

## The zinc finger protein REF-2 functions with the Hox genes to inhibit cell fusion in the ventral epidermis of *C. elegans*

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### SUMMARY

During larval development in *C. elegans*, some of the cells of the ventral epidermis, the Pn.p cells, fuse with the growing epidermal syncytium hyp7. The pattern of these cell fusions is regulated in a complex, sexually dimorphic manner. It is essential that some Pn.p cells remain unfused in order for some sex-specific mating structures to be generated. The pattern of Pn.p cell fusion is regulated combinatorially by two genes of the *C. elegans* Hox gene cluster: *lin-39* and *mab-5*. Some of the complexity in the Pn.p cell fusion pattern arises because these two Hox

proteins can regulate each other's activities. We describe a zinc-finger transcription factor, REF-2, that is required for the Pn.p cells to be generated and to remain unfused. REF-2 functions with the Hox proteins to prevent Pn.p cell fusion. *ref-2* may also be a transcriptional target of the Hox proteins.

Key words: *C. elegans*, *ref-2*, Hox genes, Cell fusion, C<sub>2</sub>H<sub>2</sub> zinc fingers, *odd-paired*

### INTRODUCTION

How complex tissues are formed from individual cells is a fundamental biological question. The *C. elegans* epidermis is composed of several different multinucleate cells (syncytia) that envelop the worm (Sulston and Horvitz, 1977; Podbilewicz and White, 1994; Shemer and Podbilewicz, 2000). These syncytia are formed by the fusion of mononucleate cells throughout embryonic and postembryonic development. The largest such syncytium is hyp7, which extends over most of the length of the worm. At hatching, hyp7 primarily covers the dorsal surface of the worm and contains 23 nuclei. hyp7 grows as the worm grows; during larval development, an additional 110 cells on the lateral and ventral surface fuse with hyp7 (Podbilewicz and White, 1994). However, not all epidermal cells join syncytia. Several cells on the ventral surface of the worm remain unfused; instead of fusing, these cells divide and generate sex-specific mating structures (Sulston and Horvitz, 1977; Sulston and White, 1980). Thus, in order for these structures to be formed, it is essential that the pattern of ventral epidermal fusions be controlled appropriately.

The pattern of ventral epidermal cell fusion is controlled by two genes in the *C. elegans* Hox gene cluster (Clark et al., 1993; Salser et al., 1993; Wang et al., 1993). However, the pattern of cell fusion is more complex than the relatively simple expression patterns of these two Hox genes would allow. This is because the activity of these Hox proteins is regulated by other factors and by each other. In most animals, relatively simple Hox gene expression patterns are elaborated into more complex cell fate patterns (Duncan, 1996). Thus, understanding how Hox proteins interact with each other and

with other factors to control anteroposterior (AP) patterning is necessary to understand fully how complex organisms are patterned. The sexually dimorphic Pn.p cell fusion pattern in *C. elegans* provides a good system in which to understand such interactions in cellular detail.

At hatching, the ventral epidermis is composed of six pairs of P blast cells that line the ventral surface of the worm (P1/2, P3/4...P11/12) (Fig. 1A) (Sulston and Horvitz, 1977). During the first larval stage (L1), these six pairs of cells rotate around each other, resulting in 12 P cells that form a single row in the ventral cord (Fig. 1A) (Sulston and Horvitz, 1977; Podbilewicz and White, 1994). As this cell rotation occurs, the nuclei of these cells migrate from the ventrolateral surface of the worm into the ventral cord. Late in L1, these P cells divide: the anterior daughters (Pn.a cells) become neuroblasts, while the posterior daughters (Pn.p cells) remain part of the epidermis (Fig. 1B). Shortly after they are generated, some of the Pn.p cells fuse with the hyp7 syncytium. In hermaphrodites, P(3-8).p in the mid-body region remain unfused (Fig. 1C). These six unfused cells are the vulval precursor cells, some of which generate the hermaphrodite vulva later in development. The remaining cells, P(1,2).p and P(9-11).p, fuse with hyp7 (P12.p behaves differently and will not be considered here). The pattern of Pn.p cell fusion is different in males, with P(1,2).p and P(7,8).p fusing with hyp7 while P(3-6).p and P(9-11).p remain unfused (Fig. 1D). These posterior unfused cells make male-specific mating structures such as the hook sensillum (Sulston and Horvitz, 1977).

*C. elegans* contains six Hox genes that, as in other organisms, direct the choice of cell fates along the AP body axis (Chisholm, 1991; Clark et al., 1993; Wang et al., 1993;

Brunschwig et al., 1999; van Auken et al., 2000). Pn.p cell fusion is regulated by two genes of the *C. elegans* Hox gene cluster: *lin-39*, a homolog of *Drosophila Sex combs reduced*, and *mab-5*, a homolog of *Drosophila Antennapedia*. *lin-39* is expressed in P(3-8).p in both sexes; *mab-5* is also expressed in both sexes, although more posteriorly, in P(7-11).p (Salser et al., 1993; Wang et al., 1993; Maloof and Kenyon, 1998). In hermaphrodites, *lin-39* prevents fusion of those cells in which it is expressed (Fig. 1C) (Clark et al., 1993; Wang et al., 1993). Although MAB-5 is expressed in Pn.p cells in hermaphrodites, it remains inactive, at least in part, because of the activity of two other transcription factors, REF-1 and EGL-27 (Ch'ng and Kenyon, 1999; Alper and Kenyon, 2001). By contrast, LIN-39 and MAB-5 are both active in males. Where either Hox protein is present alone, it can prevent Pn.p cell fusion: P(3-6).p for LIN-39 and P(9-11).p for MAB-5 (Fig. 1D) (Salser et al., 1993). Interestingly, P7.p and P8.p, in which both Hox proteins are present, fuse with *hyp7*, as do cells that contain neither Hox protein (P1.p and P2.p). This occurs because MAB-5 and LIN-39 inhibit each other's activities when they are both present in the same Pn.p cell in males. A simple model that would explain this interaction is that the two Hox proteins inhibit each other by sequestering each other within the cell. However, this does not appear to be the case because the two proteins can still inhibit each other when one Hox gene is strongly overexpressed relative to the other (Salser et al., 1993). Instead, it appears that something else is limiting in this cell fate decision. Perhaps both Hox proteins bind to regulatory sites in a cell fusion gene that encodes a direct effector of cell fusion. In this model, the binding of either Hox protein alone would inhibit expression of this gene while binding of both Hox proteins would activate expression.

To investigate the complex, sex-specific interaction between these two Hox proteins, we have analyzed mutations that alter the Pn.p cell fusion pattern by affecting Hox protein activity. We describe one such mutation, *ref-2(mu218)* (REGulator of Fusion-2), which specifically affects the ability of LIN-39 and MAB-5 to cancel each other's activities when both proteins are present in the same Pn.p cell in males. The *mu218* mutation is a dominant, non-coding mutation that affects Pn.p cell fusion by mis-regulating *ref-2*, which encodes a zinc-finger transcription factor of the *odd-paired (opa)*/Zic family. The *ref-2* gene product is required for Pn.p cells to be generated and to remain unfused.

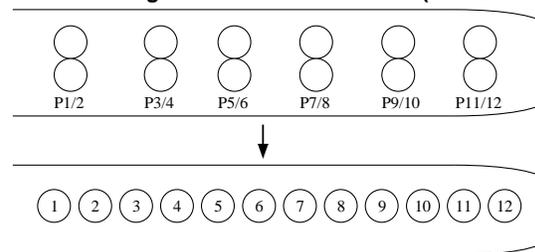
## MATERIALS AND METHODS

### Isolation and phenotypic characterization of *ref-2(mu218)*

Mutations that affect Pn.p cell fusion have been identified as described previously (Alper and Kenyon, 2001). *ref-2(mu218)* was identified among the progeny of mutagenized *lin-12(n137)*; *him-5(e1467)* worms. *lin-12(n137)*; *him-5(e1467)*; *ref-2(mu218)* males mated, albeit very inefficiently. All phenotypic characterization described in this paper was carried out in a strain that was genotypically wild type for *lin-12*, carried *him-5(e1490)* to generate males and was outcrossed twice.

To score Pn.p cell fusion, animals were stained with the monoclonal antibody MH27 (Kenyon, 1986; Francis and Waterston, 1991). Staged populations of animals were stained in early L2 after the lateral V cells had divided two to three times. P12.p behaves differently from the anterior Pn.p cells and thus was not scored for cell fusion.

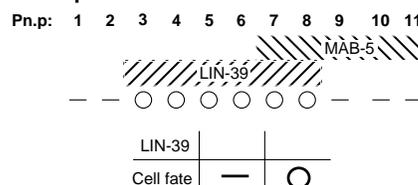
### A. P cells migrate into the ventral cord (ventral view) - early L1



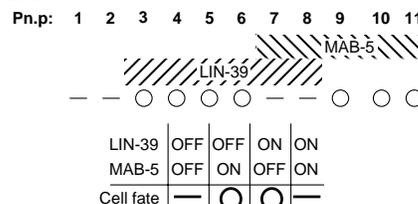
### B. 12 Pn.p cells line the ventral surface (lateral view) - late L1



### C. Hermaphrodite



### D. Male



**Fig. 1.** The Pn.p cell fusion pattern is regulated by the Hox genes *lin-39* and *mab-5*. (A) Ventral views of early-L1 (top) and mid-L1 (bottom) larvae. Six pairs of epidermal P blast cells initially lie along the ventral surface (top). During L1, the P cells migrate around each other so that a row of 12 P cells lines the ventral surface (bottom). Shortly after they migrate into the ventral cord, the P cells divide. The anterior (Pn.a) daughters become neuroblasts, while the posterior (Pn.p) daughters remain epidermal. Anterior is towards the left in this and all figures. (B) Lateral view of a late L1 larva with the 12 ventrally located Pn.p cells shown. Some of the Pn.p cells fuse with *hyp7*, which is located more dorsally along most of the mid-body region. (C,D) Pn.p cell fusion in wild-type hermaphrodite (C) and male (D). White circles indicate Pn.p cells that remain unfused, short horizontal lines indicate cells that fuse with the *hyp7* syncytium. Hox gene expression domains are similar in both sexes, as indicated in hatched regions. The tables underneath show how Hox gene expression information is interpreted in the cell fusion decision. (B-D) Reproduced, with permission, from Alper and Kenyon (Alper and Kenyon, 2001).

To construct strains containing *ref-2(mu218)*, *him-5(e1490)* and the various Hox mutations, *him-5(e1490)*; *ref-2(mu218)* males were crossed to the various Hox mutant strains. Animals homozygous for the *mu218* allele were first recovered in the F<sub>2</sub> descendants by staining families of worms with the MH27 monoclonal antibody. Animals homozygous for the Hox mutations were subsequently identified among the progeny of these *mu218* homozygotes [using the Egl phenotype for *lin-39* and the Mab (male tale defects) phenotype for *mab-5*]. *lin-39(n1760)* and *mab-5(e2088)* are both predicted to be null alleles by genetic, DNA sequence and immunofluorescence criteria (Clark et al., 1993; Salser et al., 1993; Wang et al., 1993; Maloof and

Kenyon, 1998). *mab-5(e1751)* is a gain-of-function allele that results in misexpression of wild-type *mab-5* in all Pn.p cells (Hedgecock et al., 1987; Salser et al., 1993).

All experiments (except for those with the *hs-ref-2* construct) were carried out at 20°C.

### *ref-2(mu218)* is a dominant allele

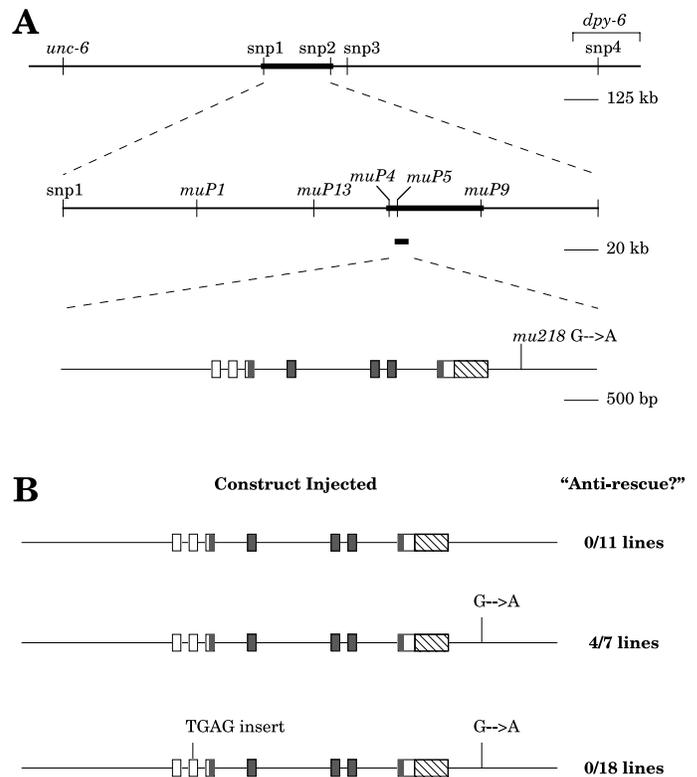
*ref-2* maps to the X chromosome (see below). Because *ref-2(mu218)* only exhibited a phenotype in males, which are genotypically XO, to test if the *mu218* allele was dominant or recessive, it was necessary to generate XX males. We did this using the mutation *tra-1(e1099)* (Hodgkin, 1987). Hermaphrodites that were *tra-1(e1099)/+; ref-2(mu218)/+* were allowed to self fertilize and the progeny were stained with MH27 to score Pn.p cell fusion. One quarter of these progeny are *tra-1(e1099)/tra-1(e1099)* XX males. If *ref-2(mu218)* was recessive, then 25% of these males would be Ref; by contrast, if *ref-2(mu218)* was dominant then 75% of these males would be Ref. We found that 71% (54/76) of these males were mutant, indicating that the *mu218* allele is dominant. This was confirmed by crossing *stDp2/+* males into *ref-2(mu218)* hermaphrodites. *stDp2* is a duplication of part of the X chromosome that includes *ref-2* (see mapping data below) fused to LG II (Meneely and Wood, 1984). All 32/32 male progeny from this cross should be *ref-2(mu218)/0*; half of these presumably carry the duplication, yet all 32 worms were phenotypically mutant, indicating that a single wild-type copy of the *ref-2* locus could not rescue the single mutant copy. Finally, injection of wild-type cosmids that cover the *ref-2(mu218)* mutation into *ref-2(mu218)* mutant worms did not rescue the mutant phenotype.

### Mapping *ref-2(mu218)*

*ref-2(mu218)* was first mapped using STS-polymorphism mapping (Williams et al., 1992) between the polymorphisms stP33 and stP129 on the center of the X chromosome. Three-factor mapping was then used to place *ref-2(mu218)* between *unc-6* and *dpy-6*, an interval of approximately 2 Mb. We then mapped *ref-2(mu218)* further using a set of single nucleotide polymorphisms (SNPs) present between our wild-type isolate N2 and another wild-type isolate of *C. elegans*, CB4856 (Koch et al., 2000; Wicks et al., 2001). We crossed CB4856 males to *unc-6(e78) ref-2(mu218) dpy-6(e14)* hermaphrodites and isolated 71 Unc-non-Dpy and 22 Dpy-non-Unc recombinants within this interval. These worm families were then stained with MH27 to score the Pn.p cell fusion phenotype. We first used a set of snip-SNPs (SNPs that alter a restriction site) to map the mutation to a 240 kb interval in between two snip-SNPs (Fig. 2A, top panel; Table 1) (Koch et al., 2000; Wicks et al., 2001). We identified 19 recombinants within this interval that were used for further mapping. To identify new SNPs in this smaller interval, we amplified by PCR small intergenic regions in this interval and sequenced these PCR products directly using a third nested primer. We identified five PCR products with polymorphisms in this way in 14 PCR sequencing attempts (Table 1). Several of these PCR products had multiple polymorphisms. We used these SNPs to map the *mu218* mutation to a 40 kb interval (Fig. 2A, middle panel).

### Cloning of *ref-2*

Worm genes are typically cloned by mapping the gene to a small region and then injecting wild-type DNA into mutant worms to rescue the mutant phenotype. Because *ref-2(mu218)* was dominant, we anticipated that this would not work. Instead, we mapped *ref-2(mu218)* to a very small interval (as described above) and then generated overlapping PCR products amplified from chromosomal DNA from a strain harboring the *mu218* mutation. These PCR products were pooled and injected into wild-type worms. Injection of some of these pools conferred the mutant phenotype on wild-type worms, what we term 'anti-rescue'. Eventually, this anti-rescuing activity was localized to a single 8 kb PCR product generated with the primers P7A and P7B (sequences below). A similar PCR product



**Fig. 2.** (A) *ref-2(mu218)* was mapped to the black regions in the top two panels, as described in Materials and Methods. Ultimately, an 8 kb PCR product was identified that induced the mutant phenotype in the progeny of injected worms (bottom panel). Only a single open reading frame is predicted on this DNA. The verified cDNA structure is indicated with exons depicted as boxes. Gray boxes indicate the region where the five zinc fingers are encoded. The 3' UTR is indicated by hatching. The G→A transition present in *mu218* DNA is also depicted. Scale is indicated in the right-hand corner of each panel. (B) Anti-rescue was achieved by injecting DNA containing the *mu218* G→A transition (middle) but not by injecting wild-type DNA (top), or DNA containing both the G→A transition and the TGAT insertion (which introduces stop codon and a frameshift prior to the first zinc finger). Anti-rescue indicates that P7.p and P8.p remained unfused in males.

amplified from wild-type chromosomal DNA did not generate a phenotype when injected into wild-type worms. These *ref-2* genomic clones were injected into worms at a concentration of 1–2 ng/μl; at higher concentrations, this DNA induced lethality. We sequenced the mutant PCR product and found a single non-coding mutation as described in the Results.

We were able to clone the *ref-2* genomic region in low copy but not high copy *E. coli* plasmid vectors, probably due to toxicity. To generate subclones of *ref-2* with various mutations, we therefore followed a PCR based strategy. Primers used were:

P7A left primer, GTCGATCGGTGTCGCGTGCCGCTGTACCAC;  
P7E left nested primer, CCATCAAATCTAACACCTTGCCGG-  
CAACTGAAC;

P7I left nested primer, GAAGTAGTGTTTTGTTTTGAATATT-  
ACCGAACGATG;

P7B right primer, GATGAATGTTGTGGGACCCACAATGT-  
GGGTGTTG;

P7G right primer, GACCCCAATTTGTTTGTAGCTGCGAGCTCAC;  
P7F right nested primer, CGGAAAACAAGATGAATGTTGTGGG-  
ACCCACAATGTGGG;

**Table 1. Primers used for SNP mapping**

snip-SNP	Primer sequences	Cosmid	Enzyme					
snp1	AGAACAGCGGACAGAGATCG TGTCACGGTACTTGCAATCG	C44E12	<i>AsnI</i>					
snp2	GGGTAGTAGCCCGTCATAAAAG TCATTTCCTATGTAGCCGGACC	C54D2	<i>AsnI</i>					
snp3	TTTCTTGACACCTCCGGTAG CTCACTCTGGTCTTTTCCG	F54E1	<i>EcoRI</i>					
snp4	GTAATCGGTACTGTGCACTG CTACATCAATGTCAACACCAGC	R04E5	<i>DdeI</i>					
SNP			Product size	Sequence	N2	CB4856		
<i>muP1</i>	CCAACGTCGGACTAGCGGACACGC GGTACCATGAACGAAGGTACCACC CGGGAGCATCAGCACAAAC	F27D9	920	5414 5849 5980	CATTTTGT CAGAA TGATG	CATT-TTGT CAAAA TGGTG	D S S	
<i>muP13</i>	GATCATTGACACATGGTCAGGCTG GAACATTGTAGATGTTAGAACTTACGAC CGTAGTCAAACCTGCAAG	K09F5	727	295	AATTT-TTTAT	AATTTTTTTAT	I	
<i>muP4</i>	GACGGCAGGCTATAAGGTAGGCG GTTTCATCGGACGTTAGCGAACTG GCCACATGGATTTGGTAC	K09F5	903	35727	ACTAT	ACAAT	S	
<i>muP5</i>	CTGCAATTTCTGCCAGCTGCCAC TTGCCACTGGTTCGGAAGTCATCG GATTGTATCTTGCCTCCG	K09F5	736	37402 38053 38061 38337 38367	TCGCT CTAAT TTGAA ACTTT TTGTA	TCCT CTGAT TTAAA ACATT TTATA	S S S S S	
<i>muP9</i>	CTCAGTGCCCAATAGCAGAAACGG GAAACCGGAATCATGCTCTTGTG CGCCAGTGATACAATGGC	ZK154	946	11337	CACAA	CATAA	S	

The four primer sets and restriction enzymes that detect RFLPs caused by snip-SNPs between the two wild-type *C. elegans* strains N2 and CB4856 are listed at the top. These four snip-SNPs were used to map *ref-2(mu218)* to a 240 kb interval as described in text. These snip-SNPs were identified by Koch et al. (Koch et al., 2000) and Wicks et al. (Wicks et al., 2000), and used for mapping as described. The SNPs identified in this work that were used to further map *ref-2(mu218)* are listed at the bottom. DNA was amplified using the first two indicated primers and then sequenced with the third nested primer. The cosmid where the SNP lies, the size of the PCR product, location in cosmid where sequence starts, sequence in N2 and CB4856, and nature of SNP are also indicated. The types of polymorphism listed on the right are: D, deletion; I, insertion; S, substitution in CB4856. As indicated, *muP1* and *muP5* have multiple SNPs in a single sequencing read.

P7J right nested primer, GGGACCCACAATGTGGGTGTTGAAAA-TTACTTTTTAAACCC;

UG1 introduces *mu218* change underlined, CAATTAATTATCTT-AAAGGAGGCACGCAACCGCATCAAAGACACCGCTACC;

UG2 reverse complement of UG1, GGTAGCGGTGCTTTGATGCGGGTTGCGTGCCTCCTTTAAGATAATTAATTG;

UG3 knocks out zinc-finger gene [inserted bases (underlined) introduce stop codon and frameshift] CAGATCCACATGCTTAC-TCTCCGAGCTGAGTTCTACATGAACATGTACAGGTGC; and UG4 reverse complement of UG3, GCACCTGTGACATGTT-CATGTAGAACTCAGCTCGGAGAGTAAGCATGTGGATCTG.

The wild-type PCR clone was generated by PCR from cosmid C47C12 using primers P7I and P7J. The PCR product in which we reintroduced the *mu218* mutation downstream of the *ref-2* gene was generated in two steps. First the left half of the final product was amplified from C47C12 with primers P7E and UG2 and the right half was similarly amplified from C47C12 with primers UG1 and P7F. These two overlapping DNA products were combined and amplified with primers P7I and P7J to generate the final *mu218*-like product. To generate a DNA product containing the *mu218* mutation and a zinc-finger gene knockout mutation, we first amplified three smaller PCR fragments from C47C12 using primers P7A and UG4 (left side), UG3 and UG2 (middle), and UG1 and P7G (right side). The left and middle DNA products were then pooled and amplified using primers P7A and UG2; similarly, the right and middle products were pooled and amplified using primers UG3 and P7G. Finally, these two DNA products were combined and amplified by PCR with primers P7I and P7J. The wild-type construct failed to anti-rescue 11 lines. The construct in which the *mu218* mutation had been re-engineered did cause anti-rescue in 4/7 lines (20-85% anti-rescue). The construct

containing both the *mu218* mutation and the zinc-finger gene knockout failed to anti-rescue 18 lines.

### Isolation of *ref-2* cDNA

We isolated and sequenced cDNA clones corresponding to the predicted open reading frame C47C12.3. These clones were generated by RT-PCR (Frohman, 1993) from wild-type DNA. The oligo(dT) primer was used to obtain the 3' end. Two gene prediction programs, Genefinder and Genie, predicted two potential 5' cDNA ends (Kent and Zahler, 2000); we detected a RT-PCR product using a primer to the 5' end predicted by Genie but not the 5' end predicted by Genefinder. While *ref-2* is not spliced to the SL1 trans-spliced leader sequence, we did obtain a fortuitous PCR product with the SL1 primer that allowed us to identify 23 bp of cDNA 5' to the translation start site and we therefore infer that this is the proper translation start site for *ref-2*. Only a single isoform was isolated from several cDNA clones. A full-length cDNA clone that extends from the ATG start site to the TGA stop site (plasmid pSA175B) was amplified and cloned into the T/A cloning vector PCR2.1TOPO (Invitrogen) using the primers RT5 ATGATGCATAGTGACTATTATACTC and RT7 TCAGTAGGAATCAGGTTTTGCC.

### RNAi of *ref-2*

dsRNA was prepared from a full-length cDNA clone (pSA175B) and injected into *mulS65* hermaphrodites. *mulS65* contains an integrated copy of *jam-1-gfp* (Mohler et al., 1998) that had been co-injected with *dpy-20(+)*. Pn.p cell fusion was scored in early to mid-L2. Unfused cells retain their cell junctions as visualized with this *gfp* fusion. The RNAi cell fusion defect was readily observed when dsRNA at a concentration of 100-500 ng/ $\mu$ l was injected. At higher concentrations (>1  $\mu$ g/ $\mu$ l), Pn.p cells were often not present in the ventral cord

(determined using Nomarski optics and *jam-1-gfp*) as described in the Results. In a typical RNAi experiment, a range of phenotypes were observed from worms containing mostly fused Pn.p cells, worms with almost all Pn.p cells missing and worms with a mixture of both, with the ratio of cells missing to cells fused or unfused increasing as the dsRNA concentration increased.

### **hs-ref-2 experiments**

To construct the *hs-ref-2* fusion, the *ref-2* cDNA was amplified by PCR from pSA175B using primers with engineered *XhoI* sites: RT21 CACACTCGAGCGATGATGCATAGTGACTATTATACTC and RT22 CACACTCGAGTCAGTAGGAATCAGGTTTTGCC. This PCR product was digested with *XhoI* and ligated into pCH15.1 (vector containing *hsp16/48*) (Rusnak and Candido, 1985; Hunter and Kenyon, 1995) that had been digested with *XhoI*. The *hs-ref-2* construct was injected at a concentration of 20 ng/μl and integrated into the chromosome. To control for changes in *hs-ref-2* copy number during outcrosses and double mutant construction, the *hs-ref-2* chromosome was made homozygous first and then the *Hox* or *rde-2* mutation was subsequently made homozygous. The wild-type siblings from homozygous *hs-ref-2* parents were scored for Pn.p cell fusion along with the relevant mutant doubles. *hs-ref-2* was induced during L1 in a manner similar to that described for *hs-lin-39* previously (Maloo and Kenyon, 1998). Briefly, newly hatched populations of worms were placed on 2.5 cm agar plates seeded with OP50. Plates were placed on an MJ research PCR machine temperature block covered with oil and heat pulsed at 31°C for 30 minutes every 3.5 hours starting 5 hours after hatching. In between pulses, the block was maintained at 20°C. Pn.p cell fusion was scored after the fourth heat pulse, when strains were in early L2. Control non-heat pulsed worms were grown at 20°C.

### **ref-2 expression experiments**

To determine where and when *ref-2* was expressed, we generated an antiserum to a region of REF-2 C-terminal to the zinc fingers (the last 72 amino acids). A PCR fragment generated using the primers HRF5 CACACTCGAGCCGGAACATGACGAGAGTAGC and HRF3 CACAAAGCTTTCAGTAGGAATCAGGTTTTGCC was digested with *XhoI* and *HindIII* and cloned into pRSETA (Invitrogen) that had been digested with *XhoI* and *HindIII*. This plasmid was transformed into the *E. coli* expression strain BL21(DE3)/plysS and His<sub>6</sub>-REF-2 protein production was induced with IPTG. The protein fusion was purified using Ni-NTA (Qiagen) affinity chromatography according to the manufacturer's instructions and injected into rabbits at Covance Research Products.

The anti-REF-2 antiserum was affinity purified as follows: 40 μg of His<sub>6</sub>-REF-2 was coupled to 50 μl affigel-15 (BioRad), according to the manufacturer's instructions and packed into a column. The column was washed in PBS and 100 μl of antiserum was then passed over the column five times. The column was washed with PBS and PBS+400mM NaCl, and the purified antiserum was eluted with 150 μl 0.2 M glycine, 1 mM EDTA (pH 2.5) directly into 30 μl 1 M Tris (pH 8.0). Staining with this purified antiserum was carried out using a modification of the procedure described by Miller and Shakes (Miller and Shakes, 1995). Briefly, worms were fixed in RFB with 0.5% paraformaldehyde for 30 minutes on ice after three freeze thaws [buffers as described by Miller and Shakes (Miller and Shakes, 1995)]. The worms were then washed in TBT, incubated in TBT + BME for 4 hours at 37°C, washed with BOBT, incubated in BOBT + 10 mM DTT for 15 minutes, washed with BOBT, incubated with BOBT + 0.3% H<sub>2</sub>O<sub>2</sub> for 15 minutes, washed with AbB, washed with AbA, and finally washed twice with distilled water. Worms were then fixed to polylysine-coated slides, freeze cracked, blocked for 1 hour in AbA, incubated with the affinity purified antiserum (diluted 1:2 – 1:4) and MH27 (1:100) in AbA, washed four times in TBST, incubated with goat anti-rabbit-rhodamine (1:100) and goat anti-mouse-FITC (1:100) in AbA, washed four times in TBST, and finally

examined in the presence of 5 ng/ml DAPI in PBS. Images were captured with a CCD. Images were colored in Photoshop so that rhodamine was red and FITC was green. Nuclei were identified based on the DAPI staining pattern. At least 30 worms were examined at each time point. Anti-REF-2 staining was scored as strong (present at easily detectable levels in most worms), weak (a weak signal was detectable in only some worms) or absent. In N2, 100% of P(3-8).p stained with the anti-REF-2 antiserum (strong staining) both before and after the posterior cells fused, while 23% and 13% of P(9-11).p had detectable levels of REF-2 (weak staining) shortly before and after cell fusion, respectively. By contrast, 58% of P(9-11).p had detectable levels of REF-2 shortly after the time of Pn.p cell fusion in *egl-27* mutant worms.

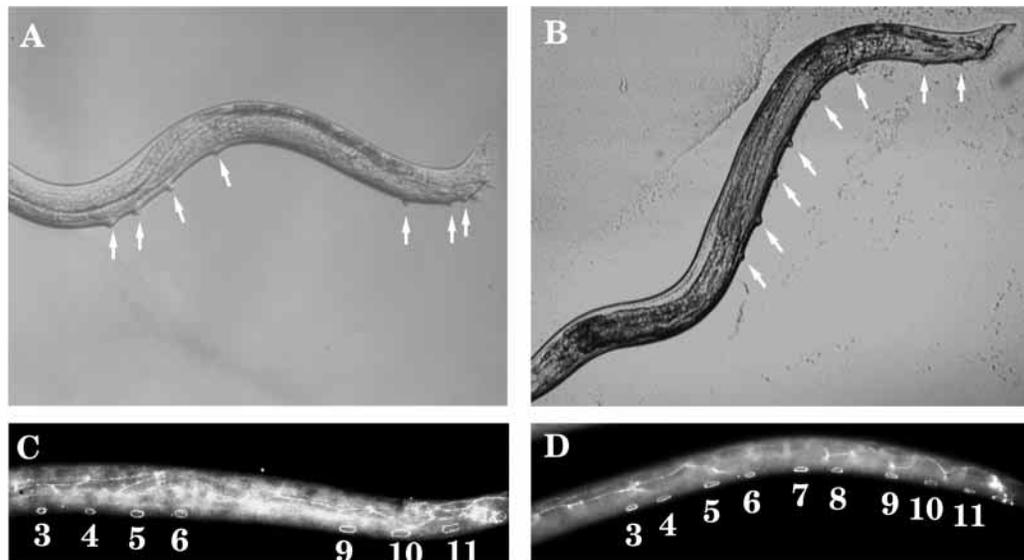
In addition to the anti-REF-2 staining described in the Results, very weak staining was rarely observed in some body wall muscle nuclei. However, this staining was inconsistent and was still present in worms in which *ref-2* expression was inhibited by transgene mediated co-suppression, suggesting that it is probably due to cross-reactivity of the antiserum with another protein.

## **RESULTS**

### **Isolation of *ref-2(mu218)***

To identify mutations that affect Pn.p cell fusion, we mutagenized worms containing mutations in *lin-12*, a homolog of the *Drosophila Notch* receptor (Yochem et al., 1988). In *lin-12(n137)* animals, all unfused Pn.p cells generate ectopic pseudovulval or hook-like structures that protrude from the ventral surface of the worm (Greenwald et al., 1983). We screened for mutations that alter the pattern of ventral protrusions in a *lin-12(n137); him-5(e1467)* background, as described previously (Alper and Kenyon, 2001) [the *him-5* mutation causes hermaphrodite worms to generate some male progeny (Hodgkin et al., 1979)]. In this screen, we identified one mutation that specifically affected the male ventral protrusion pattern. *lin-12(n137)* males have four protrusions in the mid-body region corresponding to P(3-6).p and 1-3 protrusions in the tail region corresponding to P(9-11).p (Fig. 3A) (the protrusions in the tail are sometimes obscured by other male tail structures). No protrusions are visible in between these two clusters (Fig. 3A); this corresponds to the region where P7.p and P8.p have fused with *hyp7*. In our screen, we identified *lin-12(n137); him-5(e1467); ref-2(mu218)* mutant worms in which this gap in protrusions is no longer present (Fig. 3B). To verify that the *ref-2(mu218)* mutation was affecting Pn.p cell fusion, we stained *ref-2(mu218)* mutant animals with the monoclonal antibody MH27, which recognizes a component of adherens junctions and thus outlines unfused cells (Kenyon, 1986; Francis and Waterston, 1991). As expected, whereas P(3-6).p and P(9-11).p remain unfused in early L2 in wild-type males (Fig. 3C, Fig. 4A), P(3-11).p all remain unfused in *ref-2(mu218)* mutant males (Fig. 3D, Fig. 4A). The *ref-2(mu218)* mutation did not cause any other obvious cell fusion or patterning defects in either males or hermaphrodites. While P(3-6).p and P(9-11).p remain unfused in L1 in wild-type males, P(3-6).p and P9.p do fuse with *hyp7* at a later stage of development. Similarly, in *ref-2(mu218)* mutant males that are genotypically wild type for *lin-12*, P(3-9).p, all fuse with *hyp7* later in development. Thus, *ref-2(mu218)* specifically affects the early pattern of Pn.p cell fusion but not the later pattern. The *ref-2(mu218)* mutation is also fully dominant (see Materials and Methods). This

**Fig. 3.** P7.p and P8.p remain unfused in *ref-2(mu218)* males. (A,B) Unfused Pn.p cells are inferred from the presence of ventral protrusions (indicated by white arrows) in a *lin-12(n137); him-5(e1467)* worm. *lin-12(n137)* males (A) contain a gap in the ventral protrusion pattern that corresponds to the region where P7.p and P8.p have fused with *hyp7*. No gap in the protrusion pattern is present in *lin-12(n137); ref-2(mu218)* males (B). (C,D) Unfused Pn.p cells visualized by immunostaining animals with the MH27 monoclonal antibody. P(3-6).p and P(9-11).p remain unfused in wild-type males (C), while P(3-11).p remain unfused in *ref-2(mu218)* mutant males (D).



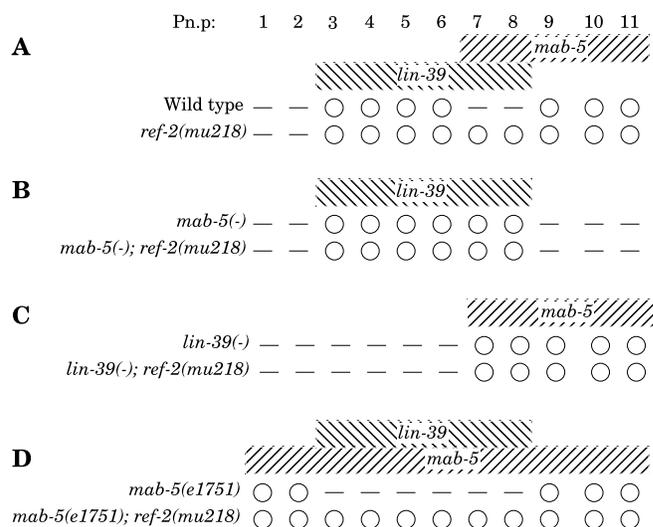
observation and those below suggest that the *mu218* mutation is an unusual regulatory mutation.

### The *ref-2(mu218)* mutation affects the ability of LIN-39 and MAB-5 to inhibit each other's activities

Why do P7.p and P8.p remain inappropriately unfused in *ref-2(mu218)* mutant males? It was intriguing that this region corresponds to the region in males where both *lin-39* and *mab-5* are expressed, and thus where the two Hox proteins inhibit each other's activities. One possibility was that one of the two Hox genes was no longer expressed in this region and thus the single remaining Hox gene could prevent cell fusion. For example, if *lin-39* were no longer expressed in P7.p and P8.p, then only MAB-5 would be present in those cells and they would remain unfused. To determine if *lin-39* expression was altered in *ref-2(mu218)* mutant animals, we examined the Pn.p cell fusion pattern in a *mab-5; him-5; ref-2(mu218)* triple mutant. The *lin-39* expression pattern should correspond to the Pn.p cell fusion pattern in *mab-5* mutant males because only the single Hox gene *lin-39* will be present in those Pn.p cells. We found that P(3-8).p remain unfused in *mab-5* mutant males in the presence or absence of the *ref-2(mu218)* mutation (Fig. 4B), suggesting that *lin-39* expression is not altered in *ref-2(mu218)* animals. This was confirmed by examining the *lin-39* expression pattern in *ref-2(mu218)* mutants using an anti-LIN-39 antiserum; *lin-39* expression was wild type in *ref-2(mu218)* mutant animals ( $n > 50$ ). Similarly, we infer that *mab-5* expression was not altered in *ref-2(mu218)* mutants because P(7-11).p remain unfused in *lin-39* mutant males, whether or not the *ref-2(mu218)* mutation was present (Fig. 4C). Thus, *ref-2(mu218)* does not appear to affect the fusion pattern by changing Hox gene expression.

An alternate possibility was that *ref-2(mu218)* was specifically affecting the ability of the two Hox proteins to inhibit each other's activities. If this hypothesis is true, then Pn.p cells expressing both *lin-39* and *mab-5* should remain unfused even if Hox gene expression patterns are altered. To test this possibility, we examined the Pn.p cell fusion pattern in worms harboring the *mab-5(e1751)* mutation, a promoter mutation that causes wild-type *mab-5* to be ectopically

expressed in all Pn.p cells (Hedgecock et al., 1987; Salser et al., 1993). In *mab-5(e1751)* males, P(3-8).p all fuse with *hyp7* because both Hox genes are now present in those cells, while P(1,2).p and P(9-11).p remain unfused because only MAB-5 is present in those cells (Fig. 4D). In *mab-5(e1751); ref-*



**Fig. 4.** The *ref-2(mu218)* mutation prevents LIN-39 and MAB-5 from canceling each other's activities in males. The indicated mutants were stained with MH27 in early L2 to score Pn.p cell fusion in males. White circles indicate unfused Pn.p cells, short horizontal lines indicate Pn.p cells that have fused with *hyp7*. Hox gene expression patterns in each set of Hox mutant strains are indicated above each set of genotypes. Alleles used: *lin-39(n1760)* and *mab-5(e2088)*, both null mutations, and *mab-5(e1751)*, in which wild-type *mab-5* is misexpressed in all Pn.p cells. All strains also harbored the *him-5(e1490)* mutation to generate males. At least 50 worms of each genotype were scored. All phenotypes listed are 100% penetrant except for: in *mab-5(e1751)*; *him-5(e1490)* worms, P1.p fused with *hyp7* in 1/52 worms and P3.p remained unfused in 1/52 worms, and in *mab-5(e1751); him-5(e1490); ref-2(mu218)* worms, P6.p fused with *hyp7* in 1/51 worms.



gene encoding a protein with five C<sub>2</sub>H<sub>2</sub> zinc-finger domains. We used RT-PCR to identify the cDNA corresponding to this predicted zinc-finger gene and found that the G→A transition in the *mu218* DNA was 399 bp downstream of the 3' end of the 3' UTR of this gene (Fig. 2A; Fig. 5A,B). This raised the possibility that the *mu218* mutation lies in an enhancer that regulates expression of this gene. The observation that the *mu218* allele is dominant is consistent with this hypothesis.

To confirm that the G→A transition was responsible for the *mu218* phenotype, we re-engineered this mutation by generating PCR products derived from the wild-type cosmid C47C12. Injection of a wild-type PCR product into wild-type worms did not affect the Pn.p cell fusion pattern, while injection of a PCR product in which the G→A transition had been re-engineered did prevent fusion of P7.p and P8.p in males (Fig. 2B). To test if this mutation was affecting Pn.p cell fusion by affecting the zinc-finger gene, we also generated and injected a construct containing this G→A mutation in which the zinc-finger gene was also knocked out (by the addition of a 4 bp insertion that introduces a stop codon and a frame shift prior to the five zinc fingers). The zinc-finger gene knockout abrogated the ability of this construct to prevent fusion of P7.p and P8.p (Fig. 2B). Thus, the *mu218* mutation affects Pn.p cell fusion by affecting this zinc-finger gene, which we now call *ref-2*. The re-engineered G→A mutation also functions in *cis*, as it did not affect the endogenous *ref-2* gene, consistent with the hypothesis that *mu218* may lie in an enhancer for *ref-2*.

What is the likely function of *ref-2*? The zinc-finger domain in *ref-2* is most similar to that of the *opa/Zic* family of zinc-finger genes (Fig. 5C) (Aruga et al., 1994; Benedyk et al., 1994; Cimbora and Sakonju, 1995; Aruga et al., 1996; Knight and Shimeld, 2001). *opa* is a *Drosophila* pair-rule gene, and the Zic genes play a role in vertebrate development. Four of the five zinc fingers in proteins of this family are very similar to the zinc fingers present in the related proteins *C. elegans* TRA-1 and *Drosophila* Cubitus interruptus (Ci). However, the first zinc finger in these family members is more diverged, containing extra amino acids in between the first two cysteines. The zinc-finger domain of REF-2 is >60% identical to the corresponding domain in the Opa/Zic family and ~50% identical to the corresponding domain in TRA-1 and Ci. The theory that *opa* and the Zic genes are derived from a common ancestral gene is supported by the observation that all of these genes share two introns that are inserted in identical locations within each gene (Aruga et al., 1996). Interestingly, these two intron insertion sites are also conserved in *ref-2* (introns 4 and 6). Many of these zinc-finger proteins that are strongly related to REF-2 act as transcription factors (Klug and Rhodes, 1987; Zarkower and Hodgkin, 1992; Klug and Schwabe, 1995; Aza-Blanc and Kornberg, 1999; Chen and Ellis, 2000; Yang et al., 2000; Yi et al., 2000) and we therefore infer that REF-2 likewise probably acts by regulating transcription.

### ***ref-2(+)* is required for Pn.p cells to be generated and to remain unfused**

Because the *mu218* mutation does not lie in the *ref-2*-coding sequence, we wished to determine if *ref-2* normally plays a role in the regulation of Pn.p cell fusion. To examine the *ref-2* (loss-of-function) phenotype, we carried out RNA interference (RNAi) (Fire et al., 1998) of *ref-2* by injecting dsRNA corresponding to the complete *ref-2*-coding sequence into worms harboring the

Pn.p:	1	2	3	4	5	6	7	8	9	10	11
Wild type	—	—	○	○	○	○	○	○	—	—	—
Weak <i>ref-2</i> (RNAi)	—	—	—	—	—	—	—	—	—	—	—
Strong <i>ref-2</i> (RNAi)	A	A	A	A	A	A	A	A	A	A	A

**Fig. 6.** Wild-type *ref-2* is required for Pn.p cells to be generated and to remain unfused. *ref-2* gene function was inhibited by RNAi as described in the Materials and Methods. A range of phenotypes was observed depending on the concentration of dsRNA injected. Weaker RNAi produced worms in which most of the Pn.p cells had fused (short horizontal lines), Stronger RNAi produced worms in which most of the Pn.p cells were absent from the ventral cord (A). White circles indicate unfused Pn.p cells. Pictured are data for hermaphrodites; similar phenotypes were also observed in males.

*jam-1-gfp* fusion (*jam-1-gfp* encodes GFP fused to the protein recognized by the MH27 antibody, allowing the Pn.p cell fusion pattern to be visualized in live animals) (Mohler et al., 1998). Many of the hermaphrodite progeny of injected worms were unable to lay eggs because they lacked a vulva. While P(3-8).p all remain unfused in wild-type hermaphrodites, few or none of the Pn.p cells remained unfused in *ref-2*(RNAi) hermaphrodites (Fig. 6). Thus, the wild-type *ref-2* gene keeps Pn.p cells from fusing. This was a striking result because it was, in a sense, a phenotype opposite to that caused by the *mu218* mutation, in which additional Pn.p cells fail to fuse. This suggests that the *mu218* mutation might be a gain-of-function mutation that causes a phenotype reciprocal to that of the loss-of-function phenotype. Likewise, few or none of the Pn.p cells remained unfused in *ref-2*(RNAi) males generated by injecting dsRNA into *jam-1::gfp; him-8(e1489)* worms, suggesting that *ref-2* is required for Pn.p cells to remain unfused in both sexes.

Interestingly, when dsRNA corresponding to *ref-2* was injected at higher concentrations (see Materials and Methods), we found that Pn.p cells (and the progeny of the Pn.a cells) were often not present in the ventral cord (Fig. 6). By lineaging and spot checking the progeny of injected worms, we found that several features of the normal P-cell lineage program were abnormal. While P cells were always present in their normal position at hatching, we observed that most P cells failed to migrate into the ventral cord during L1. The P cells that remained on the ventrolateral surface underwent a variety of fates, ranging from fusion with the hyp7 syncytium, cell death or cell division sometimes followed by cell death. The cell death phenotype did not appear similar to the canonical programmed cell death pathway as none of the characteristic features of apoptosis were observed using Nomarski optics. Instead nuclei became less distinct over time until they vanished completely.

Thus *ref-2(+)* is required both to generate Pn.p cells and to keep Pn.p cells unfused. We did not observe any other cell fusion defects in *ref-2*(RNAi) worms, suggesting that the role of *ref-2* in cell fusion is specific to the Pn.p cells.

### ***ref-2(+)* prevents Pn.p cell fusion in a Hox gene dependent manner**

To determine if *ref-2* was sufficient to prevent Pn.p cell fusion, we ectopically expressed *ref-2* using a heat-shock inducible promoter (*hs-ref-2*). We found that when *ref-2* expression was induced in hermaphrodites by several heat pulses during L1, P(3-11).p all remained unfused (Table 2). Thus, misexpression or overexpression of *ref-2* can prevent fusion of P(9-11).p.

**Table 2. *hs-ref-2* prevents Pn.p cell fusion in hermaphrodites**

Strain	Number of unfused Pn.p cells						Unfused Pn.p cells (%)										
	(-) Heat shock			(+) Heat shock			(+) Heat shock										
	P(3-8).p	P(9-11).p	<i>n</i>	P(3-8).p	P(9-11).p	<i>n</i>	1	2	3	4	5	6	7	8	9	10	11
Wild type	6.0	0	–	6.0	0	100	0	0	100	100	100	100	100	100	0	0	0
<i>muls97</i>	4.3	0	103	5.2	1.6	96	0	2	70	88	90	92	91	94	53	50	55
<i>muls99</i>	4.7	0	237	5.1	1.3	102	0	0	61	86	87	94	92	93	51	32	45
<i>muls99; rde-2</i>	5.8	0	71	5.8	1.5	30	0	0	93	97	97	97	100	100	50	47	50
<i>rde-2</i>	5.9	0	38	6.0	0	100	0	0	100	100	100	100	100	100	0	0	0

The indicated *C. elegans* hermaphrodite strains were grown at 20°C in the presence or absence of heat pulses during L1 (heat shock protocol is described in the Materials and Methods) and then stained with the MH27 antibody in early L2 to score Pn.p cell fusion. *muls97* and *muls99* are independently isolated chromosomal integrants of *hs-ref-2*. Because some Pn.p cells are not present in the ventral cord in the absence of heat shock in the strains that harbor the *hs-ref-2* construct, the identity of unfused cells could not always be determined with certainty; therefore, only a tally of the total number of unfused cells is listed in the absence of heat shock. Expression of *ref-2* using heat pulses overcame this background of missing cells; therefore, in the presence of heat shock, both the total tally as well as data for each individual Pn.p cell are listed. The *rde-2* allele used is *rde-2(ne221)* (Tabara et al., 1999).

Interestingly, no single heat pulse was able to affect Pn.p cell fusion, suggesting that *ref-2* might be acting at more than one point during Pn.p cell development.

Surprisingly, in the absence of heat shock, we found that worms harboring the *hs-ref-2* fusion exhibited a Pn.p cell defect – some Pn.p cells fused with hyp7 and some Pn.p cells were not present in the ventral cord (Table 2). This was similar to the phenotypes observed in *ref-2(RNAi)* worms. This was an unexpected result; one possibility was that uninduced *hs-ref-2* was inhibiting the endogenous *ref-2* gene via a dsRNA-like mechanism. Such an effect has been reported for a variety of transgenes and is referred to as transgene-mediated co-suppression (Dernburg et al., 2000; Ketting and Plasterk, 2000). In these other cases, the ability of the transgene to inhibit the endogenous gene copy was dependent on a subset of the RNAi machinery genes, including *rde-2* (Dernburg et al., 2000; Ketting and Plasterk, 2000). We therefore constructed the *hs-ref-2; rde-2* double mutant and found that the *rde-2* mutation was able to suppress the Pn.p cell defect in uninduced *hs-ref-2* worms (Table 2). Using an anti-REF-2 antiserum (see below), we also found reduced REF-2 levels in uninduced *hs-ref-2* worms, confirming that the transgene affected production of REF-2. By contrast, extremely high levels of REF-2 were detected with this antiserum after heat shock, indicating that heat shock can overcome this weak transgene-mediated *ref-2* loss-of-function phenotype. This has two implications. First, it confirms the results observed using *ref-2* RNAi, as similar loss-of-function phenotypes were observed by this transgene mediated co-suppression. Second, it indicates that when induced, *hs-ref-2* has to overcome this weak loss-of-function background; this explains why the penetrance of rescue in ‘wild-type’ cells is not always 100%.

While *hs-ref-2* was able to prevent fusion of P(9-11).p in hermaphrodites, the fusion of P1.p and P2.p as well as other lateral cell fusions were not affected by expression of *ref-2* using the heat shock promoter. Why were posterior Pn.p cells affected while anterior ones were not? One of the differences between these two sets of cells is that *mab-5* is expressed in the posterior, raising the possibility that REF-2 may require the activity of a Hox protein to keep a Pn.p cell unfused. To test this possibility, we examined the effect of *hs-ref-2* when induced in a strain that also harbors a *lin-39* mutation. In a *lin-39* mutant background, *hs-ref-2* was able to keep P(7-11).p unfused but P(3-6).p did fuse with hyp7 (Table 3). Thus, *ref-2* alone is insufficient to keep P(3-6).p unfused when *lin-39* is absent. However, *hs-ref-2* can keep

cells unfused in the region where *mab-5* is expressed, P(7-11).p, even though *mab-5* is normally not active in hermaphrodite Pn.p cells. Similarly, the ability of *hs-ref-2* to keep posterior Pn.p cells unfused depends on *mab-5* because the effect of *hs-ref-2* on P9.p and P10.p is strongly, but not completely, suppressed by the addition of a *mab-5* mutation (Table 3). P11.p is sometimes transformed into P12.p in a *mab-5* mutant worm (Kenyon, 1986), and thus P11.p often remains unfused in a *mab-5* mutant, even in the absence of *hs-ref-2*. A few posterior Pn.p cells still remain unfused in *hs-ref-2* worms when *mab-5* is removed. One possible explanation for this is that the more posterior Hox gene *egl-5* is misexpressed more anteriorly in *mab-5* mutants (Ferreira, 1999), and *egl-5* might therefore affect Pn.p cell fusion when *ref-2* is misexpressed in a *mab-5* mutant background.

The *ref-2(RNAi)* and *hs-ref-2* experiments together indicate that REF-2 is necessary, but not sufficient, to keep Pn.p cells unfused; a Hox gene is also required to prevent fusion. *lin-39* expression is not altered by the transgene mediated inhibition of *ref-2* or by ectopic expression of *ref-2* with heat shock ( $n > 50$  for both experiments). This suggests that *ref-2* is not functioning by altering Hox gene expression.

### ***ref-2* is expressed dynamically in the P cell lineage**

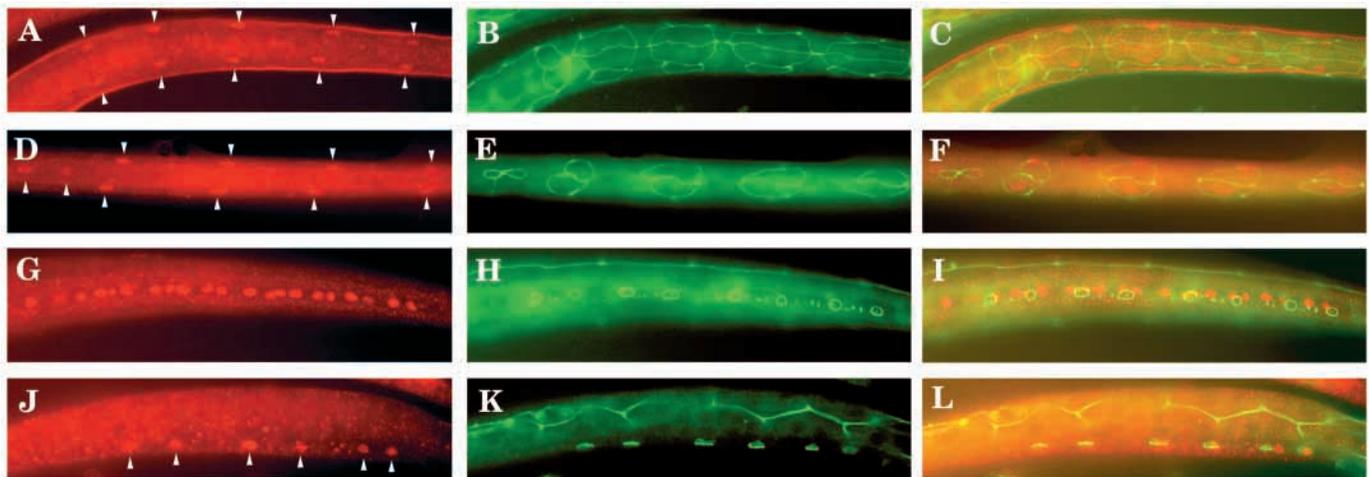
To determine where and when *ref-2* was functioning, we generated an antiserum to the C terminus of REF-2 and stained worms to examine the *ref-2* expression pattern. We found that REF-2 localized to the nucleus in cells in which it was present, consistent with the idea that it functions as a transcription factor. To ask whether the antiserum recognized REF-2 protein, we stained worms in which *ref-2* was expressed using the *hs-ref-2* construct and observed very strong nuclear expression in most cell types. We also stained worms harboring the *hs-ref-2* fusion in the absence of heat shock. As described above, these worms exhibit a moderate loss-of-function *ref-2* phenotype; when stained with the anti-REF-2 antiserum, we found that REF-2 was detected much more weakly than in wild-type worms.

The genetics suggest that REF-2 should be present in the P and Pn.p cells, as *ref-2* is required for the proper development of those cells. We therefore examined *ref-2* expression in wild-type hermaphrodites using the anti-REF-2 antiserum. REF-2 was first detected weakly in all 12 P cells just before P-cell migration (Fig. 7A-C, Fig. 8). REF-2 was present in all P cells as migration occurred and remained in both P cell daughters after division in the ventral cord (Fig. 7D-I; Fig. 8). This is also the point at which

**Table 3. *hs-ref-2* prevents fusion of posterior Pn.p cells in a Hox-dependent manner**

Strain	Number of unfused Pn.p cells						Unfused Pn.p cells (%)										
	(-) Heat shock			(+) Heat shock			(+) Heat shock										
	P(3-8).p	P(9-11).p	n	P(3-8).p	P(9-11).p	n	1	2	3	4	5	6	7	8	9	10	11
Wild type	6.0	0	–	6.0	0	100	0	0	100	100	100	100	100	100	0	0	0
<i>lin-39</i>	0	0	100	0	0	50	0	0	0	0	0	0	0	0	0	0	0
<i>mulS97</i>	4.8	0	120	5.5	1.5	72	1	0	82	94	93	92	93	93	56	42	53
<i>mulS97; lin-39</i>	0	0	101	0.3	0.9	119	0	1	1	0	1	3	13	17	26	27	34
<i>mab-5</i>	6.0	0.1	191	6.0	0.2	111	0	0	100	100	100	100	100	100	0	0	23
<i>mulS97</i>	3.0	0	255	3.7	1.5	227	0	0	30	49	57	73	85	81	50	45	56
<i>mulS97; mab-5</i>	3.3	0.1	334	3.4	0.4	305	0	0	27	51	61	68	69	67	8	11	20
<i>mulS99</i>	4.1	0	107	4.2	1.1	52	0	0	33	67	67	83	87	83	37	33	42
<i>mulS99; mab-5</i>	2.9	0.1	192	3.3	0.6	205	0	0	26	46	60	64	71	69	11	13	39

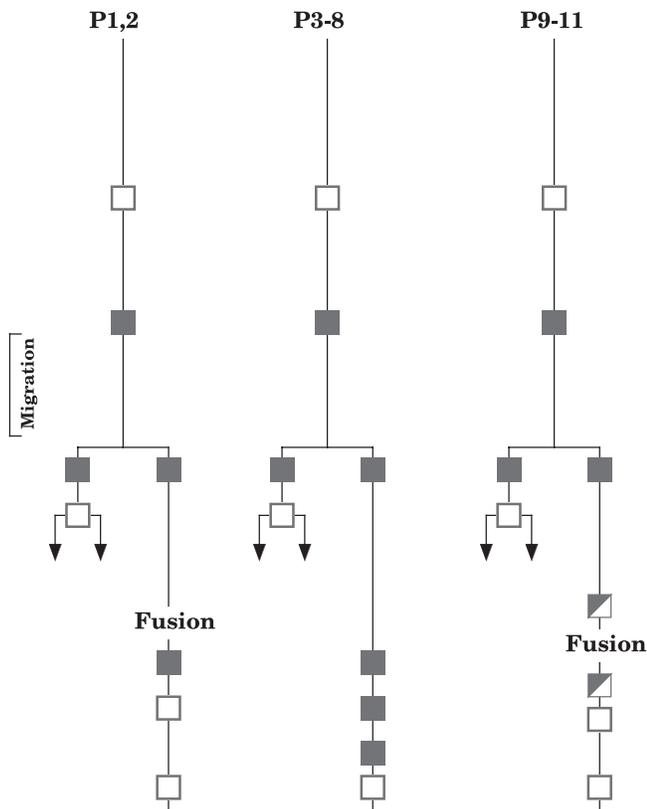
The indicated *C. elegans* hermaphrodite strains were grown at 20°C in the presence or absence of heat pulses during L1 as indicated (heat shock protocol is described in the Materials and Methods) and then stained with the MH27 antibody in early L2 to score Pn.p cell fusion. Data are presented as described in Table 2. *mulS97* and *mulS99* are independently isolated chromosomal integrants of *hs-ref-2*. The *lin-39(n1760)* and *mab-5(e2088)* alleles used are nulls (Clark et al., 1993; Salser et al., 1993; Wang et al., 1993; Maloof and Kenyon, 1998). The *hs-ref-2; Hox(+)* strain listed immediately above each *hs-ref-2; Hox(-)* strain is the *Hox(+)* sibling derived from the homozygous *hs-ref-2; Hox(-)/Hox(+)* parent strain. This controls for changes in copy number during *hs-ref-2* crosses, as detailed in the Materials and Methods. Thus, *mulS97* or *mulS99* data sets may differ slightly in individual experiments and the *Hox* mutant doubles should be compared with the *Hox(+)* strains listed immediately above them.



**Fig. 7.** *ref-2* is expressed in the P cell lineage in wild-type hermaphrodites. Staged population of L1 larvae were stained with an anti-REF-2 antiserum (red in A,D,G,J) and the MH27 monoclonal antibody (green in B,E,H,K) as described in the Materials and Methods. Merged images are shown in C,F,I,L. REF-2 is initially detected in the 12 P cells just prior to their migration into the ventral cord (A–C; ventral view) and remains on as the P cells migrate (D–F; ventral view). In this picture, P1 and P2 have migrated; the other P cells will migrate soon. REF-2 is then present in both daughters of all P cells following division (G–I; ventrolateral view). REF-2 then disappears in the Pn.a cells and their progeny. REF-2 also disappears in the Pn.p cells, although it does so at different rates. REF-2 remains on longest in the six unfused Pn.p cells P(3-8).p (J–L, lateral view). White arrowheads indicate P1/2, P3/4, P5/6, P7/8, P9/10 in A, P1 and P2 (which have migrated) and P3/4, P5/6, P7/8, P9/10 in D, and P(3-8).p in J.

anti-REF-2 staining was strongest. REF-2 then disappears in the Pn.a cell lineage (Fig. 7J–L; Fig. 8). REF-2 also disappears from the Pn.p cells, although it does so at different rates in different Pn.p cells. REF-2 is present for the longest time in the six unfused Pn.p cells P(3-8).p (Fig. 7J–L; Fig. 8). REF-2 is also present in P1.p and P2.p shortly after those cells fuse with *hyp7*, although REF-2 decreases to an undetectable level soon after (Fig. 8). REF-2 disappears most rapidly in P(9-11).p, with REF-2 being detectable in only some worms around the time of Pn.p cell fusion (Fig. 8). In summary, REF-2 protein levels decrease around the time of Pn.p cell fusion, although they do so less quickly in the cells that remain unfused. We also detected REF-2 protein in the nuclei of the B and Y cells in the tail region during L1.

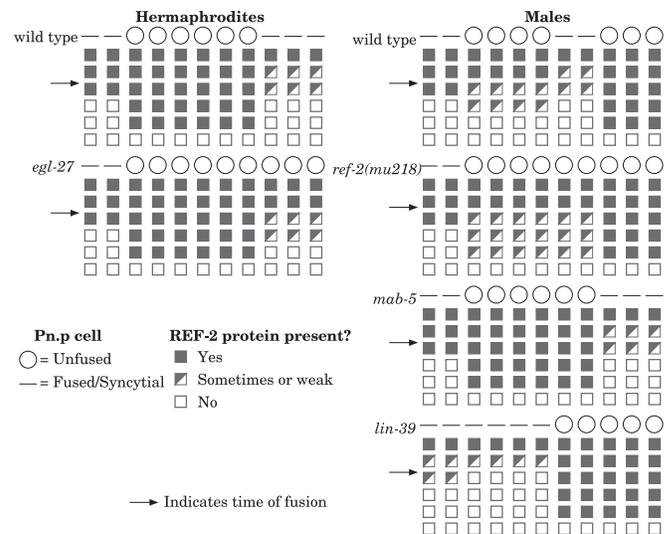
The ability of the *mu218* mutation to prevent LIN-39 and MAB-5 from canceling each other's activities suggested that *ref-2* could be a transcriptional target of the Hox genes and that the *mu218* mutation might affect Hox-mediated *ref-2* expression. To test if the Hox genes are regulating *ref-2* expression, we examined *ref-2* expression in wild-type, *lin-39* and *mab-5* mutant males (Fig. 9). In general, the early pattern of anti-REF-2 staining was the same in all three strains: on in all P cells just before P cell migration, and continued *ref-2* expression in the migrating P cells and in both P cell daughters. Expression then decreased in Pn.p cells with expression lingering longest in the cells that remain unfused. In wild-type males, *ref-2* expression lingered longest in the cells that remain



**Fig. 8.** *ref-2* is expressed dynamically in the P cell lineage. The figure summarizes the REF-2 staining pattern in the P cell lineages during L1 in wild-type hermaphrodites. Black squares indicate REF-2 present, white squares indicate REF-2 absent, squares that are half filled indicate that weak REF-2 staining can be detected at this stage in some worms.

unfused the longest, P(9-11).p. In *mab-5* mutant males, REF-2 persisted longest in P(3-8).p. By contrast, in *lin-39* mutant males, REF-2 was detected most strongly in P(7-11).p, which remain unfused, but not in anterior Pn.p cells after fusion. Thus, REF-2 protein is detected strongly in Pn.p cells expressing one Hox gene, but is expressed more weakly in Pn.p cells expressing both *lin-39* and *mab-5*. In *ref-2(mu218)* mutant males, expression of *ref-2* persisted in P(3-11).p, although *ref-2* expression was strongest in cells that remain unfused the longest, P(9-11).p. Thus, P7.p and P8.p maintain *ref-2* expression longer in *ref-2(mu218)* mutant males than in wild-type males.

We also examined the *ref-2* expression pattern in two mutants that are known to affect the Pn.p cell fusion pattern in hermaphrodites, *ref-1* and *egl-27* (Ch'ng and Kenyon, 1999; Alper and Kenyon, 2001). Both mutations prevent posterior Pn.p cell fusion by allowing MAB-5 to be inappropriately active in hermaphrodite Pn.p cells (*egl-27* also affects hermaphrodite cell fusion by altering *lin-39* expression). Again, the early pattern of *ref-2* expression was unchanged in these mutants – only the later Pn.p cell expression pattern was altered with expression lingering longer in the posterior unfused cells than in wild-type worms (Fig. 9; *ref-1* data is not pictured although it was similar to that for *egl-27*). Thus, directly or indirectly, these genes also regulate *ref-2* expression.



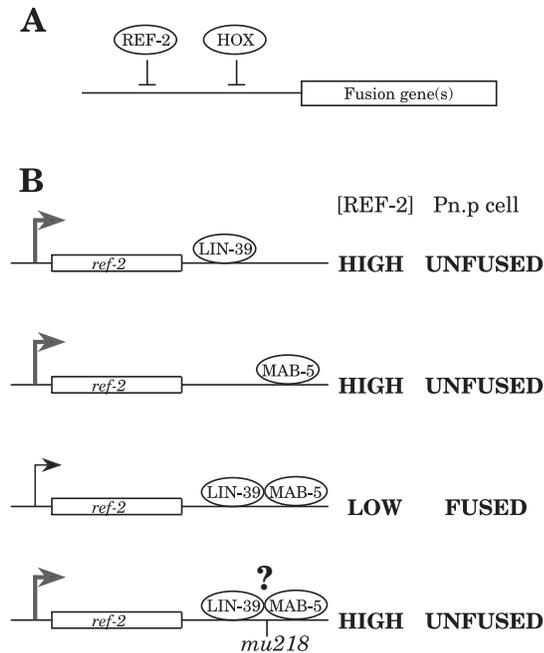
**Fig. 9.** *ref-2* expression correlates with the pattern of Pn.p cell fusion in wild-type and mutant worms. Staged populations of the indicated mutants were stained with an anti-REF-2 antiserum. The Pn.p cell fusion pattern is indicated by white circles (unfused cells) and short horizontal lines (fused cells) next to the genotype. Below each cell is the anti-REF-2 staining pattern in the Pn.p cells starting just after the Pn.p cells are formed. The first two time points (first two rows) are before Pn.p cell fusion, the last four are after the time of Pn.p cell fusion. Black squares indicate that REF-2 was detected at that time in those cells, white squares indicate no REF-2 was detected and half filled squares indicate weak expression was sometimes detected.

## DISCUSSION

The pattern of Pn.p cell fusion is regulated in a sexually dimorphic manner so that the proper Pn.p cells remain unfused and capable of generating sex-specific mating structures. This pattern of Pn.p cell fusion is regulated by two genes in the *C. elegans* Hox gene cluster. However, the pattern of cell fusion is more complex than the relatively simple Hox gene expression domains would allow. In males, this is due to the mutual inhibition of LIN-39 and MAB-5 activities. It is likely that interactions between Hox genes accounts for much of the AP diversity found in many organisms, although how this is accomplished is still poorly understood (Duncan, 1996). The interactions between LIN-39, MAB-5 and the zinc-finger transcription factor REF-2 is a good entry point to understand this subject.

### REF-2 functions with the Hox genes to prevent Pn.p cell fusion

RNAi experiments indicate that REF-2 is necessary to keep Pn.p cells unfused; however, *hs-ref-2* experiments indicate that REF-2 is not sufficient, because a Hox protein must also be present to prevent fusion. Likewise, the Hox genes are necessary but not sufficient to prevent Pn.p cell fusion. A simple model is that REF-2 and a Hox protein (be it LIN-39 or MAB-5) might together directly (Fig. 10A) or indirectly inhibit expression of a cell fusion gene or genes. Both proteins would be required for this inhibition, because if either gene is mutated, the cells fuse with *hyp7*. Alternatively, the opposite model is possible, that both proteins



**Fig. 10.** Two stages in *ref-2*-dependent patterning of Pn.p cell fusion. (A) A model for how REF-2 prevents Pn.p cell fusion. REF-2 and a Hox protein (LIN-39 or MAB-5) act by cooperatively inhibiting a common set of fusion gene targets. Alternatively, the two transcription factors could together activate a gene(s) required to prevent cell fusion. (B) A model for how *ref-2* expression might be regulated. In Pn.p cells in males, either Hox protein alone (LIN-39 or MAB-5) can activate *ref-2* transcription and thus can keep the concentration of REF-2 high in the Pn.p cells when the cell fusion decision is made. However, when both Hox proteins are present, *ref-2* expression decreases in the Pn.p cells and those cells fuse with hyp7. The *mu218* mutation allows *ref-2* to be expressed even in cells containing both LIN-39 and MAB-5 (*mu218* could affect the ability of Hox proteins to bind *ref-2* regulatory sites).

cooperatively activate transcription of a gene that encodes a fusion inhibitor.

In hermaphrodites, the REF-2 concentration remains high in P(1-8).p, yet only P(3-8).p remain unfused, probably because neither LIN-39 or MAB-5 is present in P1.p and P2.p. REF-2 is present at a lower concentration in P(9-11).p. Despite the fact that MAB-5 and at least some REF-2 are present in those posterior Pn.p cells, the cells fuse, suggesting that there may be a threshold level of REF-2 necessary to prevent fusion. The *hs-ref-2* experiments also suggest that normally REF-2 is either not expressed or is expressed below some threshold level in posterior Pn.p cells, because in wild-type worms, REF-2 does not prevent fusion of P(9-11).p. Thus, when high levels of REF-2 are generated using the *hs-ref-2* construct, posterior Pn.p cells can remain unfused where MAB-5 is present but anterior Pn.p cells cannot where MAB-5 and LIN-39 are absent.

### *ref-2* is regulated by the Hox genes

In addition to functioning with the Hox proteins to prevent Pn.p cell fusion, *ref-2* also behaves as if it were downstream of the Hox genes. *lin-39* expression is not changed in worms in which *ref-2* is inhibited by transgene mediated co-suppression or in which *ref-2* is ectopically expressed using the heat shock

promoter. By contrast, *ref-2* expression is altered in worms harboring either a *lin-39* or *mab-5* mutation. *ref-2* expression remains on stronger/longer in the Pn.p cells where a single Hox protein is present. The simplest model to explain this is that *ref-2* could be a direct (Fig. 10B) or indirect transcriptional target of the Hox proteins. In this model, when either Hox protein is present alone in the Pn.p cell in males, it can activate transcription of *ref-2* and therefore REF-2 remains above the threshold level needed to prevent fusion. When both Hox proteins are present in the same cell, *ref-2* transcription will be lower and REF-2 levels would fall below this threshold and the cells would fuse (Fig. 10B). Precisely how this occurs is still a mystery, although the location of the *mu218* mutation, which lies downstream of the *ref-2*-coding sequence, suggests the region of DNA where this regulation could be occurring. In hermaphrodites, EGL-27 and REF-1 prevent MAB-5 from affecting Pn.p cell fusion. They may do so by preventing MAB-5 from affecting *ref-2* expression. Consistent with this hypothesis, REF-2 is present at a higher concentration in posterior Pn.p cells when *egl-27* or *ref-1* is mutated.

In our model, REF-2 acts as both a Hox protein co-factor and also a Hox protein transcriptional target. Such complicated interactions are not unprecedented. For example, in *Drosophila*, it has been shown that *Ubx* regulates *wg* and *vg* and *Ubx* in turn also independently regulates genes that are regulated by *wg* and *vg* (Weatherbee et al., 1998). However, alternate models for the regulation of *ref-2* transcription are also possible. *ref-2* expression correlates with the Pn.p cell fusion pattern, consistent with the observation that REF-2 is required to keep Pn.p cells unfused. However, it is possible that upon cell fusion, REF-2 protein can diffuse into the much larger syncytium, causing an apparent decrease in the concentration of REF-2 in the nuclei of the Pn.p cells. We note, however, that REF-2 does remain at high concentration in the anterior Pn.p cells after they fuse, at least for a brief period of time, suggesting that such diffusion does not happen immediately. The fact that REF-2 can remain at high levels in these anterior Pn.p cells that do not express *lin-39* or *mab-5* also suggests that there are likely to be other factors affecting *ref-2* expression.

Several experiments indicate that the sex-specific regulation of the Hox proteins acts by affecting *ref-2* expression. The properties of the *mu218* mutation suggest that the expression of *ref-2* is downstream of the ability of LIN-39 and MAB-5 to cancel each other's activities. This is demonstrated by the ability of the *mu218* mutation to bypass this mutual Hox inhibition in both wild-type and *mab-5(e1751)* mutant worms. The hypothesis that this effect could be operating at the level of *ref-2* transcription is reinforced by the altered *ref-2* expression pattern observed in a strain harboring the *mu218* mutation. The observation that P(3-11).p all remain unfused in hermaphrodites in the *hs-ref-2* experiments is also consistent with the idea that REF-2 acts downstream of the Hox proteins. The ability of REF-2 to keep posterior Pn.p cells unfused in these worms is still dependent on MAB-5. Thus, MAB-5 is active in hermaphrodites in which the normal regulation of *ref-2* expression is bypassed using the heat-shock promoter, suggesting that the inhibition of MAB-5 in hermaphrodites by *ref-1* and *egl-27* also ultimately acts at the level of regulation of *ref-2* expression. As MAB-5 is now active in these *hs-ref-2* hermaphrodites, we might have predicted that MAB-5 and

LIN-39 would inhibit each other and that P7.p and P8.p would now fuse with hyp7. However, these cells still remain unfused in these *hs-ref-2* experiments, again suggesting that the interaction between LIN-39 and MAB-5 ultimately acts at the level of *ref-2* expression.

### REF-2 is required for the proper execution of Pn.p cell fate

RNAi experiments indicate that REF-2 is required for several other aspects of Pn.p cell development, including P cell migration and P and Pn.p cell survival. While the late pattern of REF-2 expression does change in the various Hox mutants examined, the early pattern in the P cell and young Pn.p cell remains unchanged, suggesting that other factors also regulate *ref-2* transcription. In a sense, REF-2 can be thought of as a P/Pn.p cell identity factor that acts during L1 to control the various aspects of early P/Pn.p cell development, including Pn.p cell fusion. REF-2 transcriptional targets probably include genes that encode factors required for many aspects of these cell fates including cell survival, division, migration and fusion. REF-2 could act at a single time to control all aspects of Pn.p fate, but we favor the hypothesis that it functions at multiple times in the P and Pn.p cell lineage. Consistent with this hypothesis, we observed that REF-2 is present in the P and Pn.p cells throughout the time in L1 when the RNAi experiments cause a phenotype. Additionally, while we could prevent Pn.p cell fusion and overcome the transgene-mediated inhibition of *ref-2* activity with numerous pulses of *hs-ref-2* during L1, no single pulse at any point during L1 could overcome these defects and prevent fusion, even though a single pulse was sufficient to generate very high levels of REF-2.

Although *ref-2* and the *opa/Zic* genes probably share a common ancestor, they have quite different phenotypes. The *Zic* genes affect various aspects of vertebrate development, including the establishment of left-right asymmetry of internal organs and the development of the central nervous system (Aruga et al., 1994; Aruga et al., 1996; Nakata et al., 1997; Nakata et al., 1998). Mutations identified in some members of the *Zic* gene family have been implicated in several human developmental abnormalities (Gebbia et al., 1997; Brown et al., 1998). *opa* is a *Drosophila* pair-rule gene; *opa* also plays a role in *Drosophila* midgut morphogenesis (Benedyk et al., 1994; Cimbora and Sakonju, 1995). Interestingly, like *ref-2*, *opa* expression is regulated by two Hox genes (*Antennapedia* and *abdominal-A*) in the mesoderm (Cimbora and Sakonju, 1995), raising the possibility that some aspects of the regulation of this class of transcription factors might be conserved.

The interactions between REF-2 and the two Hox proteins are quite complex. This is not unexpected because the cell fate decision that they regulate is also quite complicated. *ref-2* is a key integrator of developmental information in the Pn.p cells: *ref-2* integrates AP positional information from the Hox proteins in a combinatorial manner, sex-specific information from *ref-1* and *egl-27*, and a P-lineage specific signal. It is likely that as the details of such regulation become clear in other systems, similar features will be observed. Although few examples of how Hox genes interact to control cell fate have been studied in detail, it has been shown that *Hoxa1* and *Hoxb1* act synergistically in mammals to pattern the cranial nerves and second branchial arch (Gavalas et al., 1998; Studer et al., 1998). Several proteins that regulate Hox protein activity (either directly or indirectly) have

been identified in *Drosophila*, including *extradenticle* and *homothorax*, which regulate several Hox proteins, and *teashirt* and an isoform of *cap 'n' collar*, which regulate *Sex combs reduced* and *Deformed*, respectively (Andrew et al., 1994; de Zulueta et al., 1994; McGinnis and Ragnhildstveit, 1998; Pinsonneault et al., 1997; Rauskolb et al., 1993; Rieckhof et al., 1997; Veraksa et al., 2000). The characterization of factors like REF-2 that function with the Hox genes to regulate Pn.p cell fusion will allow us to study such complicated interactions in molecular and cellular detail. Moreover, while global regulators of development such as the Hox genes have been identified in many organisms, identification of the targets of these genes that carry out morphogenesis have proven to be more elusive (Botas, 1993; Graba et al., 1997). REF-2 is a good candidate to identify such targets.

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