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The *lin-3/let-23* pathway mediates inductive signalling during male spicule development in *Caenorhabditis elegans*

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SUMMARY

During *Caenorhabditis elegans* male spicule development, four pairs of precursor cells respond to multiple positional cues and establish a pattern of fates that correlates with relative anterior-posterior cell position. One of the extracellular cues is provided by the F and U cells, which promote anterior fates. We show that the genes in the *lin-3/let-23* signalling pathway required for hermaphrodite vulval induction also mediate this F/U signal. Reduction-of-function mutations in *lin-3*, *let-23*, *sem-5*, *let-60* or *lin-45* disrupt the fate of anterior cells. Likewise, activation of the pathway with ubiquitously produced signal results in posterior cells inappropriately adopting the anterior fates even in the absence of F and U. We have further used this genetic pathway to begin to understand how multiple positional cues are integrated to specify cell fate. We demonstrate that *lin-15* acts in spicule development as it does in vulval induction, as a negative regulator of *let-23* receptor activity. A second extracellular cue, from Y.p, also acts antagonistically to the *lin-3/let-23* pathway. However, this signal is apparently integrated into the *lin-3/let-23* pathway at some step after *lin-45* raf and is thus functionally distinct from *lin-15*. We have also investigated the role of *lin-12* in forming the anterior/posterior pattern of fates. A *lin-12* gain-of-function defect is masked by redundant positional information from F and U.

Key words: cell lineage, cell interaction, cell fate specification, pattern formation

INTRODUCTION

Cell interactions play a central role in the development of many organisms. As the specific proteins and cellular processes involved in classic vertebrate inductions become identified, the underlying complexities of multiple signals that act in parallel to specify fates are striking (reviewed in Kimelman et al., 1992; Davidson, 1993). A key issue in understanding how fate is specified by multiple signals is understanding how the information content of such signals is integrated in the responding cells to produce a unified outcome. In *C. elegans*, the essentially invariant and precisely described cell lineage provides a reproducible background to study cell interactions. The set of characterized interactions required during the development of the male spicules indicate that they may serve as a model that combines complex signal integration with the precision of single cell analysis (Chamberlin and Sternberg, 1993).

During *C. elegans* male postembryonic development, several precursor cells divide in a sex-specific manner to produce the cells of copulatory structures in the tail. One such cell, B, is the precursor of all of the cells of the male spicules (Fig. 1A). During male development, the anterior daughter of B (B.a) divides to produce eight progeny (Fig. 2). These eight progeny make up four pairs: ventral (**aa**), dorsal (**pp**) and two identical lateral pairs (**ap**/**pa**). For each pair, there is an anterior and a posterior fate that differ in the subsequent cell lineage produced by each cell, as well as the differentiated fates of the progeny produced by that lineage (Sulston and Horvitz, 1977; Sulston et al., 1980). The choice of fate within each pair is responsive to extracellular cues provided by neighboring cells. Cell ablation experiments suggest that the other male-specific blast cells, or their progeny, provide distinct positional cues and that the eight B.a greatgrandprogeny may also interact (Chamberlin and Sternberg, 1993) (Fig. 3). For example, ablation of two other male-specific blast cells, called F and U, disrupts the fates of positionally anterior cells. In some cases they produce the cell lineage normally associated with their posterior neighbors. In contrast, ablation of the blast cell Y.p disrupts the fate of some positionally posterior cells.

To identify the genes that mediate the cell interactions required for proper development of the B cell, we have begun to characterize the role of genes required for other cell interactions in *C. elegans*. In this study, we have focused on a set of genes that play a role in vulval development. *C. elegans* hermaphrodite vulval development requires a signal from the anchor cell (AC) in the gonad that acts on three of six epidermal blast cells termed vulval precursor cells (VPCs) (Kimble, 1981). In normal development, the three AC-proximal VPCs produce vulval tissue, whereas the three distal VPCs produce nonspecific epidermis. The AC signal (an epidermal growth factor (EGF)-like protein encoded by the gene *lin-3* (Hill and Sternberg, 1992)) is both necessary and sufficient to promote the VPCs to initiate vulval development. Genes that are necessary for the response to *lin-3* include *let-*

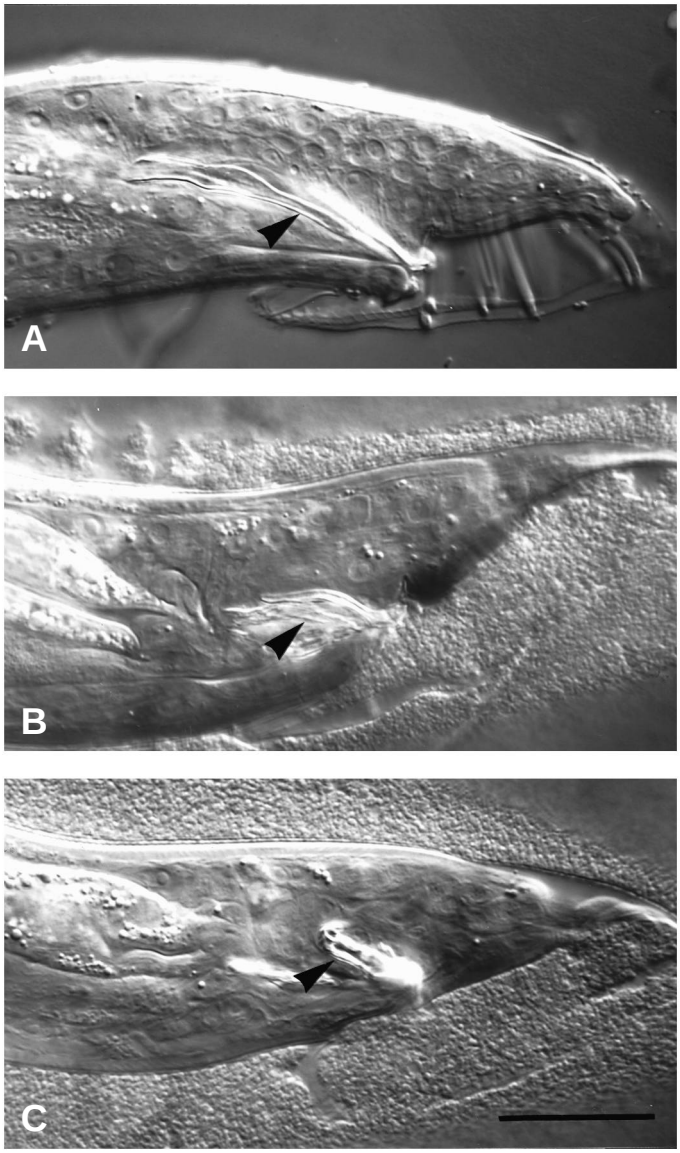

Fig. 1. Comparison of the adult male spicules in wild-type (A), *lin-3(sy53)/lin-3(n1058)* (B) and *syEx21(hsp::lin-3)* (C) animals. Arrow points to the left spicule. Nomarski photomicrographs, anterior left, ventral down. (A) In wild-type animals, the spicules are long and straight. (B,C) In animals with *lin-3* activity reduced or with *lin-3* activity increased, the spicules are short and crumpled. However, the B lineage defects responsible for the morphological defect are opposite from each other (see text). Scale, 20 μm.

23 (receptor) (Aroian et al., 1990), *sem-5* (adaptor) (Clark et al., 1992), *let-60* (ras) (Han and Sternberg, 1990) and *lin-45* (raf) (Han et al., 1993). Reduction-of-function mutations in any of these genes result in a Vulvaless (Vul) phenotype where all six VPCs may produce non-vulval epidermis at the expense of vulval tissue. Gain-of-function mutations in *let-60* (Beitel et al., 1990) and over-production of LIN-3 (Hill and Sternberg, 1992) result in a Multivulva (Muv) phenotype in which all six VPCs may produce vulval tissue.

A second potential cell interaction is represented by the gene *lin-15*. Loss-of-function mutations in *lin-15* result in a Muv phenotype. In vulval development, genetic analysis suggests that *lin-15* acts in parallel to *lin-3*, as a negative regulator of *let-23* (Ferguson et al., 1987; Huang et al., 1994). Genetically, *lin-15* represents two independently mutable functions both of which must be mutant to produce an observable phenotype (Ferguson and Horvitz, 1989). Mosaic analysis indicates that *lin-15* activity in the VPCs or in the AC is not sufficient for normal vulval development, and it is proposed that *lin-15* may function in the surrounding epidermis (hyp7) (Herman and Hedgecock, 1990). The cloning of *lin-15* revealed a complex locus with two transcriptional units that each have the potential to encode a novel protein (Huang et al., 1994; Clark et al., 1994).

In normal vulval development, the induced VPCs adopt one of two vulval fates. The cell most proximal to the AC adopts a 1° fate, whereas the two more distal adopt a 2° vulval fate. A lateral interaction between VPCs ensures that two adjacent cells do not adopt the 1° fate (Sternberg, 1988). The gene *lin-12* mediates this lateral interaction and acts in several cell pairs as a genetic 'switch' between two fates (Greenwald et al., 1983). *lin-12* acts in a cell-autonomous manner, likely on the receiving end of a cell interaction (Seydoux and Greenwald, 1989). The cloning of *lin-12* revealed a transcript with the potential to encode a transmembrane protein with thirteen EGF-like repeats in the presumed extracellular domain, six copies of a cdc10/SWI6 motif in the presumed intracellular domain and overall similarity to the *Drosophila* gene *Notch* (Yochem et al., 1988).

In hermaphrodite vulval development, the three cell interactions represented genetically by the *lin-3/let-23* pathway, *lin-15* and *lin-12* act coordinately to produce the normal pattern of cell fates (reviewed by Sternberg, 1993). In this paper, we describe experiments that investigate the role of these genes in specifying fate in the male B lineage. We also investigate the relationship between the cell interactions identified by cell ablation experiments and the genetic pathways. Although the specifics differ between the two developmental processes, the function of these genes in the B lineage parallels that of vulval development: the genes of the *lin-3/let-23* pathway mediate a positional signal from the F and U cells, *lin-15* likely acts as a negative regulator of *let-23* (receptor) activity and *lin-12* mediates a lateral interaction. Furthermore, in the B lineage, ablation of the Y.p cell defines an additional cell interaction distinct from these three genetic pathways.

MATERIALS AND METHODS

Strains

Nematode strains were cultured according to standard techniques (Brenner, 1974; Sulston and Hodgkin, 1988). Loss-of-function mutations in *lin-3*, *let-23*, *sem-5*, *let-60* and *lin-45* are known or believed to be lethal, with the death phase prior to the time of spicule development. However, we believe the genotypes in Table 1B represent a reduction of normal gene function based on two criteria. First, for male tail development each allele is recessive to a wild-type copy of the gene (except for *let-60(sy95dn)* and *let-60(sy100dn)*; data not shown). Second, more stringent genetic tests indicate that the alleles represent a reduction of normal function of the gene for vulval development (Aroian and Sternberg, 1991; Clark et al., 1992; Ferguson and Horvitz, 1985; Han et al., 1990, 1993). For *lin-3*, *let-23* and *let-60*, we have tested several alleles or allelic combinations in order to verify that the observed phenotypes reflect common pleiotropies of the locus rather than unrelated second mutations or

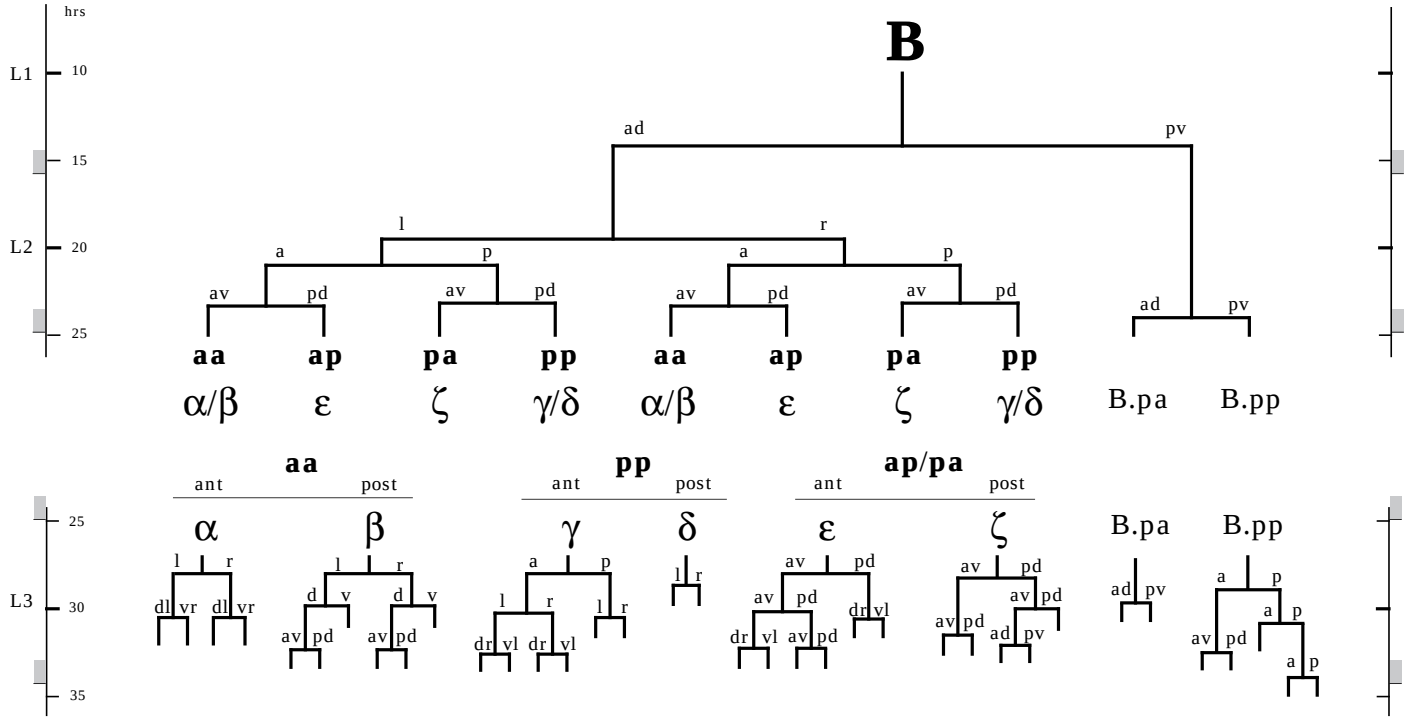

Fig. 2. The lineage of the *C. elegans* male B cell, after Sulston and Horvitz (1977) and Sulston et al. (1980). Vertical lines indicate a cell, horizontal lines indicate a cell division. Larval stage and approximate developmental time post-hatching are indicated in the left margin. Division axes are as indicated: a, anterior; p, posterior; d, dorsal; v, ventral; l, left; r, right.

unusual mutations. Also, we wanted to identify genotypes that may represent severe reduction of gene function for the male tail. The extent of the lineage defects can be variable both between and within given genotypes. This variability may be due to the alleles retaining partial gene activity.

Mutations used are described by Brenner (1974), Hodgkin et al. (1988) and as noted below.

Linkage Group (LG) II: *clr-1(e1745)*. *let-23(sy97)*, *(sy278)* and *(n2020)* (Aroian and Sternberg, 1991; H. M. C. and P. W. S., unpublished; S. Clark and R. Horvitz, unpublished). *unc-4(e120)*.

LG III: *lin-12(n137)* and *(n137n720)* (Greenwald et al., 1983).

LG IV: *unc-24(e138)*. *mec-3(e1338)*. *lin-3(n378)*, *(n1058)*, *(n1059)* and *(sy53)* (Ferguson and Horvitz, 1985; Hill and Sternberg, 1992). *lin-45(sy96)* (Han et al., 1993). *let-60(n2021)*, *(sy95dn)*, *(sy100dn)*, *(n1046gf)* and *(sy103gf)* (Beitel et al., 1990; Han and Sternberg, 1991; G. Jongeward, unpublished). *dpy-20(e1282)*. *unc-22(s7)*. *unc-31(e169)*. *nT1[unc(n754) let]* (=*DnT1* balancer; Ferguson and Horvitz, 1985).

LG V: *him-5(e1467)* and *(e1490)* (Hodgkin et al., 1979).

LG X: *sem-5(n1619)* (Clark et al., 1992). *lin-15(e1763)*, *(n309)* and *(n377)* (Ferguson and Horvitz, 1985).

Transgenic strains: PS1226 *unc-31(e169); syEx21(hsp::lin-3)*. PS1238 *unc-31(e169); syEx23(hsp::lin-3)* (R. J. Hill, unpublished). These strains include multiple copies of a transgene with the EGF-encoding domain of *lin-3* and a synthetic signal sequence under control of the *hsp16* promoter/enhancer (Stringham et al., 1992). The transgenes are maintained as extrachromosomal arrays. Although the transgenes produce only a portion of the *lin-3* gene, for simplicity we often refer to the product as LIN-3 (rather than the more accurate LIN-3 EGF domain) as it represents *lin-3* activity. Genomic *unc-31* DNA was also included as a selectable marker.

Full genotypes of animals in Tables 1, 2 and 3 are as follows: *him-5(e1490)* doubles were constructed for *let-23(sy97)*, *let-23(sy278)*, *let-60(n2021)*, *lin-45(sy96)*, *let-60(n1046gf)*, *let-60(sy103gf)*, *lin-15(e1763)*, *lin-15(n309)*, *lin-15(n377)* and *lin-12(n137)*. Data originally summarized in Greenwald et al. (1983) (Tables 2 and 3) are from *lin-12(n137); him-5(e1467)* and *lin-12(n137n720); him-5(e1467)* animals. *let-23(n2020)* is *let-23(n2020) unc-4(e120); him-5(e1490)*.

Other genotypes and construction:

lin-3(sy53)/lin-3(n1058): *unc-24(e138) mec-3(e1338) lin-3(sy53) dpy-20(e1282) / lin-3(n1058)*. Construction: *unc-24(e138) mec-3(e1338) dpy-20(e1282)/+* males were crossed with *lin-3(n1058)/DnT1* hermaphrodites. Single non-Unc cross males (genotype: *unc-24(e138) mec-3(e1338) dpy-20(e1282)/lin-3(n1058)* or *+/lin-3(n1058)*) were crossed with *unc-24(e138) mec-3(e1338) lin-3(sy53) dpy-20(e1282)/DnT1* hermaphrodites. non-Dpy non-Unc animals from crosses that yield Dpy Unc progeny (indicating paternal genotype of *unc-24(e138) mec-3(e1338) dpy-20(e1282)/lin-3(n1058)*) are the desired genotype.

lin-3(n378)/lin-3(n1059): *lin-3(n378) / unc-24(e138) lin-3(n1059) dpy-20(e1282); him-5(e1490)/+*. Construction: *lin-3(n378); him-5(e1490)* males were crossed with *lin-3(n1059)/DnT1* hermaphrodites. non-Unc male cross progeny are the desired genotype.

sem-5(n1619): *clr-1(e1745)/+; sem-5(n1619)*. Construction: N2 males were crossed with *clr-1(e1745); sem-5(n1619)*. *clr-1(e1745)* suppresses the lethality associated with *sem-5(n1619)* and maternally rescues for lethality. However, the male tail defect is still observed.

let-60(dn): *let-60(sy95)/dpy-20(e1282); him-5(e1490)* or *unc-24(e138) let-60(sy100) dpy-20(e1282)/unc-22(s7); him-5(e1490)*.

Double mutant strains of *let-23* or *lin-45* and *lin-15* were constructed according to standard methods (Ferguson et al., 1987). In addition to the two mutations, double mutant strains include *him-5(e1490)*. *let-23(sy278); lin-15(e1763)* is *let-23(sy278) unc-4(e120); him-5(e1490); lin-15(e1763)*.

Cell lineage and ablation

Cell nuclei divisions in living animals were directly observed using Nomarski differential interference contrast optics as described by Sulston and Horvitz (1977). Nomenclature follows the standard of Sulston and Horvitz (1977), with modifications of Chamberlin and

Sternberg (1993). All lineages were followed from the first divisions of the B.a(l/r)xx cells (early to mid L3 larval stage; x represents both progeny of a division) through the L3 molt. Cell nuclei were destroyed by a laser microbeam as described by Avery and Horvitz (1987). F, U and Y.p were ablated at the stage when B had divided to produce two progeny (early L2), according to the procedures in Chamberlin and Sternberg (1993). Ablation of B.a progeny was during mid to late L2 stage, soon after the targeted cells were generated. Ablation of the 'B.a positional cue' for the **aa** cells in Table 3 represents the ablation of B.a(l/r)p, followed by the ablation of B.a(l/r)ap.

Heat-shock induction of *lin-3* transgenes

Transgenic animals received heat shock at late L2 larval stage as follows. Individual animals were anaesthetized on pads of 5% noble agar in water containing 5 μM sodium azide, staged and allowed to recover one hour on a standard 5 cm NGM agar Petri plate seeded with *E. coli* OP50. Plates were then sealed with parafilm and floated in a 33°C water bath for 90 minutes to induce the heat-shock response.

RESULTS

The *lin-3/let-23* signalling pathway mediates the F/U signal during male spicule development

We have examined the effects of increasing and decreasing the activity of the *lin-3/let-23* pathway using chromosomal mutations and extrachromosomal transgenes. Taken together the results indicate that activation of the pathway is both necessary and sufficient to promote anterior fates, and it likely mediates the positional cue provided by F and U.

Mutations in some genes in the *lin-3*/*let-23* pathway disrupt fates of anterior cells in the male B lineage

Some mutations in a subset of genes in the *lin-3/let-23* signalling pathway result in an abnormal male spicule phenotype (see Fig. 1; also Aroian and Sternberg, 1991). These genes include *lin-3*, *let-23*, *sem-5*, *let-60* and *lin-45*. In the B lineage, reduction-of-function mutations in any of these genes disrupt the lineages of the anterior cells (Table 1B,2-10). For instance, of seven *lin-3(sy53)/lin-3(n1058)* animals followed, none had a normal α or a normal γ lineage and only one had normal ε lineages (Table 1B,2). The phenotypes are similar to the abnormalities that result from ablation of both F and U in wild-type males (Table 1B,1). In some cases the anterior cells produce a lineage similar to their posterior neighbors. For instance, in all seven *lin-3(sy53)/lin-3(n1058)* animals both **aa** cells produced β lineages.

Although the mutant phenotypes resemble the effects of F/U ablation, the F and U cells appear normal in the mutants. The F and U lineages are normal in mutant animals (lineages normal in 2/2 *lin-3(sy53)/lin-3(n1058)* and 2/2 *lin-45(sy96)* animals). In addition, the linker cell in the gonad dies as in wild-type animals (10/10 *lin-3(sy53)/lin-3(n1058)* and 8/8 *lin-45(sy96)* animals). Since U.(l/r)p 'murder' the linker cell in intact animals (Sulston and White, 1980), these U progeny are still capable of at least one of their normal functions.

The **pp** cell lineage defect in *lin-3* and *lin-45* mutants is enhanced by ablation of F and U (Table 1B,11,12). For example, in F⁻U⁻ *lin-3(sy53)/lin-3(n1058)* animals (Table 1B,11) both anterior and posterior **pp** cells produced δ lineages (posterior fate) in all seven animals. In contrast, although anterior **pp** lineages were disrupted, this pattern was observed in only one of seven *lin-3(sy53)/lin-3(n1058)* intact animals (Table 1B,2). However, ablation of F and U in mutant animals does not result in a consistent transformation of all anterior cells to posterior fate. In particular, although the lineages of anterior **ap** cells were disrupted, the **ap** cells did not produce ζ lineages.

Ectopic expression of the ligand, LIN-3, disrupts the fates of posterior cells

To test further the role of this signalling pathway in the B lineage, we used a transgenic construct that includes the EGF-coding domain of *lin-3* under control of a broadly expressed heat-shock promoter (R. J. Hill, personal communication) that expresses in many tissues (Stringham et al., 1992). In the B lineage, heat-shock treatment of transgenic animals results in disruption of the lineage of the posterior cells (Table 1C,1,2). In the **aa** and **ap**/**pa** pairs, the posterior cells produce lineages normally associated with their anterior neighbors. For example, in five of six heat-shocked *syEx21* animals followed, both **aa** cells produced α lineages and both **ap** and **pa** cells produced ε lineages (Fig. 4).

In the **pp** pair, the posterior cells produce more anterior-like lineages. However, lineages of both anterior and posterior cells in this pair can be disrupted to produce up to eight progeny. Such abnormal lineages are observed following ablation of the **ap**/**pa** cells in wild-type (Chamberlin and Sternberg, 1993) as well as in the transgenic animals. Although abnormal, we believe these lineages may represent a transformation of all four **pp** daughters to the fate of the anterior daughter of γ (γ.a; see Fig. 2). Specifically, cell ablation experiments suggested

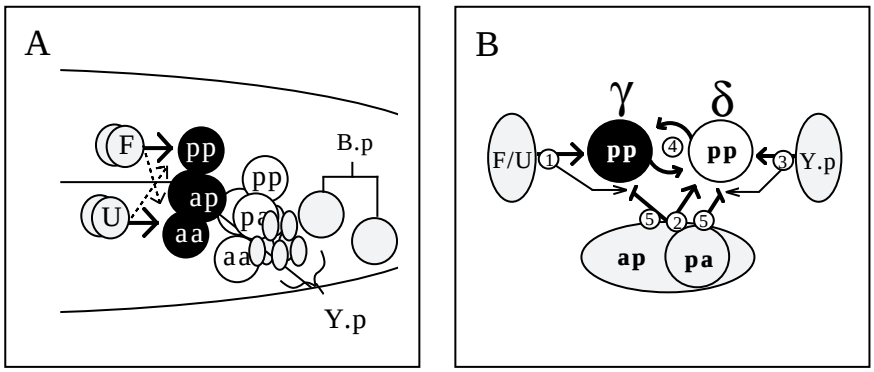

Fig. 3. (A) Diagram illustrating the approximate positions of the B, F, U and Y.p progeny in a mid-L3 larval stage male (anterior left, ventral down). Arrows indicate the signal from F and U that promotes the anterior fates in the **aa**, **ap**/**pa** and **pp** pairs of B.a progeny. (B) The signal from F and U (1) represents one of several cell interactions that specify fates in the B.a progeny, as illustrated in this model for the **pp** cells (after Chamberlin and Sternberg, 1993). Other positional cues (arrows) are provided by Y.p, or its progeny (3) and the other neighboring B.a progeny (2). In addition, the neighboring B.a progeny act to prevent the F/U and Y.p cues from acting on inappropriate cells (bars, 5). This interaction may be passive (Chamberlin and Sternberg, 1993). The two **pp** cells may also interact (4) (Greenwald et al., 1983; this work). There is no evidence that B.p provides any positional cue. Cells in black represent anterior fate (**aa**, α; **ap**, ε; **pp**, γ), white represent posterior fate (**aa**, β; **pa**, ζ; **pp**, δ).

Table 1. Effects of disruption of cell interactions on the B lineage

			aa						**pp**										**ap/pa**									
			ant			post			ant					post					ant				post					
	genotype	**ablations**	α	abn	β	α	abn	β	γ	abn>4	γ*/δ*	abn<4	δ	γ	abn>4	γ*/δ*	abn<4	δ	ε	ε*	abn	ζ	ε	ε*	abn	ζ		
A. Normal																												
	wild type	intact	**all**	.	.	.	.	**all**	**all**	.	.	.	.	.	.	.	.	**all**	**all**	.	.	.	.	.	.	**all**		
B. Activity of the pathway reduced																												
1	wild type	F-U-	.	**4**	**3**	.	.	**7**	**2**	.	**3**	.	**2**	.	.	.	.	**7**	**9**	**4**	**1**	.	.	.	.	**14**		
2	*lin-3(sy53)/lin-3(n1058)*	intact	.	.	**7**[2]	.	.	**7**[2]	.	**1**	**5**	.	**1**	.	.	.	.	**7**	**2**	**11**	**1**	.	.	.	.	**14**		
3	*lin-3(n378)/lin-3(n1059)*	intact	**1**	.	**2**[1]	.	.	**3**[1]	**3**	.	.	.	.	.	.	.	.	**3**	**6**	.	.	.	.	.	.	**6**		
4	*let-23(sy97)*	intact	.	**1**	**4**	.	.	**5**	**3**	.	**1**	.	.	.	.	.	.	**4**	**1**	**7**	.	.	.	.	.	**8**		
5	*let-23(n2020)*	intact	.	**1**	**1**	.	.	**2**	**2**	.	.	.	.	.	.	.	.	**2**	**3**	**1**	.	.	.	.	.	**4**		
6	*let-23(sy278)*	intact	.	.	**4**	.	**1**	**3**	**2**	.	**2**	.	.	.	.	.	.	**4**	.	**6**	**1**	**1**	.	.	.	**8**		
7	*sem-5(n1619)*	intact	.	**1**[1]	**4**	.	.	**5**[1]	**1**	.	**4**	.	.	.	.	.	.	**5**	**3**	**6**	**1**	.	.	.	.	**10**		
8	*let-60(n2021)*	intact	.	.	**5**[1]	.	.	**5**[1]	**1**	.	**1**	.	**3**[3]	.	.	.	.	**5**[3]	**7**	**3**	.	.	.	.	.	**10**		
9	*let-60(dn)/+*	intact	**3**	**4**	**1**	.	.	**8**	**4**	.	**1**	.	.	.	.	.	.	**5**	**8**	.	**1**	.	.	.	.	**9**		
10	*lin-45(sy96)*	intact	.	**3**	**4**[1]	.	**1**	**6**[1]	**2**	.	**4**	.	**1**	.	.	.	.	**7**	**3**	**10**	**1**	.	.	.	.	**14**		
11	*lin-3(sy53)/lin-3(n1058)*	F-U-	.	**1**	**4**[1]	.	**1**	**4**[1]	.	.	.	.	**7**[4]	.	.	.	.	**7**[4]	**1**	**9**	**2**	.	.	.	.	**12**		
12	*lin-45(sy96)*	F-U-	.	**1**	**2**[1]	.	.	**3**[1]	.	.	**2**	**1**	**1**	.	.	.	.	**4**	.	**6**	.	.	.	.	.	**6**		
C. Activity of the pathway increased																												
1	*syEx23(hsp::lin-3)*	intact	**5**[1]	.	.	**2**[1]	**1**	**2**	**5**	.	.	.	.	**1**	.	**3**	**1**	.	**9**	.	**1**	.	**7**	**2**	**1**	.		
2	*syEx21(hsp::lin-3)*	intact	**6**	.	.	**5**	.	**1**	**1**	**4**	.	.	.	.	**4**	**1**	.	.	**12**	.	.	.	**11**	**1**	.	.		
3	*let-60(gf)*	intact	**8**	.	.	**1**	**1**	**6**	**8**	.	.	.	.	.	.	**3**	**2**	**3**	**11**	.	.	.	.	.	**1**	**10**		
4	*syEx21(hsp::lin-3)*	F-U-	**3**[1]	.	**2**	**1**	**1**[1]	**3**	**4**[1]	**1**	.	.	.	.	**1**	**3**[1]	**1**	.	**8**	**1**	**1**	.	**5**	**1**	**3**	**1**		
D. Role of *lin-15*																												
1	*lin-15(n309)*	intact	**5**	.	**1**	.	.	**6**	**3**	.	.	.	.	.	.	**1**	.	**2**	**5**	.	.	.	.	.	.	**5**		
2	*lin-15(n377)*	intact	**3**	.	.	.	**1**	**2**	**3**	.	.	.	.	.	.	**2**	.	**1**	**6**	.	.	.	.	.	.	**6**		
3	*lin-15(e1763)*	intact	**7**	.	.	.	.	**7**	**7**	.	.	.	.	.	.	**2**	.	**5**	**14**	.	.	.	.	.	.	**14**		
4	*let-23(sy97); lin-15(n309)*	intact	.	.	**5**	.	.	**5**	**5**	.	.	.	.	.	.	.	.	**5**	**4**	**6**	.	.	.	.	.	**10**		
5	*let-23(sy278); lin-15(e1763)*	intact	.	.	**4**[3]	.	.	**4**[3]	**3**	**1**	.	.	.	.	.	.	.	**4**	.	**8**	.	.	.	.	.	**8**		
6	*lin-45(sy96); lin-15(e1763)*	intact	.	.	**4**	.	.	**4**	.	**1**	**1**	**1**	**1**	.	.	.	.	**4**	**2**	**5**	**1**	.	.	.	.	**8**		
E. Interaction with Y.p cue																												
1	wild type	Y.p-	**7**	.	.	.	**1**	**6**	**7**	.	.	.	.	.	.	**3**	**3**	**1**	**12**	.	.	.	.	.	.	**12**		
2	wild type	F-U- Y.p-	**3**[2]	**2**[2]	.	.	**1**[1]	**4**[3]	**4**	**1**	.	.	.	.	.	**2**	**2**	**1**	**3**	**6**	**1**	.	.	.	.	**10**		
3	*lin-3(sy53)/lin-3(n1058)*	Y.p-	.	**1**	**4**	.	**1**	**4**	**1**	**1**	**3**	.	.	.	**1**	**3**	.	**1**	**1**	**9**	.	.	.	.	.	**10**		
4	*let-23(sy97)*	Y.p-	.	**3**[2]	.	.	.	**3**[2]	**2**	.	**1**	.	.	.	.	**3**	.	.	**2**	**2**	.	.	.	.	.	**4**		
5	*let-60(n2021)*	Y.p-	.	**1**	**4**[1]	.	.	**5**[1]	**3**	**1**	**1**	.	.	.	.	**3**	**1**	**1**	**6**	**4**	.	.	.	.	.	**10**		
6	*lin-45(sy96)*	Y.p-	.	**2**	**2**	.	**1**	**3**	**1**	**1**	**1**	**1**	.	.	.	**2**	**2**	.	**3**	**5**	.	.	.	.	.	**8**		

Each line represents all of the animals observed under a specific experimental condition. Each of the pairs of B.a progeny cells (**aa**, **pp**, **ap**/**pa**) are indicated, along with the lineage produced by the positionally anterior and the positionally posterior member of the pair. The numbers represent the number of animals followed that produced the indicated lineage. For instance, in all seven *lin-3(sy53)/lin-3(n1058)* mutant animals (line B.2), both **aa** cells produced a β lineage. In some animals, the left and right cells of a pair fail to migrate to anterior and posterior positions. Superscript numbers indicate the lineage associated with cells that fail to migrate and the number of animals. Thus the **aa** cells failed to migrate in two *lin-3(sy53)/lin-3(n1058)* of the seven total animals examined and both cells produced β-like lineages. In cases where the two cells that fail to migrate produce dissimilar lineages, they are aligned in the table to most closely approximate wild type. Cases where a cell produced a lineage in which the timing and number of divisions are consistent with a normal lineage but the axes are skewed are included as a normal lineage. '.' indicates no animals produced the lineage. Data for B.1, E.1 and E.2 are from Chamberlin and Sternberg (1993). γ*/δ* and ε* are commonly observed abnormal lineages. Each results in four progeny. 'abn' indicates any other abnormal lineage. Combination of all non-normal lineages of a cell for comparison to wild-type, intact animals provides a conservative interpretation of the data. For example, in F⁻U⁻ animals (line B.1), 0/7 anterior **aa** cells were normal (although only 3 produced a β-like lineage and 4 produced a non-α, non-β, abnormal lineage). In general, the specifics of the abnormal lineages are not essential for this type of interpretation. 'abn>4' and 'abn<4' indicate abnormal with more than, or less than four progeny, respectively. 'abn>4' lineages are more anterior (γ) -like and include lineages that result in up to eight progeny (see text). 'abn<4' are more posterior (δ) -like. The nature of the abnormal lineages is further discussed in Chamberlin and Sternberg (1993). In some cases the numbers of **aa**, **pp** and **ap**/**pa** cells do not follow a 1:1:2 ratio because all cells were not followed to the completion of the lineage for some animals.

that patterning of γ and δ fates may occur in two steps: first, the F and U signal promotes anterior versus posterior fate in the **pp** cells; second, it promotes γ.a versus γ.p fate in the daughters of the anterior **pp** cell. According to this model, if the signal is not localized or 'modulated' both **pp** cells may adopt the anterior fate and both daughters may adopt the γ.a fate (Chamberlin and Sternberg, 1993). The observed result is up to eight progeny from each **pp** cell. The lineages observed in the transgenic animals that are presumably expressing the LIN-3 EGF domain ubiquitously (and hence the signal is not localized) are consistent with this model.

Ubiquitous LIN-3 is also sufficient to compensate for the absence of F and U (Table 1C,4), suggesting that activation of the *lin-3/let-23* pathway is sufficient to promote anterior fates. Gain-of-function *let-60* mutations that result in an activated protein also disrupt posterior fate, but to a lesser extent than ubiquitously expressed LIN-3 (Table 1C,3).

Two activities that act antagonistically to *lin-3/let-23* are integrated at functionally distinct steps in the pathway

Mutations in *lin-15* disrupt the fate of posterior **pp** cells

In the hermaphrodite vulva, reduction-of-function mutations in the *lin-15* locus result in a phenotype opposite from reduction-of-function mutations in the *lin-3/let-23* genes required for vulval fates. In the male tail, mutations in *lin-15* disrupt some posterior fates (Table 1D,1-3). However, even in *lin-15* null mutants (*e1763* represents a deletion of the *lin-15* locus (Huang et al., 1994)), the lineage defect is only observed in some animals and only the lineage of the posterior **pp** cell is usually disrupted: it divides to produce up to four progeny instead of the normal two. Thus, in the B lineage mutations in *lin-15* result in a phenotype opposite from those seen in *lin-3/let-23* mutants. However, the effect is partial: spicule morphology in 31/63 (49%) *lin-15(n309)*, 23/55 (42%) *lin-15(n377)* and 23/57 (40%) *lin-15(e1763)* adult males is abnormal, consistent with the observation that the B lineage is abnormal in only 5/13 (38%) *lin-15* mutant animals followed. The effect is also primarily in the **pp** cell pair.

lin-15 likely acts as a negative regulator of *let-23* in the male B lineage

We followed the B lineage in a subset of double mutants to confirm the position of *lin-15* in the *lin-3/let-23* genetic pathway. Mutations in both *let-23* and *lin-45* block the requirement for functional *lin-15* (Table 1D,4-6). Specifically, whereas in *lin-15* mutants the lineage of the posterior **pp** cell can be disrupted, it is not disrupted in the *let-23; lin-15* or *lin-45; lin-15* double mutants. In addition, the lineage of the anterior **pp** cell can be disrupted as it is in *let-23* or *lin-45*, but never *lin-15*, mutants. These results are consistent with *lin-15* acting in the B lineage as it does in vulval development, where it negatively regulates *let-23*.

The positional cue from Y.p represents a signalling pathway distinct from *lin-15*

Y.p, or its progeny, produces a positional cue that promotes posterior fate, especially in the **pp** pair (Chamberlin and Sternberg, 1993). Ablation of Y.p disrupts the lineage of the posterior **pp** cell and the lineage defect is similar to that observed in *lin-15* mutants (Table 1E,1). This suggested that Y.p may be the source of a signal mediated by *lin-15*. Ablation of F, U and Y.p together results in a disruption of both anterior and posterior **pp** cell lineages (Table 1E,2). Ablation of Y.p in *lin-3*, *let-23*, *let-60* or *lin-45* mutants resembles $F^{-}U^{-}Y.p^{-}$ animals in that the fate of both anterior and posterior **pp** cells are commonly disrupted (Table 1E,3-6). In no case is a genetic mutation epistatic to the requirement for the presence of the Y.p cell. The similar results obtained for *lin-3*, *let-23*, *let-60* and *lin-45* mutants following Y.p ablation indicate that the positional information from Y.p does not act on the *lin-3/let-23* signalling pathway upstream of *lin-45* raf. Thus, *lin-15* and the Y.p signal are functionally distinct.

lin-12 can mediate a lateral interaction between the pp cells

Reduction-of-function mutations in *lin-12* (*lin-12(0)*) result in both **pp** cells producing γ-like lineages (anterior fate). In contrast, no B.a lineage defects are observed in animals bearing *lin-12* gain-of-function mutations (*lin-12(d)*) (Greenwald et al., 1983). To better understand the role of *lin-12* in **pp** fate specification, we have carried out cell ablation experiments in *lin-12(d)* mutants. Our results suggest that *lin-12(d)* mutations result in the opposite transformation from *lin-12(0)* mutations if F and U are ablated (Table 2). In *lin-12(d)* mutants both **pp** cells produce δ lineages when F and U are removed. However, since this result is at least similar to the effect observed when F and U are ablated in genotypically wild-type animals, the

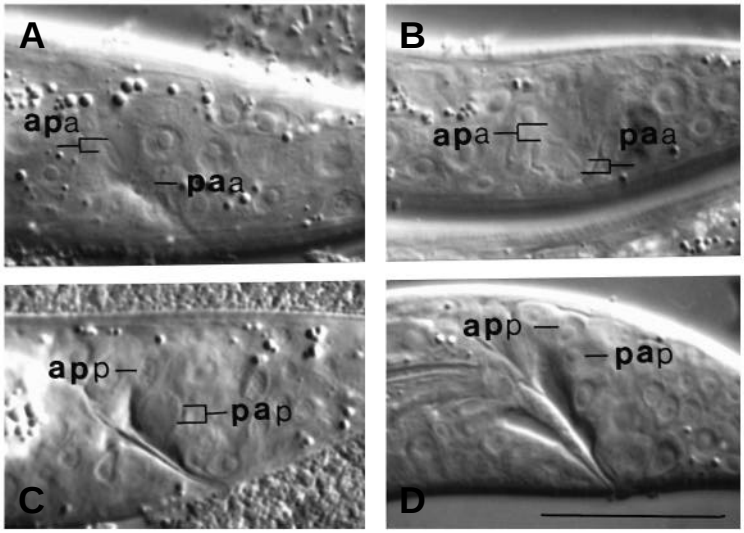

Fig. 4. Transformation of **pa** cells to ε fate in animals with the generally expressed *hsp::lin-3* transgene. Nomarski photomicrographs compare wild type to heat shocked, transgenic animals carrying *syEx21* (anterior left, ventral down). Differences between ε and ζ lineages are apparent in the timing of division of the progeny of **ap** and **pa**. In wild-type animals (A,C), **ap** cells produce ε lineages and **pa** cells produce ζ lineages. In a normal ε lineage, the anteroventral daughter (**ap**a, A; metaphase plate is visible) divides prior to the posterodorsal daughter (**ap**p, C). In a normal ζ lineage, **pa**p divides prior to **pa**a. Ectopic LIN-3 can promote posterior cells to produce the lineages normally associated with their anterior neighbors. In such animals, the anteroventral daughters of both **ap** and **pa** (B; metaphase plates are visible) divide prior to the posterodorsal daughters (D) and both cells produce ε lineages. Scale, 20 μm.

Table 2. The role of *lin-12* in the pp **pair**

				pp					
				anterior			posterior		
	F/U	*lin-12*	Y.p	γ	abn	δ	γ	abn	δ
a.	+	+	+	**all**	.	.	.	.	**all**
b.	+	-	-	**3**^{2}	.	.	**2**^{2}	**1**	.
c.	+	d	+	**3**	**1**	.	.	.	**4**
d.	-	+	+	**2**	**3**	**2**	.	.	**7**
e.	-	d	+	.	.	**3**^{1}	.	.	**3**^{1}
f.	-	+	-	**4**	**1**	.	.	**4**	**1**
g.	-	d	-	.	.	**5**^{3}	.	.	**5**^{3}

In *lin-12(0)* animals, both **pp** cells adopt γ fate (b). In *lin-12(d)* animals, the **pp** cells are normal. Ablation of the positional cues from F/U (e) uncovers the defect in *lin-12(d)* animals and both **pp** cells adopt δ fate. The contrast between *lin-12(d)* and wild type is best seen when F, U and Y.p are all removed (compare f to g). Each line indicates the presence of the F/U positional cue, *lin-12* genotype (+, wild type; –, loss-of-function (*lin-12(0)*); d, gain-of-function (*lin-12(d)*) and presence of Y.p positional cue. Data in b and c (and b and c of Table 3) were originally summarized in Greenwald et al. (1983). Data in d and f (and d and f of Table 3) are from Chamberlin and Sternberg (1993). In *lin-12(0)* animals (b), the Y cell adopts a neuronal fate and thus the Y.p cue is absent. In *lin-12(d)* animals (c,e,g) there are two Y.p-like cells. Both cells were ablated in the animals of line g. Notation is as in Table 1.

Table 3. The role of *lin-12* in the aa **pair**

					aa					
					anterior			posterior		
	F/U	*lin-12*	Y.p	B.a	α	abn	β	α	abn	β
a.	+	+	+	+	**all**	.	.	.	.	**all**
b.	+	-	-	+	**2**	.	.	.	.	**2**
c.	+	d	+	+	**3**	.	.	.	.	**3**
d.	-	+	-	+	**3**^{2}	**2**^{2}	.	.	**1**^{1}	**4**^{3}
e.	-	d	-	+	**2**^{2}	**2**^{2}	.	.	**2**^{2}	**2**^{2}
f.	-	+	-	-	**6**^{5}	.^{1}	.	.	**3**^{3}	**3**^{2}
g.	-	d	-	-	**1**	**1**^{1}	**1**^{1}	.	**1**	**2**^{2}

aa lineages are normal in both *lin-12(0)* and *lin-12(d)* mutants. Removal of F/U, Y.p and B.a positional cues in wild-type animals uncovers a possible lateral interaction between **aa** cells (Chamberlin and Sternberg, 1993; f). Removal of F/U, Y.p and B.a positional cues in *lin-12(d)* mutants indicates that *lin-12* does not appear to play an essential role in the specification of **aa** fates even in the absence of these positional cues. Notation is as in Tables 1 and 2.

contrast between *lin-12(d)* and wild type is best seen when F, U and Y.p are removed. In wild-type animals, this ablation results in γ and γ*/δ* lineages from both **pp** cells, whereas in *lin-12(d)* animals both **pp** cells produce δ lineages (posterior fate; compare Table 2f,g). Thus, the presence of F and U can override the effect of the *lin-12(d)* mutation. An additional defect in *lin-12(0)* animals is that the presumptive Y cell is transformed to a neuronal fate similar to its lineal homolog DA9 (Greenwald, et al., 1983). Since *lin-12(0)* animals are also missing the positional cue from Y.p, we cannot yet establish if the presence of Y.p is likewise sufficient to compensate for the absence of *lin-12* activity.

Ablation experiments in wild-type males suggested that the **aa** cells may also interact with each other (Chamberlin and Sternberg, 1993). However, ablation experiments in *lin-12(d)* mutants carried out for the **aa** pair failed to provide evidence of an essential role for *lin-12* (Table 3).

DISCUSSION

The genes in the *lin-3/let-23* signalling pathway mediate the F/U signal defined by cell ablation

Cell ablation experiments suggest that four distinct cell interactions are essential for normal anterior/posterior patterning of fates for four pairs of cells in the *C. elegans* male B lineage (Chamberlin and Sternberg, 1993). The male-specific blast cells F and U mediate one of these signals that is necessary for normal anterior fates. The *lin-3/let-23* pathway is both necessary and sufficient to promote anterior fates, and thus likely mediates the F/U signal.

The lineage defect in *lin-3/let-23* mutants with F and U ablated is more severe than either mutant or ablation alone. This result suggests that not only might the analyzed mutations not represent null mutations for male tail function, but also that all signalling activity may not be eliminated from animals in which F and U have been ablated. Such residual activity may come from the debris of the ablated cells, or may come from other, unidentified sources. However, if such other sources exist they are not sufficient to promote the normal anterior fates in the absence of F and U, and likely do not play a significant role in normal development.

The role of *lin-3/let-23* in fate specification

What specific role in fate specification does the *lin-3/let-23* pathway play in *C. elegans* spicule development? Our results suggest that the same genes mediate the F/U signal for all four pairs of B.a progeny. Since mutations in a single gene of the *lin-3/let-23* pathway can result in all of the defects observed in animals with F and U ablated, we can exclude the possibility

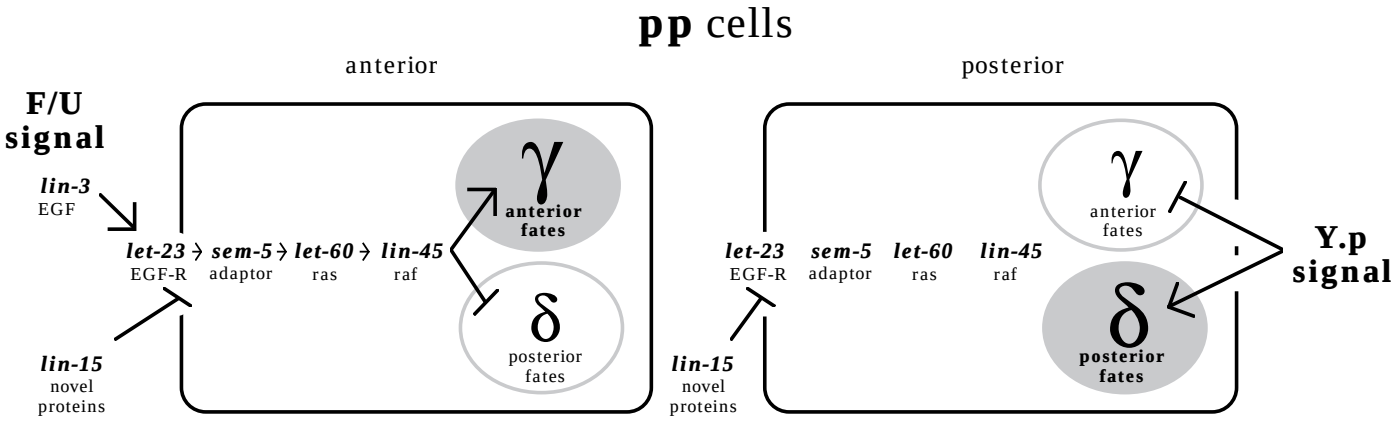

Fig. 5. Site of action of *lin-15* and the Y.p cue relative to the *lin-3/let-23* pathway. *lin-15* likely acts antagonistically and in parallel to *lin-3* as a negative regulator of *let-23* activity to specify fates in the B lineage. The Y.p signal acts on the pathway downstream of *lin-45* and may represent an independent signalling pathway that acts in parallel to, or possibly after, the *lin-3/let-23* pathway. The gene order in the pathway is based on the epistasis established for these genes in hermaphrodite vulval induction (reviewed by Sternberg, 1993). It is not known if the genes are expressed in the indicated cells.

that F and U produce three different signals, with one for the **aa** pair, one for the **pp** pair and one for the **ap**/**pa** pairs. Nevertheless, the cellular response is distinct for each pair. In response to generally expressed LIN-3, the **pp** cells produce more progeny than normal while **aa** cells produce fewer progeny. LIN-3 also does not appear to induce a specific differentiated cell type (Chamberlin and Sternberg, 1993), since neuronal and epidermal progeny arise from both anterior and posterior blast cells (Sulston et al., 1980). These differences among the three pairs suggest that the *lin-3/let-23* pathway promotes a particular choice among possible responses. The final outcome depends upon functional differences among the responding cells.

The role of *lin-15* in the B lineage

During *C. elegans* vulval development, loss-of-function, molecular null mutations in *lin-15* result in all VPCs adopting vulval fates (Huang et al., 1994). The defect in the B lineage, in contrast, is less extreme and less than 50% penetrant. Both vulval development and spicule development, however, have a necessary requirement for the positive acting genes of the *lin-3/let-23* pathway. One possible reason for this difference in the requirement for *lin-15* is that development of the B cell includes additional specification mechanisms, such as positional cues from Y.p and the other B.a progeny, that also act antagonistically to the *lin-3/let-23* pathway. We believe that although the function of *lin-15* may be similar in both developmental processes, its role is diminished in the B lineage as it is partially redundant with other activities.

The role of *lin-12* in the B lineage

Our results support the findings of Greenwald et al. (1983) and suggest that *lin-12* may mediate a lateral interaction between the two **pp** cells. However, *lin-12* is not sufficient for δ fate, as the presence of the F and U cells can promote γ fate in *lin-12(d)* mutants. This result is similar to the relationship between *lin-12(d)* mutations and the AC signal in vulval development. Normally all VPCs adopt 2° vulval fate in *lin-12(d)* mutants. However, these animals also lack an AC due to an earlier function of *lin-12*. In rare *lin-12(d)* animals with an AC, the AC signal can override the *lin-12(d)* defect and promote 1° fate in the most proximal VPC (Sternberg and Horvitz, 1989). In the B lineage, *lin-12* may also not be necessary for δ fate, as we have not tested if a *lin-12(0)* animal with a normal Y cell can produce a normal pattern of **pp** cell fates. Thus, *lin-12* functions in this cell pair, but is at least partially redundant with other positional cues.

Integration of multiple signals

Two activities that act antagonistically to the *lin-3/let-23* pathway are required for normal fate specification in the dorsal **pp** pair (Fig. 5). If *lin-15* acts in the B lineage as it does in vulval development and is required in cells other than the responding cells (Herman and Hedgecock, 1990), then both *lin-15* and the Y.p positional cue represent extracellular cues that must be integrated by the responding cells. We have characterized the probable relationship of both these activities relative to the *lin-3/let-23* pathway. If the function of a gene (like *lin-15*) or a signal (like the Y.p cue) is to negatively regulate the activity of the receptor *let-23*, mutations in the receptor or any downstream gene such as *lin-45* should render the pathway insensitive to the removal of the regulator. Consequently, the lineage defect observed in *let-23* or *lin-45* mutants with the activity removed should be the same as when the activity is intact. This is the case with *lin-15*. In particular *let-23; lin-15* and *lin-45; lin-15* double mutants resemble the single *let-23* and *lin-45* mutants, with the posterior **pp** cell always producing a normal δ lineage. However, ablation of Y.p in *let-23*, *let-60* or *lin-45* mutants results in abnormal lineages similar to $F^{-}U^{-}Y.p^{-}$ or *lin-3* $Y.p^{-}$ animals. In all cases, the posterior **pp** cell produces abnormal lineages rather than the normal δ lineage. Therefore, the Y.p cue does not act by negatively regulating the *lin-3/let-23* pathway upstream of *lin-45* raf. Furthermore, the anterior **pp** cell produces abnormal lineages as in the single mutants rather than the normal γ lineage. Thus, disruption of the Y.p signal is not strictly epistatic to disruption of the F/U signal. We propose that the Y.p cue represents a distinct, parallel signalling pathway that acts at the same time or later than the *lin-3/let-23* pathway.

The positional cues from F/U and Y.p represent two of the

four active cell interactions identified by cell ablation experiments (see Fig. 3B). F and U provide an anterior positional cue (labeled 1 in Fig. 3) that is mediated by the *lin-3/let-23* signalling pathway. Our results and the results of Greenwald et al. (1983) suggest that, at least in the **pp** pair, the lateral interaction (4 in Fig. 3) is mediated by the gene *lin-12*. Our results indicate that *lin-15* does not mediate the Y.p cue (3 in Fig. 3). However, our data do not rule out the possibility that *lin-15* plays a role in mediating the positional cue from the other B.a progeny (2 in Fig. 3).

We have found that, in the *C. elegans* male B lineage, the fate of the **pp** cells results from coordinate integration of multiple signals, including three defined genetically by the *lin-3/let-23* pathway, *lin-15* and *lin-12* genes initially identified for their role in hermaphrodite vulval development. Not only do the **pp** cells respond to the same signalling pathways as do the hermaphrodite VPCs, but the integration of these signals appears to be similar: *lin-15* acts as a negative regulator of *let-23* and the signal mediated by the *lin-3/let-23* pathway can override the effect of a *lin-12(d)* mutation. Yet the cellular responses, such as cell division pattern and the differentiated fates of progeny that result in response to these signals are very different in male spicule development compared to hermaphrodite vulval development. An exciting possibility is that, in insect and vertebrate development, as well as in nematode development, not only are signalling pathways conserved, but so is the coordination of multiple signal integration.

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