh and Rothman, 2001) (data not shown), we identified an ~800 bp region surrounding intron 2 of *egl-18* that includes a vulval enhancer. We found that a reporter construct (pKK63) containing this ~800 bp element and an ~200 bp basal promoter fragment of *egl-18* is sufficient to drive GFP expression in the VPCs and their descendants as well as in the six VC motoneurons that innervate vulval muscles (Fig. 4A-C), which are likely to be co-regulated with vulval cells. Similar vulval expression was observed when GFP was fused to the start codon of either *egl-18* or *elt-6* in a reporter containing ~8 kb of contiguous genomic sequence that includes this ~800 bp region (Fig. 4D and not shown), suggesting that the ~800 bp region is likely to be a vulval enhancer for both genes. As the expression levels and patterns of pKK63 showed substantial variability, even among chromosomal integrants of the transgene, our characterization of the spatial and temporal pattern of *egl-18/elt-6*::GFP expression is based on the composite pattern that emerged from examination of many animals.

When expression of *egl-18/elt-6*::GFP is first detected in VPCs around the mid-L2 stage, all six VPCs are equally likely to express GFP (Fig. 4A). However, beginning at around the late-L2/early-L3 stage, until the VPCs divide in the mid-L3 stage, the expression in P5.p-P7.p is generally higher than in the other VPCs, and P6.p often shows the strongest expression (Fig. 4B). Expression persists in the descendants of P5.p-P7.p (Fig. 4C) through the L4 stage, and P6.p descendants typically show stronger expression than descendants of P5.p and P7.p. This pattern is similar to that of *lin-39* expression in the developing vulva (Maloof and Kenyon, 1998), and suggests that, like *lin-39*, *egl-18/elt-6* may be upregulated by Ras-mediated vulval induction.

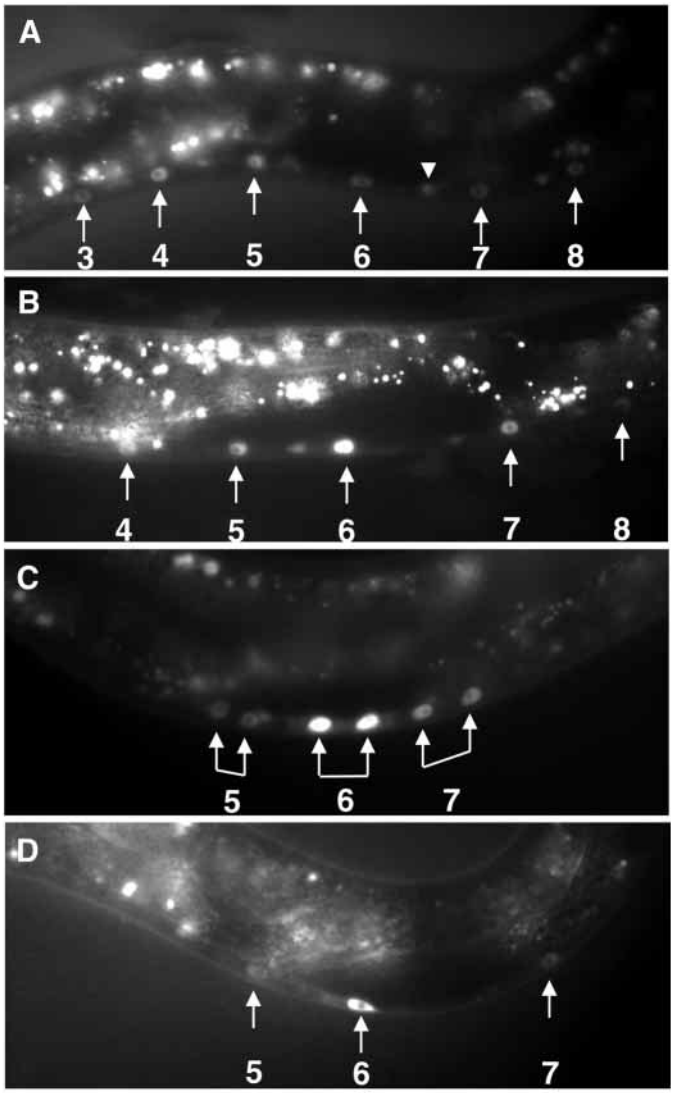


Fig. 4. Expression of *egl-18/elt-6*::GFP. (A-C) Expression of pKK63 containing an ~800 bp enhancer and a basal promoter. (A) L2 larva, in which all VPCs (P3.p-P8.p) show approximately equal levels of GFP expression (arrows). All six VC neurons show GFP expression, although not all are visible in this focal plane. Arrowhead indicate a VC neuron. (B) L3 larva shortly before VPC divisions, showing stronger GFP expression in P5.p-P7.p cells than in P4.p and P8.p. (C) L3 larva after the first VPC cell divisions. Daughters of P6.p show higher GFP expression than daughters of P5.p and P7.p. GFP expression is not detectable in P4.px and P8.px cells. (D) Early-L3 larva showing expression of an *elt-6* transcriptional fusion pKK41 in P5.p-P7.p cells. pKK41 contains ~8 kb of genomic sequence upstream of the *elt-6* ATG, including the ~800 bp enhancer in pKK63 (Koh and Rothman, 2001).

egl-18/elt-6 act downstream of *lin-39* activity in the VPCs

We tested the relationship between *lin-39* and *egl-18/elt-6*, by analyzing the effect of reducing *lin-39* activity on expression of the latter. We found that *lin-39(RNAi)* animals show virtually undetectable expression of *egl-18/elt-6*::GFP (not shown). However, almost all VPCs adopt the fused fate in *lin-39(RNAi)* animals; the lack of *egl-18/elt-6* expression might simply be a

consequence of fusion per se. We therefore examined the effects of partial reduction of *lin-39* function on *egl-18/elt-6*::GFP expression, conditions in which at least some VPCs remained unfused (see Materials and Methods). We found that while 76% (n=160) of unfused P5.p-P7.p cells in *lin-39(+)* worms expressed GFP, only 42% (n=85) did so in animals carrying *n709*, a *lin-39* reduction-of-function allele; this is a highly significant difference ($\chi^2(1)=26.7$, $P<0.0001$). Furthermore, we found that soaking of L1 larvae in *lin-39* dsRNA significantly reduced the fraction of unfused P5.p-P7.p cells that express *egl-18/elt-6*::GFP from 92% (n=110) for the control soak to 55% (n=128) ($\chi^2(1)=39.0$, $P<0.0001$). In both experiments, reduction of *lin-39* activity resulted in lowered *egl-18/elt-6*::GFP expression.

Consistent with the model in which *egl-18* and *elt-6* act downstream of LIN-39 Hox during vulval development, we found that overexpression of *egl-18* from heat-shock promoters is sufficient to partially rescue vulval defects in *lin-39(RNAi)* animals. Among *lin-39(RNAi)* control animals subjected to heat-shock (five 15 minute pulses at 37°C), only 36% (n=92) showed wild-type vulval invaginations at the L4 stage. By contrast, significantly more (76%; n=98; $\chi^2(1)=30.3$; $P<0.0001$) *lin-39(RNAi)* animals carrying *hs-egl-18* had normal invaginations following the same heat-shock regimen. In the absence of heat-shock, *lin-39(RNAi)* had approximately equal effects on both wild-type (17% with normal invaginations; n=93) and *hs-egl-18*-bearing (16% with normal invaginations; n=93) animals.

A Hox/PBC-binding site is essential for vulval expression of *egl-18/elt-6*::GFP reporters

Hox proteins appear to require co-factors to achieve DNA-binding specificity (for reviews, see Mann and Affolter, 1998; Mann and Chan, 1996). The most extensively studied of the Hox co-factor genes are the *Drosophila extradenticle* (*exd*) and mammalian *pre-B cell homeobox 1* genes, collectively referred to as PBC genes. Hox and PBC proteins form heterodimers that bind DNA in vitro. *C. elegans* contains one known Exd homolog, CEH-20, which appears to act as a Hox co-factor (Liu and Fire, 2000). Consistent with the possibility that *egl-18* and *elt-6* are direct targets of LIN-39 Hox, we found several consensus Hox/PBC-binding sites (TGATNNAT) in the *egl-18* and *elt-6* genomic region (Fig. 5). Two of these [site 1 (TGATATAT) and site 2 (TGATTGAT)] are present in intron 2 of *egl-18*, which is included in the ~800 bp promoter element that directs GFP expression in the VPC lineages and VC neurons. Several lines of evidence indicate that site 1, but not site 2, is important for vulval-specific expression of *egl-18/elt-6*. First, alteration of 6 bp in site 1 eliminated expression in the VPC lineages and VC neurons, whereas a similar mutation that alters 4 bp of site 2 had no obvious effect on reporter expression (Fig. 5; Table 4). Second, a reporter in which 544 base pairs surrounding only Site 1 is present showed expression in the vulva and VC neurons (Table 4), albeit at an attenuated level compared with the reporter containing both sites. Mutation of Site 1 from this construct eliminated vulval and VC expression (Table 4). Finally, comparison of the *egl-18* sequence of *C. elegans* and *C. briggsae* revealed a highly

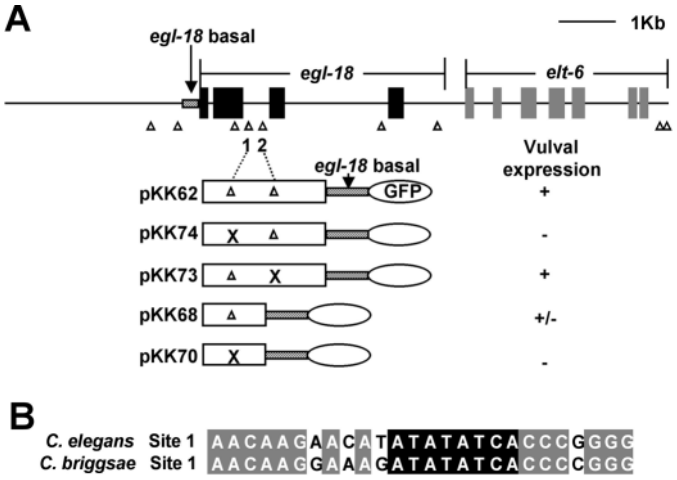


Fig. 5. (A) Hox/PBC-binding sites in the *egl-18* and *elt-6* genomic region and GFP reporter constructs. Open reading frames of *egl-18* and *elt-6* are indicated by black and gray boxes, respectively. Introns and 5′ and 3′ UTRs are indicated by lines. Triangles indicate consensus Hox/PBC-binding sites, two of which in the second intron of *egl-18* are labeled sites 1 and 2. (B) Alignment of *C. elegans* site 1 and the corresponding *C. briggsae* sequence. The region corresponding to *C. elegans* site 2 is not conserved in *C. briggsae*.

Table 4. Effects of changes in potential Hox/PBC binding sites on reporter expression

Construct	Site 1	Site 2	Co-injection marker	% vulval expression	% VC expression	Number of worms*	Number of lines
pKK62	Wild type	Wild type	*ceh-22*::GFP†	49	92	49	2
pKK62	Wild type	Wild type	*unc-119(+)*	35	95	109	5
pKK74	Mutated	Wild type	*ceh-22*::GFP	0	0	119	5
pKK74	Mutated	Wild type	*unc-119(+)*	0	0	81	4
pKK73	Wild type	Mutated	*unc-119(+)*	35	99	102	6
pKK68	Wild type	Deleted	*ceh-22*::GFP	6	82	50	2
pKK68	Wild type	Deleted	*unc-119(+)*	8	93	119	2
pKK70	Mutated	Deleted	*unc-119(+)*	0	0	105	8

Only transgenic animals expressing the co-injection marker were scored for expression of GFP in the developing vulva or VC neurons during the L3 and L4 stages. The animals were scored positive for GFP if at least one cell had a detectable level of GFP expression. Wild-type site 1, TGATATAT; mutated site 1, TctcgagT; wild-type site 2, TGATTGAT; mutated site 2, aGcTcGAg.

*Approximately equal numbers of worms were analyzed from each line.

†For convenience, only *ceh-22*::GFP is listed in this column, even though these strains were obtained using both *ceh-22*::GFP and *gcy-5*::GFP as co-injection markers.

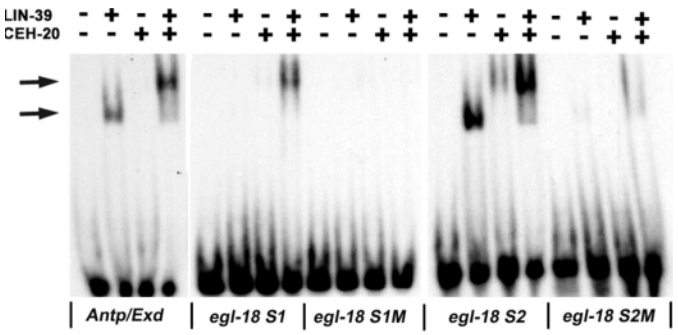


Fig. 6. LIN-39 and CEH-20 bind cooperatively to two consensus Hox/PBC binding sites in vitro. The bottom arrow indicates bands corresponding to probes bound to LIN-39 alone and the top arrow indicates bands corresponding to probes bound to LIN-39/CEH-20 heterodimers. In the first four lanes, an oligonucleotide (Antp/Exd) containing a binding site for a *Drosophila* Hox protein, Antennapedia, and its co-factor, Extradenticle, is used as a positive control. LIN-39 and CEH-20 bind the wild-type site 1 (S1) and site 2 (S2) efficiently, but not the mutated sites (S1M and S2M). Site 2 shows greater binding affinity for LIN-39/CEH-20 heterodimers than does site 1, and LIN-39 alone can bind site 2 but not site 1.

conserved 27 bp element surrounding Site 1 (Fig. 5B) but no conservation of site 2. Thus, the site 1 Hox/PBC site is apparently necessary and sufficient for vulva-specific expression of *egl-18/elt-6*::GFP.

LIN-39/CEH-20 dimers bind Hox/PBC sites in the *egl-18/elt-6* vulval enhancer

We performed electrophoretic mobility shift assays to test the hypothesis that *egl-18* and *elt-6* are direct targets of LIN-39/CEH-20 heterodimers in the vulva. Indeed, we found that LIN-39 and CEH-20 heterodimers bind in vitro to 30 bp oligonucleotides centered on either the Hox/PBC site 1 or site 2 (Fig. 6). Whereas binding of LIN-39/CEH-20 to site 1 oligos could be competed away with excess unlabeled site 1 or 2 oligos, unlabeled site 1 oligos could not compete with site 2 oligos (not shown), implying that site 2 has a higher in vitro affinity for LIN-39/CEH-20 than does site 1. Our results indicate that LIN-39/CEH-20 heterodimers can bind cooperatively to site 1, which is essential for expression of the *egl-18/elt-6* reporter in the vulva. Based on these results and the phenotypes of *egl-18*/*elt-6* mutants, it seems likely that LIN-39 regulates vulval development by directly activating EGL-18 and ELT-6, which in turn repress epidermal fusion and activate vulval differentiation.

DISCUSSION

Control of vulval cell fates, division and fusion by the EGL-18 and ELT-6 GATA factors

We have demonstrated that ELT-5 is encoded by the *egl-18* gene, which was previously identified only by mutations, and that the EGL-18 and ELT-6 GATA factors function in several aspects of vulval development, including regulation of cell fusion and cell fate specification. In *egl-18(RNAi)* animals rescued for lethality by *wEx1070*, which drives ELT-6 under a partial *egl-18* promoter lacking a vulval enhancer, many VPCs inappropriately fuse with the surrounding syncytium, some adopt the tertiary fate instead of primary or secondary fates, and some stop dividing after two division rounds. Thus, these genes apparently activate vulval differentiation programs, repress cell fusion and provide mitogenic information.

All five alleles of *egl-18* eliminate the zinc-finger DNA-binding domain of the protein and may represent null or strong loss-of-function alleles. However, they exhibit phenotypes considerably milder than those seen in the *egl-18(RNAi); wEx1070* strain. This can be explained by proposing that, while *egl-18* chromosomal mutations eliminate only *egl-18* activity, *egl-18(RNAi)* abolishes the activity of both *egl-18* and *elt-6* because of their dicistronic transcription. The vulval phenotypes observed in *egl-18(RNAi); wEx1070* animals are somewhat variable; it is possible that the strongest phenotype seen (i.e., all VPCs adopting the F fate) represents the null phenotype of *egl-18* and *elt-6* double mutants.

We did not obtain evidence that EGL-18 and ELT-6 control one important aspect of vulval development: the generation of the VPCs during the L1 stage (i.e. by preventing fusion of the midbody Pn.p cells). Although inappropriate fusion of the P3.p-P8.p cells occurred during the late L2 and L3 stages in *egl-18(RNAi); wEx1070* animals, we never observed fusion of P3-8.p cells in late L1 larvae, the stage at which they fuse in *lin-39* null mutants and when other Pn.p cells fuse in wild-type animals (Clark et al., 1993; Wang et al., 1993). One possible interpretation of this result is that *egl-18* and *elt-6* might regulate Pn.p cell fusion specifically during the L2 and L3 stages. Several other genes, e.g. *bar-1* and *sem-4*, regulate Pn.p cell fusion only during the L2 and L3 stages (Eisenmann et al., 1998; Gleason et al., 2002; Grant et al., 2000). Alternatively, *egl-18* and *elt-6* may regulate early Pn.p cell fusion, but such a role was not apparent in our experiments because the partial promoter used to rescue *egl-18(RNAi)* lethality drives detectable levels of ELT-6 expression in P cells of embryos as a component of the widespread AB lineage expression (Koh and Rothman, 2001). Thus, residual levels of ELT-6 in Pn.p cells of late L1 larvae may be sufficient to repress P3.p-P8.p fusion.

The possibility that EGL-18/ELT-6 might repress cell fusion in the early Pn.p cells would not be unexpected given the role of these genes in regulating fusion in other epidermal cells (Koh and Rothman, 2001) earlier in development. In fact, it is conceivable that EGL-18/ELT-6 might function broadly to repress fusion in non-syncytial epidermal cells.

egl-18 and *elt-6* are likely to be direct targets of the LIN-39 Hox protein in the vulva

The ~800 bp vulval enhancer surrounding intron 2 of *egl-18* is sufficient to drive *egl-18/elt-6*::GFP expression in the VPC lineages and the VC neurons. This function requires an intact Hox/PBC consensus site, which binds LIN-39/CEH-20 heterodimers in vitro. The effect of *lin-39* activity on expression of *egl-18/elt-6*::GFP, and the observation that overexpression of EGL-18 rescues vulval defects in animals with reduced *lin-39* activity, further suggest that *egl-18* and *elt-6* are direct targets of *lin-39* in the vulva and VC neurons and may mediate the positional regulatory information provided by this Hox gene.

Although site 1 appears to be necessary for vulval expression of reporter constructs, other results indicate that site 1 is not

strictly necessary for *egl-18/elt-6* expression in vulval development. In *ok290* deletion mutants both sites 1 and 2 are removed, but they show relatively mild vulval phenotypes (64% wild-type vulval invagination at the L4 stage) compared with *egl-18(RNAi); wEx1070* animals (8% wild-type; see Table 2). This observation implies that site 1 is not the only regulatory site responsible for *egl-18/elt-6* expression in the vulva. Other sites, either the potential Hox/PBC binding sites found throughout the *egl-18* and *elt-6* genomic region (Fig. 5B), or sites controlled by other regulatory factors, may contribute to *egl-18/elt-6* expression during vulval development.

It is interesting to note that while site 2 shows higher affinity in vitro, site 1 appears to be more critical than site 2 for in vivo reporter expression and is the only one of the two that is conserved in *C. briggsae*. These observations are consistent with previous findings suggesting that in vivo specificity may be more important than affinity (Mann and Affolter, 1998). As the conservation of sequences between *C. elegans* site 1 and the corresponding site in *C. briggsae* extends beyond the Hox/PBC octamer consensus binding site (23/27 base pairs are identical) (Fig. 5), there are likely to be other, as yet unidentified factors that bind the element and that are important in vivo for specificity of *lin-39* activity. Such additional factors may be required to restrict *egl-18/elt-6* expression to a subset of *lin-39*-expressing cells. Although the expression patterns of *lin-39* and *egl-18/elt-6* overlap, they are not identical: for example, *lin-39* is expressed in all ventral cord neurons in the mid-body region, whereas *egl-18/elt-6*::GFP is expressed only in VC neurons. In addition, widespread expression of LIN-39 by a heat-shock promoter does not cause ectopic expression of *egl-18/elt-6*::GFP (not shown). These results suggest that LIN-39 is insufficient to activate *egl-18/elt-6* expression and that other VPC- and VC-specific transcription factors may be required for their expression. The 27-mer conserved enhancer element we have identified may prove useful in discovering such tissue-specific factors as well as additional binding partners of the LIN-39 Hox protein.

Despite systematic attempts at discovering targets of Hox genes in the fly (e.g. Gould et al., 1990; Mastick et al., 1995), homeotic response elements of only a few target genes have been characterized in detail (e.g. Capovilla et al., 1994; McCormick et al., 1995; Pederson et al., 2000; Regulski et al., 1991). In *C. elegans*, only one Hox-responsive element, that of *hlh-8*, which functions in postembryonic mesoderm development, has been characterized (Liu and Fire, 2000). Comparison of multiple Hox-responsive elements, including the *lin-39*-responsive element we have identified, may be helpful in understanding how Hox genes regulate their target genes.

Our finding that the GATA factors regulate fusion in the development of two different cell types (seam and vulva) suggests the possibility that they are key regulators of fusion more generally, acting on the same set of target genes in multiple cell types. For example, they may be intermediaries that integrate developmental cues and repress genes that promote cell fusion, such as the recently identified *eff-1* gene, which is required for all cell fusions in the *C. elegans* epidermis (Mohler et al., 2002; Witze and Rothman, 2002). However, as they promote distinct differentiated cell fates depending on the context (e.g., seam fate versus vulval fate), they must also have distinct sets of target genes. Discovery of such common and distinct targets of *egl-18* and *elt-6* may help to elucidate how multiple signaling pathways and Hox genes achieve diverse developmental tasks.

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