lin-25, a gene required for vulval induction in *Caenorhabditis elegans*

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During vulval development in the *Caenorhabditis elegans* hermaphrodite, the fates of six vulval precursor cells (VPCs) are influenced by distinct cell signaling events. In one event, a somatic gonadal cell, the anchor cell, induces the three nearest VPCs to adopt vulval cell fates. In another event, lateral signaling between adjacent VPCs specifies one of two different vulval fates, 1° and 2°. Induction of vulval fates by the anchor cell is mediated by a signal transduction pathway involving *let-60* Ras, *lin-45* Raf, and *mpk-1/sur-1* MAP kinase, whereas lateral signaling is mediated by *lin-12*. We have shown that the mutant phenotype of *lin-25*, a gene required for VPC fate specification, results from a defect in vulval induction. Genetic epistasis experiments indicate that *lin-25* is required in the inductive signaling pathway downstream of *let-60* Ras and the Raf/MAP kinase cascade. A decrease in induction also appears to decrease lateral signaling. We have cloned and sequenced the *lin-25* gene and shown that it encodes a novel protein that may be a target of the *mpk-1/sur-1* MAPK.

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Signaling between cells is required for a variety of developmental processes including cell-fate determination, cell migration, cell proliferation, and cell shape changes. In recent years, it has become apparent that many of the molecules that mediate signaling have been very highly conserved during evolution. In many different signal transduction pathways, binding of a ligand to a tyrosine kinase type receptor leads to the sequential activation of a group of proteins: Ras, Raf, MAP kinase kinase (MEK), and MAP kinase (MAPK). Biochemical studies have shown that these proteins can direct cell proliferation or differentiation of cultured mammalian cells (for review, see Khosravi and Der 1994). Genetic analysis has shown that the same cascade mediates many different signaling events in both unicellular and multicellular organisms including the response to mating pheromones in *Schizosaccharomyces pombe* (Fukui et al. 1986; for review, see Kurjan 1993), growth control in *Saccharomyces cerevisiae* (for review, see Broach and Deschenes 1990), the induction of vulval cell fates in *Caenorhabditis elegans* (Beitel et al. 1990; Han and Sternberg 1990; Han et al. 1990), the establishment of dorsoventral polarity and development of the terminal structures in the *Drosophila* embryo (Ambrosio et al. 1989; Brand and Perrimon 1994), and the determination of photoreceptor cell fate in the eye imaginal disc (Simon et al. 1991).

A great deal of progress has been made recently in

elucidating the events leading up to the activation of Ras by upstream components (for review, see Khosravi and Der 1994). In contrast, much less is known at the present time about the events taking place at the end of the cascade, downstream of MAPKs. Many changes induced by activation of the Ras/MAPK pathway undoubtedly result from changes in gene expression. Precisely how these come about, however, is not well understood. Furthermore, it is not known whether changes in transcription are sufficient to mediate the effects of the Ras pathway or whether changes brought about independently of immediate effects on gene expression are also important.

Work on other organisms has shown that MAPKs mediate their effects at least in part by phosphorylating transcription factors. For example in both mammalian cells and *Drosophila*, MAPKs are able to modulate the activities of some members of the Ets family of DNA-binding proteins (Gille et al. 1992; Marais et al. 1993; Brunner et al. 1994b; O'Neill et al. 1994). However, several other substrates have been identified for MAPK, some of which are themselves kinases (Ray and Sturgill 1987; Sturgill et al. 1988; Chou et al. 1992; Stokoe et al. 1992), suggesting that changes in gene expression might also result from the indirect activation of other factors. For example, activation of Ras in PC12 cells leads to the rapid stimulation of the CREB kinase which, in turn, activates the DNA-binding protein, CREB, which is necessary for full induction of the immediate early gene, *c-fos* (Ginty et al. 1994).

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In yeast, some changes induced by MAPKs occur independently of immediate changes in transcription. For example, in *S. cerevisiae*, the MAPK, FUS3, has at least two separate functions. One is to activate the transcription factor STE12 (Song et al. 1991), which is required for the transcription of genes in response to mating pheromones, and another is to activate the product of the FAR1 gene (Peter et al. 1993), a cyclin-dependent kinase inhibitor, which is required for growth arrest in the G₁ phase of the cell cycle (Chang and Herskowitz 1992).

During development of the vulva in the *C. elegans* hermaphrodite, a signal from a somatic gonadal cell, the anchor cell, causes three nearby hypodermal cells, P5.p, P6.p, and P7.p, to adopt vulval fates (Sulston and White 1980; Kimble 1981). These three cells are part of a group of six cells, P3.p–P8.p, that are collectively termed vulva precursor cells (VPCs). The VPCs are initially developmentally equivalent: Each has the potential to adopt vulval or hypodermal fates (Sulston and White 1980; Sternberg and Horvitz 1986). Classical and molecular genetic analyses have shown that the anchor cell signal, encoded by the *lin-3* gene (Hill and Sternberg 1992), promotes vulval induction by activating a signal transduction pathway involving Let-23, a receptor-type tyrosine kinase (Aroian et al. 1990), Sem-5, an SH2- and SH3-domain containing protein (Clark et al. 1992), Let-60 (Ras), and Lin-45 (Raf) proteins (Han and Sternberg 1990; Han et al. 1993), and a MAPK, Mpk-1/Sur-1 (Lackner et al. 1994; Wu and Han 1994).

Precisely how the activation of *mpk-1/sur-1* MAPK causes cells to adopt vulval fates is not well understood. Two genes, *lin-1* and *lin-31*, have been shown to function downstream of *mpk-1/sur-1* in the genetic pathway for VPC fate specification (Lackner et al. 1994; Wu and Han 1994), but it is not yet clear how they act at a molecular level. *lin-1* is a negative regulator of vulval induction (Horvitz and Sulston 1980; Ferguson and Horvitz 1985). Loss-of-function mutations in *lin-1* cause cells that normally adopt a hypodermal fate, P3.p, P4.p, and P8.p, to instead adopt vulval fates (Horvitz and Sulston 1980; Sulston and Horvitz 1981; Ferguson et al. 1987). *lin-31*, which encodes a transcription factor of the HNF3/forkhead family, is involved in both positive and negative regulation (Miller et al. 1993). Mutations in *lin-31* lead to defects in fate specification in all six cells, P3.p–P8.p (Ferguson et al. 1987), and the Lin-31 protein is thought to function by regulating the activities of other proteins, both positively acting determinants and negatively acting ones (Miller et al. 1993). However, until this study, no strictly positively acting factors acting at this step in vulval induction had been identified.

In this paper we describe the genetic and molecular genetic characterization of the *lin-25* gene, which is required for VPC fate specification. We have shown by a combination of laser ablation experiments and genetic epistasis tests that *lin-25* is a positive regulator of vulval induction and that it functions downstream of *let-60 ras* in the genetic pathway for induction. We have cloned and sequenced the gene and shown that it encodes a

novel protein that may be a substrate for the Mpk-1/Sur-1 MAPK.

Results

Background

The *C. elegans* vulva, the structure linking the uterus to the exterior, is generated from the 22 descendants of 3 ventral hypodermal cells, P5.p, P6.p, and P7.p (Sulston 1976; Sulston and Horvitz 1977). These three cells are part of a group of six cells, P3.p–P8.p, that are initially developmentally equivalent (Fig. 1) (Sulston and White 1980). The fates of these six cells, termed collectively the vulval precursor cells (VPCs), are influenced by two or possibly three distinct cell signaling events (Kimble 1981; Sternberg and Horvitz 1986, 1989; Herman and Hedgecock 1990). The anchor cell is thought to induce the three VPCs closest to it, P5.p, P6.p, and P7.p, to adopt vulval fates (Sulston and White 1980; Kimble 1981) (Fig. 1). Two different vulval fates are seen in wild-type worms, 1° and 2°. P6.p, which lies closest to the anchor

Figure 1. (A) A model for signaling in the developing vulva (based on Horvitz and Sternberg 1991). A gonadal cell, the anchor cell (AC), induces nearby cells to adopt vulval cell fates (Kimble 1981). VPCs induced to adopt a vulval fate signal laterally to one another causing P5.p and P7.p to adopt the 2° fate (Sternberg 1988). The precise order of the signaling events is not yet known. The putative inhibitory signal between hyp7 and the VPCs (Herman and Hedgecock 1990) is not shown. (B) Each fate is to give rise to a lineage, that is to undergo a particular pattern of cell divisions and to generate a characteristic set of cell types. For the VPCs, the pattern formed by the planes of cell division and the adhesion properties of the granddaughters is a useful marker for the VPC fates (Sternberg and Horvitz 1986; see Table 2 legend). These planes are designated T or L: (T) a transverse (left–right) division (with respect to the symmetry of the animal); (L) a longitudinal division (anterior–posterior); (N) the cell does not divide. If the cells produced by a longitudinal division adhere to the ventral cuticle this is denoted by underlining (i.e., L). P5.p and P7.p generate mirror symmetric lineages both of which are considered 2°. (S) The cell fuses with the large hypodermal syncytium, hyp7, which surrounds the VPCs.

cell, adopts the 1° fate, whereas P5.p and P7.p adopt the 2° fate. The choice between 1° and 2° fates is thought to be controlled by a second signaling event in which VPCs signal laterally to one another (Sternberg 1988). In wild-type hermaphrodites P3.p, P4.p, and P8.p do not give rise to cells contributing to the vulva but, instead, generate hypodermal cells [the hypodermal (3°) fate]. There is evidence that a large syncytial cell, *hyp7*, which surrounds the VPCs participates in a third signaling event that inhibits the VPCs from adopting vulval fates (Herman and Hedgecock 1990).

Mutations affecting vulval development are of two types, vulvaless and multivulva (Horvitz and Sulston 1980; Sulston and Horvitz 1981; Ferguson and Horvitz 1985). Vulvaless mutations prevent P5.p, P6.p, and P7.p from adopting vulval fates: Animals carrying such mutations have no vulva and are therefore unable to lay eggs. Multivulva mutations cause the opposite phenotype. In animals carrying these mutations, cells that normally adopt the hypodermal fate, P3.p, P4.p, and P8.p, instead adopt a vulval fate (1° or 2°) (Ferguson et al. 1987). Multivulva mutations, therefore, cause vulval tissue to be generated at ectopic sites. The pseudovulvae so formed are clearly visible under the dissecting microscope as protrusions from the ventral hypodermis (Horvitz and Sulston 1980; Sulston and Horvitz 1981).

The lin-25 null phenotype

Ten *lin-25* alleles are presently known. *lin-25(e1446)* and *lin-25(n545ts)* were found in genetic screens for mutations affecting egg-laying (Trent et al. 1983; Ferguson and Horvitz 1985). Two additional alleles, *ar90* and *n1063*, were isolated in noncomplementation screens that made no assumptions about the nature of the null phenotype (see Materials and methods; K. Edwards and H.R. Horvitz, pers. comm.), and six other alleles were isolated in a number of other genetic screens (S. Clark and H.R. Horvitz; H. Chamberlin and P. Sternberg; D. Eisenmann and S. Kim; M. Han; all pers. comm.). The phenotypes conferred by the seven strongest alleles, *e1446*, *ar90*, *n1063*, *ga67*, *ku70*, *ku77*, and *ku78*, are indistinguishable (Table 1A). Approximately 6% of hermaphrodites homozygous for any of these alleles die as larvae, a slightly greater proportion are sterile, and the remainder (the majority) are fertile but completely egg-laying defective. All 10 *lin-25* mutant alleles are recessive to wild type. Furthermore, animals homozygous for *n1063* but bearing a duplication of the region are able to lay eggs (Table 1B).

The seven strong *lin-25* mutations appear to be null alleles by the following criteria. First, for all seven alleles neither the larval lethality nor the sterility defects are markedly enhanced in *trans* to a deficiency of the region, *arDf1* (Table 1B; data not shown). Second, *lin-25(n1063)* behaves like a deficiency in *trans* to *lin-25(n545ts)*, at 20°C. At this temperature, *lin-25(n545ts)* is a hypomorphic (reduced activity) allele (Table 1B). Third, *lin-25(ar90)* is suppressed by the amber suppressor tRNA

encoded by *sup-7(st5)* (Waterston 1981; Wills et al. 1983) (see Materials and methods). Finally, we have sequenced *lin-25(ar90)* and found that the mutation creates an amber codon that would lead to a severely truncated protein (see below).

Ferguson et al. (1987) have described three different effects of *lin-25* mutations on postembryonic development. First, during the L1 stage, in ~75% of the animals, one of the six cells, P3.p–P8.p, divides to produce two daughters that subsequently both become VPCs. *lin-25* mutant hermaphrodites, therefore, often contain seven VPCs instead of the normal six. Second, during the L3 stage, *lin-25* mutant animals show defects in VPC fate specification. Third, temperature-shift experiments indicate that *lin-25* is required for the development of some component of the egg-laying apparatus during the early L4 stage. In this paper we have focused on the role of *lin-25* in VPC fate determination. Some additional observations on other defects are described in Materials and methods.

In *lin-25(e1446)* mutant hermaphrodites P5.p and P7.p, which normally adopt the 2° fate, instead adopt the 3° fate (Ferguson et al. 1987). In many animals P6.p adopts the 1° fate but in others the fate adopted by P6.p does not correspond to any seen in wild-type animals. We have analyzed the VPC lineages in animals carrying a different allele, *lin-25(n1063)*, (Table 2) and found a similar pattern. In these animals P5.p and P7.p undergo the 3° lineage and P6.p often generates a hybrid lineage, which either has characteristics of two different vulval lineages (1° and 2°) or of a vulval lineage and the nonvulval (3°) one.

lin-25 is not required for specification of the 2° fate if Lin-12 is activated

The absence of cells expressing the 2° fate could arise for two quite different reasons. First, it could result from a defect in lateral specification. For example, mutations affecting the production of the lateral signal or its reception or transduction in P5.p and P7.p might result in P5.p and P7.p failing to adopt the 2° fate. Alternatively, it might indicate a defect in vulval induction. In hermaphrodites carrying certain hypomorphic mutations in *lin-3*, *let-60*, and *sem-5*, genes encoding components of the inductive signaling pathway, the pattern of fates adopted by the VPCs is similar to that seen in *lin-25* mutants (Sulston and Horvitz 1981; Beitel et al. 1990; Clark et al. 1992).

To investigate the possibility that *lin-25* might be required for lateral signaling we constructed *lin-12(d)/+*; *lin-25(0)* double-mutant animals. *lin-12* is thought to be the receptor for the lateral signal. Dominant, hypermorphic mutations in *lin-12*, such as *lin-12(n137)*, cause all six VPCs to adopt the 2° fate, and hermaphrodites carrying such mutations are Multivulva (Greenwald et al. 1983). *lin-25(e1446)* had no effect on the Multivulva phenotype of *lin-12(n137)/+*. One hundred percent (215/215) of the *lin-12(n137)/+*; *lin-25(e1446)* double-mutant

Table 1. *lin-25* alleles and gene dosage studies with *lin-25*

A. Comparison of <i>lin-25</i> alleles			
	Percent		
Allele ^a	Egl ^b	sterile ^b	larval arrest ^c
<i>e1446</i>	100 (231/231)	10 (28/278)	7 (19/278)
<i>ar90</i>	100 (273/273)	14 (48/337)	5 (16/337)
<i>n1063</i>	100 (159/159)	14 (28/199)	6 (12/199)
<i>ku78</i>	100 (172/172)	12 (25/211)	7 (14/211)
<i>ku70</i>	100 (many)	—	—
<i>ku77</i>	100 (156/156)	12 (23/191)	6 (12/191)
<i>ga67</i>	100 (287/287)	9 (30/342)	7 (25/342)
<i>sy29</i>	100 (374/374)	5 (18/393)	<1 (1/393)
<i>n545ts</i>	67 (170/255)	0 (0/255)	<1 (1/255)
<i>n1722ts</i>	2 (5/246)	0 (0/246)	0 (0/246)

B. Gene dosage studies with <i>lin-25</i>			
	Percent		
Genotype	Egl ^b	sterile ^b	larval arrest ^c
<i>n1063/arDf1^{b,d}</i>	100 (167/167)	20 (46/224)	5 (11/224)
<i>n1063/+</i>	0 (0/185)	0 (0/185)	0 (0/185)
<i>n1063/n1063/+^c</i>	4 (6/168)	2 (3/171)	0 (0/171)
<i>+/+/+^c</i>	3 (7/212)	2 (5/217)	0 (0/217)
<i>n545ts/n1063^f</i>	85 (231/271)	0 (0/271)	0 (0/271)
<i>n545ts/arDf1^f</i>	89 (193/217)	0 (0/217)	0 (0/217)

^aAll alleles are EMS induced. The isolation of *e1446* and *n545ts* has been described elsewhere (Ferguson and Horvitz 1985). The isolation of *ar90* is described in Materials and methods. *n1063* and *n1722ts* were obtained from K. Edwards, S. Clark, N. Young, and H.R. Horvitz (MIT, Boston, MA). *ku70*, *ku77*, and *ku78* were obtained from M. Han (University of Colorado, Boulder). *ga67* was obtained from D. Eisenmann and S. Kim (Stanford University, CA). *sy29* was obtained from H. Chamberlin and P. Sternberg (Caltech, Pasadena, CA). The complete genotype of *ku70* was *dpy-11(e224) lin-25(ku70)*.

^bThe complete broods of 5–8 hermaphrodites were scored first for larval lethality. Animals that survived were then scored for fertility and for the ability to lay eggs. (Egl) Egg-laying defective. Percentages of Egl animals given are the percentages of surviving and fertile animals that were Egl rather than of the whole population. Animals were scored as sterile only if they had no progeny. Many other animals had much reduced brood sizes. Animals homozygous for *e1446*, *ar90*, *n1063*, *ku70*, *ku77*, *ku78*, and *ga67* are completely egg-laying defective providing their parent was also homozygous; partial maternal rescue is seen when the parent is heterozygous (see Materials and methods).

^cIt is not known why some *lin-25* animals die as larvae (see Materials and methods).

^dComplete genotype *unc-42(e270) arDf1/sma-1(e30) lin-25(n1063)*. *arDf1* deficiency homozygotes die before hatching (Ahnn and Fire 1994; R. Barstead, pers. comm.). Unhatched eggs were therefore assumed to be of the genotype *arDf1/arDf1* rather than *arDf1/lin-25(n1063)*. The *Sma* phenotype of *sma-1(e30)* is easily scored in newly hatched L₁ larvae so that it was possible to distinguish dead larvae of the genotype *lin-25(n1063)/lin-25(n1063)* from those of the genotype *lin-25(n1063)/arDf1*. The proportion of *Sma* to non-*Sma* animals in the population surviving to become adults was not significantly different from the expected ratio of 1:3. Furthermore, the proportion of unhatched eggs to hatched was 1/4. These results suggest that the large majority of *lin-25(n1063)/arDf1* animals hatched.

^eComplete genotypes *sma-1(e30) lin-25(n1063)*; *ctDp8* and *unc-42(e270) sma-1(e30)*; *ctDp8*, respectively. Duplications and deficiencies often cause low penetrance egg-laying defects for reasons that are not understood.

^fThe percentages given are for *trans* heterozygotes arising from a cross in which one parent carried a wild-type *lin-25* allele. Animals of these genotypes coming from hermaphrodites lacking a wild-type *lin-25* allele are 100% Egl.

animals were Multivulva. We followed the lineages generated by the VPCs in eight double-mutant hermaphrodites. In most individuals, most of the VPCs adopted the 2° fate (Table 2). Furthermore, in double-mutant animals carrying a molecular marker for the 2° fate, *lacZ* under the control of *lin-11* gene regulatory sequences (Freyd et al. 1990; G. Freyd and H.R. Horvitz, pers. comm.), the pattern of expression of the reporter was consistent with all cells having adopted the 2° fate (data

not shown). Thus *lin-25* is not required for the specification of the 2° fate in the presence of activated Lin-12.

lin-25 is required for vulval induction in the absence of *lin-15* activity

We reasoned that the *lin-25* mutant phenotype might be explained if the gene product were required upstream of *lin-12* for the production or function of the lateral signal, or were itself the signal. To test this possibility we con-

Table 2. Vulval fates in *lin-25* mutant animals

Genotype	AC	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	Pn.p ^a
Wild type ^b	+	3°	3°	2°	1°	2°	3°	
<i>lin-25(n1063)</i> ^c	+	SS	SS	<u>LLTN</u>	TTTT	<u>NTLL</u>	SS	
	+	3°	3°	3°	1°/ind	3°	3°	3°
	+	SS	SS	SS	TTTS	SS	SS	
	+	SS	SS	TTTT	TO S	S	SS	
	+	SS	SS	SS	OSOL	SS	SS	
	+	SS	SS	SS	SS	STLT	SS	SS
<i>lin-25(n1063)</i>	+	SS	SS	SS	S SS	SS	SS	SS
	+	SS	SS	S TO	TOLT	SS	S	
	— ^d	3°	3°	3°	3°	3°	3°	3°
	—	S	SS	SS	SS	S	SS	
	—	S	SS	SS	SS	SS	S	SS
	—	SS	SS	SS	SS	SS	SS	SS
<i>lin-12(n137)/+</i> ^e	— ^f	2°	2°	2°	2°	2°	2°	
<i>lin-12(n137)/+;</i> <i>lin-25(e1446)</i>	— ^f	2°	2°	2°	2°	2°	2°	2°
		<u>LLON</u>	<u>LLLO</u>	<u>LLTN</u>	<u>LLTN</u>	<u>LLOO</u>	<u>NSLL</u>	<u>LLOO</u>
		<u>LOTT</u>	<u>OLLL</u>	<u>LLTN</u>	<u>LLTT</u>	<u>NOSS</u>	<u>LLTN</u>	
		<u>LLTO</u>	<u>LLTN</u>	<u>LLTT</u>	<u>LOLL</u>	<u>NTLL</u>	<u>LLTN</u>	<u>LLOT</u>
		<u>LLLT</u>	<u>LLON</u>	<u>LLLL</u>	<u>LLLN</u>	<u>SSTN</u>	<u>LLTT</u>	<u>LLLO</u>
		<u>LLLL</u>	<u>LLTT</u>	<u>OOLL</u>	<u>TLLL</u>	<u>LLLL</u>	<u>NOLT</u>	
		<u>LLOT</u>	<u>LLTO</u>	<u>LLLN</u>	<u>LTLL</u>	<u>OTLL</u>	<u>LNLL</u>	
		SS	<u>LLTT</u>	<u>OLTT</u>	<u>NTLL</u>	<u>LLLL</u>	<u>LOLL</u>	
		<u>LLTT</u>	<u>LLTO</u>	<u>LLON</u>	<u>LLNT</u>	<u>NTLL</u>	<u>NSLL</u>	<u>LLOT</u>

Each line showing lineages represents the vulva lineages of an individual animal. See Fig. 1 legend for the meaning of S, L, T and N. (O) Oblique division. Consensus fates derived from several lineages are shown above each group of lineages, using the criteria of Sternberg and Horvitz (1986). (3°) S and SS; (2°) LLTN, LLTT, LLTO, LLOT (or their mirror images); (1°) TTTT (or any lineage in which all four descendants of a VPC generate cells that fail to adhere to the ventral cuticle); (ind) cells were induced, but lineage generated was not clearly 1° or 2°. Lineages were followed from the mid-L3 stage onwards.

^aIn hermaphrodites carrying strong *lin-25* mutations one of the cells, P3.p–P8.p, often divides during the L1 stage to produce two cells both of which subsequently become VPCs (Ferguson et al. 1987). *lin-25* mutant hermaphrodites therefore often have seven rather than six VPCs. We did not determine which cell had divided precociously in these animals: Cells are therefore numbered arbitrarily starting at P3.p, the most anterior VPC.

^bFrom Sulston (1976) and Sulston and Horvitz (1977).

^cThe lineages generated in *lin-25(e1446)* are described in Ferguson et al. (1987). The position of the AC with respect to the VPCs in *lin-25* mutant hermaphrodites is somewhat variable.

^d(–) Animals with no anchor cell. The anchor cell was removed by ablating the progenitors of the somatic gonad, Z1 and Z4, during the L1 stage.

^eFrom Mango et al. (1991).

^fMost *lin-12(n137)/+* hermaphrodites have no anchor cells (Greenwald et al. 1983).

structed double mutant animals containing a mutation in *lin-25* and a mutation in another gene required for correct VPC fate specification, *lin-15*. *lin-15* is a negative regulator of vulval induction (Ferguson et al. 1987; Clark et al. 1994; Huang et al. 1994). In *lin-15(n309)* mutant hermaphrodites, all six VPCs adopt a vulval fate even in the absence of the anchor cell (and the rest of the somatic gonad) (Ferguson et al. 1987; Sternberg and Horvitz 1989). In these animals, lateral signaling between adjacent VPCs results in a pattern in which cells adopting the 1° and 2° fates alternate along the ventral hypodermis (Ferguson et al. 1987; Sternberg 1988). This pattern requires *lin-12* activity: In *lin-12(0); lin-15(n309)* double-mutant hermaphrodites, all six VPCs adopt the 1° fate and the animal is Multivulva, even in the absence of the gonad (Sternberg and Horvitz 1989). If *lin-25* were required specifically for lateral signaling in the vulva, the

lin-25(0); lin-15(n309) double mutant should resemble the *lin-12(0); lin-15(n309)* double mutant. All six VPCs should adopt the 1° fate, and such animals would be Multivulva. We found instead that *lin-25* mutations strongly suppressed the Multivulva phenotype of *lin-15(n309)* (Fig. 2). The effect of *lin-25* mutations on the fates adopted by the VPCs in *lin-15* mutant animals was most clearly seen in the absence of the gonad. In *lin-25(0); lin-15(n309)* double-mutant hermaphrodites in which the gonad had been ablated most VPCs adopted the non-vulval (3°) fate, or expressed hybrid lineages, suggesting that they had been only partially induced (Table 3). These results suggest that *lin-25* is required for induction of vulval fates rather than specifically for lateral specification.

It was not possible to examine the efficiency of lateral signaling in *lin-25(0); lin-15(n309)* double-mutant her-

Figure 2. Phenotypes of adult hermaphrodites carrying mutations affecting vulval development. All animals are viewed by Nomarski differential interference contrast microscopy. (N2) The wild-type strain. The arrowhead indicates the position of the vulva. (*lin-25*) Complete genotype *lin-25(e1446)*. This animal has a small protrusion at the vulva characteristic of *lin-25* mutant hermaphrodites (Ferguson and Horvitz 1985). (*lin-12(d)/+*) Complete genotype *dpy-19(e1259)lin-12(n137)/unc-32(e189)*. *lin-12(d)/+* hermaphrodites are Multivulva (Greenwald et al. 1983). Ectopic pseudovulvae are marked with arrowheads. *lin-12(d)/+; lin-25(0)*, complete genotype *dpy-19(e1259)lin-12(n137)/unc-32(e189); lin-25(e1446)*. *lin-25* does not affect the Multivulva phenotype caused by *lin-12(d)/+*. (*lin-15(0)*) Complete genotype *lin-15(n309)*. *lin-15* mutant animals display a Multivulva phenotype distinct from that shown by *lin-12(d)* mutant animals (Ferguson and Horvitz 1985; Ferguson et al. 1987). The pseudovulvae are larger and less regularly spaced. (*lin-25(0); lin-15(0)*) Complete genotype *lin-25(e1446); lin-15(n309)*. *lin-25* mutations suppress the Multivulva phenotype caused by *lin-15* mutations. (*let-60(gf)*) Complete genotype *let-60(n1046)*. The Multivulva phenotype caused by *let-60(gf)* is similar to that caused by *lin-15* mutations (Ferguson et al. 1987; Han et al. 1990). (*let-60(gf); lin-25(0)*) Complete genotype *let-60(n1046); lin-25(e1446)*. *lin-25* mutations suppress the Multivulva phenotype caused by *let-60(gf)*. *lin-1(e1777)* is a strong reduction-of-function mutation (Ferguson and Horvitz 1985). The Multivulva phenotype is suppressed by mutations in *lin-25*. The *lin-1(e1777); lin-25(n545ts)* double mutant animal was raised at 20°C, a temperature at which *lin-25(n545ts)* behaves as a hypomorph. *lin-1(sy254)* is a null allele (G. Beitel and H.R. Horvitz, pers. comm.). The *lin-1(sy254); lin-25(e1446)* hermaphrodite has a reduced number and size of pseudovulvae.

maphrodites in which the gonad had been ablated because in very few cases did two adjacent cells both adopt a vulval fate. However, in *lin-25(0); lin-15(0)* double-mutant hermaphrodites in the presence of the gonad, the cells closest to the anchor cell are often induced to adopt vulval fates. In these animals, described in more detail below, we found that lateral signaling was indeed compromised. However, in view of the fact that *lin-25* is clearly required for vulval induction we favor the explanation that the inefficiency of lateral signaling seen in these animals is probably an indirect result of defects in induction.

lin-25 is required for vulval induction by activated *let-60 Ras*

Many hypomorphic alleles of genes encoding compo-

nents of the inductive signaling pathway (including *let-23*, *sem-5*, *let-60*, *lin-45*, and *mpk-1/sur-1*) suppress the Multivulva phenotype of *lin-15(n309)* (Sternberg and Horvitz 1989; Beitel et al. 1990; Han et al. 1990, 1993; Clark et al. 1992). The fact that *lin-25* mutations also suppress the phenotype caused by this allele, therefore, suggested that *lin-25* might be part of the same pathway. To test this idea we constructed a number of double-mutant hermaphrodites containing an activated allele of *let-60 Ras*, *n1046*, and various alleles of *lin-25*. Activated *let-60 Ras* alleles such as *let-60(n1046)* cause a Multivulva phenotype similar to that caused by *lin-15(n309)* (Ferguson and Horvitz 1985; Beitel et al. 1990; Han et al. 1990). We found that the *lin-25* mutations *ar90*, *n1063*, *e1446*, *n545ts*, and *n1722ts* all suppressed the Multivulva phenotype caused by *let-60(n1046)* (Fig. 2). Consistent with this observation is the fact that three

Table 3. *lin-25* mutations reduce vulval induction

Genotype	AC	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	Pn.p
<i>lin-15(n309)</i> ^a	—	2°	2°/1°	2°	1°	2°	2°/1°	
<i>lin-25(e1446); lin-15(n309)</i>	—	3°	3°/ind	3°/ind	3°/ind	3°/ind	3°/ind	3°
	—	SS	SS	LOTT	S OT	SS	SLLO	SS
	—	SLTS	SSSS	LOTO	S SS	S LL	SSSL	
	—	SS	S	SS	LTTO	NTLL	SS	
	—	SS	S LS	S	SS	LLSS	S TS	SS
<i>let-60(n1046)</i>	—	3°	3°/ind	2°	3°/ind	2°	3°	
	—	SS	SS	LLTN	S TO	NOLL	SS	
	—	SS	LLOO	LLL?	OTTO	NLLL	SS	
	—	SS	SS	LLLN	S OT	LLOO	SS	
	—	S LO	S OT	LLLN	TTOT	NTLL	S SS	
	—	SS	SS	LLOT	S OO	NOOL	SS	
<i>let-60(n1046); lin-25(e1446)</i>	—	3°	3°	3°	3°	3°	3°	3°
	—	SS	SS	SS	SS	SS	SS	
	—	SS	SS	SS	SS	SS	SS	
	—	SS	SS	SS	SS	SS	SS	SS
	—	SS	SS	SS	SS	SS	SS	SS
	—	SS	SS	OTTT	SS	SS	SS	SS

See Fig. 1 legend and Table 2 footnote for meaning of S, N, T, L, and O.

(?) Division not observed.

^aFrom Ferguson et al. (1987).

lin-25 alleles, *ku70*, *ku77* and *ku78*, were isolated in genetic screens for suppressors of the *let-60(n1046)* phenotype (M. Han, pers. comm.).

Because the expressivity of the Multivulva phenotype of *let-60(n1046)* is partially dependent on the anchor cell signal (P. Sternberg, pers. comm.), to determine the order in which *lin-25* and *let-60* function in the pathway for vulval induction, we compared the phenotype of the *let-60(n1046); lin-25(e1446)* double mutant, with that of the single mutant, *let-60(n1046)*, in the absence of the gonad. Twenty two of twenty three hermaphrodites of the genotype *let-60(n1046)* in which the gonad had been ablated were Multivulva. In contrast, all 35 animals of the genotype *let-60(n1046); lin-25(e1446)* in which the gonad had been ablated were non-Multivulva. The suppression of the Multivulva phenotype of *let-60(n1046)* was reflected in the fates adopted by the VPCs. In *let-60(n1046)* animals in which the gonad had been ablated, many VPCs still adopted vulval fates (Table 3), whereas in *let-60(n1046); lin-25(e1446)* double-mutant animals in which the gonad had been ablated almost all VPCs adopted the nonvulval fate (Table 3). These results suggest that *lin-25* functions after *let-60* in the genetic pathway for vulval induction.

lin-25 may be required for the induction of a component of the lateral signaling pathway

lin-25 mutations do not completely abolish the ability of the VPCs to respond to the anchor cell signal. In the *lin-25* single mutant, and in double-mutant hermaphrodites of the genotypes *let-60(n1046); lin-25(e1446)*, and *lin-25(e1446); lin-15(n309)* in which the gonad is intact, cells close to the anchor cell are often induced to adopt

vulval cell fates (Table 4). In the double-mutant hermaphrodites, we observed a number of instances in which two adjacent VPCs both adopted the 1° fate. Of 19 pairs of adjacent cells (in 13 different animals) in which both cells adopted a vulval fate we observed 5 pairs in which both members of the pair adopted the 1° fate (Table 4). In hermaphrodites of the genotype *lin-15(n309)* the frequency with which adjacent VPCs both adopt the 1° fate is considerably lower (1/69 pairs of cells) (Sternberg 1988; Sternberg and Horvitz 1989). Therefore, *lin-25* mutations affect the efficiency with which VPCs signal to one another laterally. One possible explanation for this observation is that *lin-25* is required for the efficient expression of some component of the lateral signaling pathway in response to Lin-3. Alternatively, *lin-25* could be required at two separate points during VPC fate specification, first as a component of the vulval induction pathway and second as a component of the lateral signaling pathway.

Genetic interactions between *lin-25* and genes acting after *mpk-1/sur-1* MAPK in the pathway for vulval induction

All existing alleles of *lin-25* reduce the efficiency of vulval induction. Because this is also true for both *mpk-1/sur-1* MAPK and *lin-45* Raf it was not possible to perform epistasis tests between *lin-25* and either of these genes. Multivulva mutations exist, however, in two genes, *lin-1* and *lin-31*, that function after *mpk-1/sur-1* in the genetic pathway for VPC fate specification.

lin-1 encodes a negative regulator of vulval induction: Loss-of-function alleles of *lin-1* display a Multivulva phenotype similar to that caused by *lin-15(n309)* (Hor-

Table 4. *lin-25* may be required for the induction of a component of the lateral signaling pathway

Genotype	AC	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	Pn.p
<i>let-60(n1046)</i> ^a	+	1°/2°/3°	1°/2°	2°	1°	2°	1°/2°/3°	
<i>let-60(n1046);</i> <i>lin-25(e1446)</i>	+	SS	SS	SS	<u>TLTT</u>	<u>TTTT</u>	SS	SS
	+	SS	SS	<u>LLTN</u>	TTTO	<u>NTLL</u>	SS	
	+	SS	SS	S OT	TTTT	<u>NOLL</u>	SS	
	+	SS	SS	S TT	OOTO	SS	SS	SS
	+	S	SS	<u>TOTT</u>	<u>TTOL</u>	<u>OLLL</u>	SS	SS
	+	SS	SS	S TT	OOTO	SS	SS	SS
	+	SS	SS	LNT O	TTTT	<u>NTLL</u>	SS	SS
	+	SS	S	SS	L?TT	TT S	SS	S
<i>lin-15(n309)</i>	+	2°	2°/1°	2°	1°	2°	1°/2°	
<i>lin-25(e1446);</i> <i>lin-15(n309)</i>	+	S	SS	<u>OTLO</u>	<u>TTTT</u>	<u>OTTT</u>	SS	
	+	SS	SS	<u>LLTN</u>	TTTT	<u>NTLL</u>	S	
	+	S	SS	S TO	<u>LOTT</u>	TTTT	<u>NTLL</u>	SS
	+	S	SS	<u>LLTT</u>	<u>TTTT</u>	<u>TTTL</u>	<u>NOLL</u>	SS
	+	S	SS	<u>OTTT</u>	<u>TOTT</u>	<u>TTLL</u>	SSSS	SS

See Fig. 1 legend and Table 2 footnote for the meaning of S, N, T, L, and O.

(?) Division not observed.

^aFrom Ferguson et al. (1987). For animals of the genotypes *lin-25(e1446)*; *lin-15(n309)* and *let-60(n1046)*; *lin-25(e1446)*, boxes highlight cases of adjacent 1° lineages. Not all 1° lineages are boxed.

vitz and Sulston 1980; Ferguson et al. 1985). Reduction of *lin-1* activity causes a Multivulva phenotype even if *let-60* Ras, *lin-45* Raf, or *mpk-1/sur-1* MAPK activity is reduced (Han et al. 1990, 1993; Lackner et al. 1994; Wu and Han 1994; S. Tuck and I. Greenwald, unpubl.). We constructed many different *lin-1*; *lin-25* double mutants with alleles of varying severity of each gene. We found that all *lin-25* alleles tested, including the weakest allele, *lin-25(n1722)*, suppressed the Multivulva phenotype caused by *lin-1(e1275)* and *lin-1(e1777am)* (Horvitz and Sulston 1980; Ferguson and Horvitz 1985). *lin-1(e1777am)* strongly reduces *lin-1* activity (Horvitz and Sulston 1980; Ferguson and Horvitz 1985). However, *lin-25(e1446)* only weakly suppresses the Multivulva phenotype of the *lin-1* molecular null alleles *lin-1(ar147)* and *lin-1(sy254)* (kindly provided by G. Beitel and H.R. Horvitz, MIT, Boston, MA). Double mutants of genotype *lin-1(sy254)*; *lin-25(e1446)* display a slight reduction in the size and number of pseudovulvae (Fig. 2), although lineage analysis indicates that the double mutant has considerably more induction than does *lin-1(e1777)*; *lin-25* (data not shown).

lin-31 mutations cause a complex phenotype in which aspects of the Multivulva and Vulvaless phenotypes may both be expressed within the same animal. One or more of the VPCs that normally adopt a nonvulval fate, P3.p, P4.p, and P8.p, instead adopt a vulval fate, and one or more of the VPCs that normally adopt a vulval fate, P5.p, P6.p, and P7.p, instead adopt the nonvulval fate. It is not yet known where in the pathway for VPC fate specification *lin-31* acts in relation to *lin-1*. Most (77%) *lin-31(n301)* hermaphrodites are Multivulva (Miller et al. 1993). We found that in *lin-31(n301)*; *lin-25(e1446)* double-mutant hermaphrodites, the size and number of pro-

trusions were slightly reduced but that the majority of animals (73%, *n* = 186) were still Multivulva.

lin-25 encodes a novel protein

To begin to explore the biochemical function of *lin-25* we cloned and sequenced the gene. *lin-25* mapped to an ~2.5 Mb interval between *myo-3* and *TcPAR2*, a restriction fragment length polymorphism (RFLP) (Fig. 3A). We further correlated the physical and genetic maps in the region by identifying, mapping, and cloning a number of *Tc1*-associated RFLPs. Two of these, *arP2* and *arP5*, lying respectively to the right and left of *lin-25* (Fig. 3A and B), defined an interval of ~250 kb. Cosmid and λ phage clones (Coulson et al. 1986, 1988, pers. comm.) in this interval were pooled and injected into the germ line of *lin-25* mutant hermaphrodites and tested for their ability to complement the egg-laying defect (Fig. 3C). Ultimately, a 10.1-kb *SpeI* fragment from the λ phage clone YSL59 was found that complemented efficiently. Deletion of ~2 kb from either end of this fragment abolished the rescuing activity (Fig. 3C).

When the 10.1-kb fragment was used to probe Northern blots containing RNA from a mixed stage population of *C. elegans* hermaphrodites, two poly(A)⁺ transcripts were detected: One of ~3.6 kb (Fig. 3D) and a second of ~800 bp (not shown). No transcripts were detected in the poly(A)⁻ fraction. The 3.6-kb transcript appeared likely to correspond to the *lin-25* gene because probes throughout the 10.1-kb rescuing fragment hybridized to the 3.6-kb transcript, whereas only those containing an 850-bp fragment from one end (3' of the 3.6-kb transcript) hybridized to the 800-bp transcript (not shown). The 10.1-kb fragment was completely sequenced on both strands.

Figure 3. Genetic and physical maps of regions around *lin-25*. (A) Partial genetic map of the *lin-25* V region. The duplication *ctDp11*, not shown, complements *unc-42*, *unc-76*, and all the markers lying between [Hunter and Wood 1992]. *arP2*, *arP4*, and *arP5* are Bristol (N2)/Bergerac (BO) Tc1-associated RFLPs. (B) Physical map of the region around *lin-25*. YSL59 is a λ phage clone. All others are cosmid clones. Not all clones on the physical map are shown. (C) Plasmid, cosmid, and λ phage clones in the immediate vicinity of *lin-25*. Their ability to rescue the temperature sensitive mutation, *lin-25(n545ts)* is indicated on the right. W05B10 and YSL59 are cosmid and λ phage clones, respectively; the rest are plasmid clones containing inserts derived from YSL59. pSNY1 contains a linker insertion at a unique *Bgl*III site in exon 7. (D) The structure of the *lin-25* gene. Large boxes represent exons. Solid regions represent the putative protein coding region starting at an ATG in exon 1 and ending at TGA in exon 13. Products resulting from the splicing of the SL1 spliced leader RNA to the 5' ends of exons 1, 2, and 3 have been observed as have those resulting from the splicing of SL2 to the same exons. Smaller boxes below represent cDNA clones. Shaded areas represent regions of each clone that were sequenced. All *Spe*I, *Pst*I, *Bgl*III, and *Xba*I sites are shown.

Several cDNAs corresponding to the 3.6-kb transcript were also isolated and sequenced (see Materials and methods). We confirmed that the 3.6-kb transcript corresponds to the *lin-25* gene in two ways. First, we introduced a linker-insertion mutation into a unique *Bgl*III site in an exon on the 10.1-kb rescuing fragment (Fig. 3D). The linker contained a stop codon in all three frames and completely abolished the rescuing activity (Fig. 3C). Second, we have identified molecular lesions in two *lin-25* alleles, *ar90* (codon 197: CAG $\rightarrow$ TAG) and *ga67* (codon 284: TTC $\rightarrow$ TAC).

A conceptual cDNA 3659 nucleotides in length appears to span the entire gene. A putative translation initiation codon at position 55 lies in a favorable context (Kozak 1986) and is preceded by a stop codon, in frame, at position 49. At the 3' end, there are 15 dA residues, not present in the genomic sequence, located 16 nucleotides from a potential polyadenylation signal sequence, TATAAA (Birnstiel et al. 1985). The 5' end contains spliced leader sequences (see Materials and methods).

Comparison of the cDNA and genomic sequences revealed that all 13 exons are present on the 10.1-kb rescuing fragment (Fig. 3D). The size of the composite cDNA is consistent with the size of the mRNA estimated by Northern blot analysis (Fig. 4).

Conceptual translation of the open reading frame generated a polypeptide sequence of 1139 amino acids. Both the nucleotide sequence and deduced amino acid sequence are shown in Figure 5. The protein sequence was compared to that of all proteins in the GenBank and EMBL data bases using both the BLAST (Altschul et al. 1990) and FASTA (Pearson and Lipman 1988) programs. No significant similarity to other proteins was detected. With the exception of potential sites for posttranslational modification, no matches to known peptide sequence motifs were detected by use of the computer programs from the GCG (Devereux et al. 1984) and MacVector (IBI) software packages.

Lin-25 contains a single match to the full MAPK phosphorylation site consensus, PX(S/T)P (Clark-Lewis et al.

Figure 4. Northern blot analysis of the *lin-25* transcripts. RNA was isolated from a mixed developmental stage population of wild-type *C. elegans* hermaphrodites. The positions to which the 28S (3.5 kb) and 18S (1.75 kb) rRNAs migrated is indicated at left. The blot was probed under high stringency conditions with radiolabeled RNA made in vitro with *lin-25* cDNAs as templates for transcription. *lin-25* sequences represented in the probe extend from position 245 (Fig. 5) to position 3565.

1991; Gonzalez et al. 1991], and three additional matches to the core consensus, (S/T)P. A single phosphorylation site is sufficient for the regulation of some proteins by MAPK (O'Neill et al. 1994; Brunner et al. 1994b) but the probability that the consensus occurs purely by chance in a protein the size of Lin-25 is quite high (~0.3 for the full consensus, assuming a random distribution of amino acids occurring at the frequency they are found in Lin-25). Lin-25 also contains a potential nuclear localization signal (Boulikas 1993) (Fig. 5).

As mentioned above, the *lin-25(ar90)* allele, which is suppressed by the amber suppressor tRNA *sup-7(st5)* (Waterston 1981; Wills et al. 1983) is associated with an amber mutation at codon 197. This mutation would lead to the production of a severely truncated protein <20% the size of the predicted 1139-amino-acid Lin-25 protein. This observation strongly supports the genetic arguments that *lin-25(ar90)* is a null allele.

Discussion

Genetic analysis of vulval development in the *C. elegans* hermaphrodite has shown that the induction of vulval fates is mediated by a signal transduction pathway involving *let-60* Ras, *lin-45* Raf, and the *mpk-1/sur-1* MAPK (Han and Sternberg 1990; Han et al. 1993; Lackner et al. 1994; Wu and Han 1994). In this study, we have shown that the absence of *lin-25* activity causes a de-

crease in the amount of vulval induction. The genetic interactions with other mutations affecting vulval induction suggest that *lin-25* acts after the Raf/MAPK protein kinase cascade and that a decrease in vulval induction may lead to a decrease lateral signaling, a *lin-12*-mediated signaling event.

lin-25 appears to function after *let-60* Ras in the genetic pathway for vulval induction

Dominant mutations in *let-60* Ras, resulting from changes in codon 13, constitutively activate the biochemical pathway that mediates the response to the vulva inducing signal Lin-3 (Beitel et al. 1990; Han et al. 1990; Han and Sternberg 1990). Hermaphrodites carrying such mutations display a Multivulva phenotype resulting from fate transformations in which cells in the ventral hypodermis that normally adopt the hypodermal fate instead adopt vulval fates. Like partial loss-of-function mutations in *lin-45* Raf (Han et al. 1993) and the *mpk-1/sur-1* MAPK (Miller et al. 1993; Wu and Han 1994), mutations in *lin-25* that reduce or eliminate the activity of the protein suppress the Multivulva phenotype of *let-60(n1046)* activated Ras.

It seems unlikely that *lin-25* is an immediate target of *Let-60* Ras during vulval induction. Biochemical experiments on cultured cells and genetic experiments in *Drosophila* have shown that two immediate targets of Ras in both mammals and flies are the proteins Raf and MEK (Tsuda et al. 1993; Van Aelst et al. 1993; Vojtek et al. 1993; Warne et al. 1993; Zhang et al. 1993). Furthermore, evidence from many different studies suggests that the pathway between Ras and MAPKs is a simple linear one (Ambrosio et al. 1989; Crews et al. 1992; Kyriakis et al. 1992; Thomas et al. 1992; Wood et al. 1992; Lu et al. 1993; Ogiso et al. 1993; Tsuda et al. 1993; Brunner et al. 1994a; Leever et al. 1994; Stokoe et al. 1994). Both Raf and a MAPK are involved in vulval induction (Han et al. 1993; Lackner et al. 1994; Wu and Han 1994), suggesting that components of this highly conserved biochemical pathway perform similar functions. It therefore seems likely that *lin-25* functions further downstream in the pathway, after *mpk-1/sur-1* MAPK. Although we could not perform epistasis experiments to test this idea directly, because all existing mutations in *lin-45* Raf, *mpk-1/sur-1* MAPK, and *lin-25* reduce vulval induction, this interpretation is supported by the genetic interactions between *lin-25* and *lin-1*, a gene that is thought to act after *mpk-1/sur-1* (Lackner et al. 1994; Wu and Han 1994). While *lin-1* is epistatic to mutations in *let-60* Ras, *lin-45* Raf, and *mpk-1/sur-1* MAPK, *lin-25* mutations suppress the multivulva phenotype caused by certain alleles of *lin-1*. The simplest interpretation of our results therefore is that *lin-25* acts downstream of the *mpk-1/sur-1* MAPK, after or at the same time as *lin-1*.

One explanation for the observation that *lin-25* is epistatic to *let-60(n1046)* would be that *lin-25* is necessary for the VPCs to be intrinsically capable of adopting vulval fates. We think it unlikely, however, that *lin-25* is involved solely in establishing the identity of the VPCs

Figure 5. *lin-25* cDNA sequence and deduced amino acid sequence. Amino acid numbers are shown at the *top* and nucleotide numbers at the *bottom*. The termination codon TGA is denoted by an asterisk. The SL1 trans-spliced leader sequence is underlined. A potential polyadenylation signal sequence is shown in bold. (Δ) Intron-exon boundaries. A potential MAPK phosphorylation site, PX[S/T]P (Clark-Lewis et al. 1991; Gonzalez et al. 1991), is denoted by Ξ , and a potential nuclear localization signal sequence (Boulikas 1993) is overlined with dashes. Codon 197, which is altered by *lin-25(ar90)* to an amber codon, is indicated by a solid square positioned underneath.

the changes induced by the Ras/MAPK pathway (in all cells) undoubtedly result from changes in gene expression it is possible that Lin-25 is a transcription factor required for the induction of genes in response to Lin-3. Another possibility is that Lin-25 does not itself bind DNA but modulates the activity of a protein which does; for example, we predict that there will be a *C. elegans* protein similar to MCM1 and SRF (Treisman and Ammerer 1992). Finally, it is possible that Lin-25 and other

proteins acting after Let-60 in the vulval induction pathway may act to cause changes in cell physiology directly rather than through changes in gene expression.

It is possible that another gene is partially functionally redundant with *lin-25*. Alleles that strongly reduce or eliminate *lin-25* activity lead to high-penetrance reduction in vulval induction (and an apparently unrelated defect in egg-laying; see Materials and methods) but only low-penetrance sterility, larval lethality, and extra VPCs formation from P3.p–P8.p doublings (see Table 1 and Materials and methods). In contrast, alleles that strongly reduce or eliminate *let-60* Ras, *lin-45* Raf, or *mpk-1/sur-1* MAPK lead to sterility or a highly penetrant larval arrest with a characteristic rod-like morphology (Ferguson et al. 1985; Beitel et al. 1990; Han et al. 1990, 1993; Lackner et al. 1994; Wu and Han 1994). These observations may indicate that another gene is functionally redundant with *lin-25* for the sterility and larval lethal defects. There may also be functional redundancy for vulval induction because P6.p is generally induced in *lin-25* mutants.

lin-25, *lin-1*, and *lin-31* may not function in a simple linear pathway

Besides *lin-25* and *lin-1*, one other gene, *lin-31*, has been shown to act after *mpk-1/sur-1* in the genetic pathway for VPC fate specification. It is not likely, however, that *lin-1*, *lin-25*, and *lin-31* function in a simple linear biochemical pathway. The phenotypes caused by mutations in *lin-1*, *lin-25*, and *lin-31* and the interactions observed among these mutations suggest that the Lin-1, Lin-25, and Lin-31 proteins may not directly regulate one another. Hermaphrodites carrying null mutations in *lin-31* show aspects of both the Vulvaless and Multivulva phenotypes (Ferguson et al. 1987; Miller et al. 1993). P5.p, P6.p, or P7.p sometimes adopts the nonvulval fate and P3.p, P4.p, or P8.p sometimes adopts a vulval fate. This phenotype implies that Lin-25 cannot function solely by regulating the activity of Lin-31 (or vice versa) because *lin-25*, unlike *lin-31*, is not required for the specification of the 3° fate. Similarly it is not possible that Lin-1 acts solely by regulating the activity of Lin-31 (or vice versa) because *lin-1*, unlike *lin-31*, does not appear to be required for the specification of the vulval fates. A further reason to think that *lin-1*, *lin-25*, and *lin-31* might not directly regulate one another is the fact that there is evidence to suggest that more than one target may exist for the *mpk-1/sur-1* MAPK (G. Beitel, S. Tuck, I. Greenwald and H.R. Horvitz, in prep.).

While *lin-1*, *lin-31*, and *lin-25* may not act in a linear pathway, it is possible that they act together in certain aspects of VPC fate specification. *lin-31* encodes a member of the HNF3/*forkhead* family of transcription factors (Miller et al. 1993). To account for the *lin-31* mutant phenotype Miller and co-workers have proposed that transcription complexes exist in the VPCs that can activate vulval specific genes and repress hypodermal ones and that others exist that can repress vulval specific genes and activate hypodermal ones (Miller et al. 1993).

The deregulation of cell fate specification seen in animals carrying *lin-31* mutations could be explained if Lin-31 were part of both types of complex. There would be inefficient repression of vulval specific genes in P3.p, P4.p, and P8.p but at the same time inefficient activation of such genes in P5.p, P6.p, and P7.p. This model requires that Lin-31 acts in concert with other proteins to specify cell fates. It is possible that Lin-25 and Lin-1 each function together with Lin-31 to control the expression of the vulval and nonvulval fates, respectively.

Genetic epistasis experiments between *lin-25* and *lin-1* suggest a complex, dosage-sensitive relationship between the two genes. All alleles of *lin-25*, including *lin-25(n1722)*, which itself has little effect on vulval induction, suppress the Multivulva phenotypes caused by *lin-1(e1275)* and *lin-1(e1777)*, which strongly reduce *lin-1* activity (Ferguson et al. 1985). However, *lin-25(e1446)* has only mild effects on the ectopic vulval induction caused by *lin-1* molecular null alleles (G. Beitel and H.R. Horvitz, pers. comm.; data not shown). One possible explanation for these interactions would be that downstream of *mpk-1/sur-1* MAPK *lin-1* and *lin-25* do not regulate one another directly but instead act on separate branches of a forked pathway. MAPKs are capable of phosphorylating a number of different proteins, and it has been suggested that MAPK may act at a point at which signal transduction pathways frequently branch (Stokoe et al. 1992). Vulval induction, therefore, might be the result of two different activities of *sur-1/mpk-1* MAPK: inactivation of Lin-1 and activation of Lin-25. This model and a number of others also consistent with the experimental data are presented in Figure 6.

The interactions between *lin-1* and *lin-25* are reminiscent of the interactions between *yan* and *pointed (ptd)*, two genes involved in R7 photoreceptor cell fate determination in *Drosophila* (Lai and Rubin 1992; Brunner et al. 1994b). O'Neill et al. (1994) and Brunner et al. (1994b) have suggested that the combination of positive modulation of PtdP2 and negative modulation of Yan provides more delicate control of induction than could be achieved if just one target existed. The combination of the negative factor Lin-1 and the positive factor Lin-25 may similarly provide sensitive control over vulval induction in *C. elegans*.

lin-25 may help link two different signaling pathways in the VPCs

In the wild-type hermaphrodite the vulva is formed from the descendants of the three VPCs closest to the anchor cell, P5.p, P6.p, and P7.p. P6.p adopts one vulval fate, the 1° fate, whereas P5.p and P7.p adopt a different vulval fate, the 2° fate (Sulston and Horvitz 1977). The specification of the 2° fate is controlled by lateral signaling, an event thought to occur between adjacent VPCs (Sternberg 1988; Sternberg and Horvitz 1989). Lateral signaling, which is mediated by the receptor *lin-12*, ensures that adjacent VPCs do not both adopt the 1° fate (Greenwald et al. 1983; Sternberg and Horvitz 1989).

The phenotypes of certain double mutants suggests

Figure 6. Models for how Lin-25 might function during VPC fate specification. In models A, B, and C, Lin-25 could either be a direct target of Mpk-1/Sur-1 (or some intervening kinase) or be required for the activity of the direct target. The phenotypes of mutations in *lin-1*, *lin-25*, and *lin-31* imply that Lin-25 is formally a positive regulator of vulval fates (Ferguson et al. 1987), Lin-1 is a negative regulator (Horvitz and Sulston 1980; Sulston and Horvitz 1981; Ferguson and Horvitz 1985) and Lin-31 is required for both vulval and nonvulval fates (Ferguson et al. 1987; Miller et al. 1993). (A) Lin-25 is a substrate for the Mpk-1/Sur-1 MAPK (or some intervening kinase). Lin-1 negatively regulates Lin-25 activity. Lin-31 acts in conjunction with Lin-25 to specify vulval fates and with some other protein to specify nonvulval fates (not shown). (B) Both Lin-1 and Lin-25 are targets of Mpk-1/Sur-1 (or some intervening kinase). This model is similar to the one proposed for the function of Yan and PtdP2 in the determination of the R7 photoreceptor cell fate in *Drosophila* (O'Neill et al. 1994; Brunner et al. 1994b). (C) Lin-1, a target of the pathway, negatively regulates Lin-25 activity. Because *lin-25* mutations only weakly suppress null mutations in *lin-1* (data not shown), for this model to be correct, *lin-1* must also regulate another gene, here denoted *lin-x*, with which *lin-25* is partially redundant. No genes with this characteristic have yet been identified. (D) Lin-25 acts in a pathway parallel to Lin-1 and Lin-31.

that *lin-25* may help link the inductive and lateral signaling pathways. *lin-25* mutations do not completely abolish the ability of the VPCs to respond to the inducing signal, Lin-3. In *lin-25* mutant hermaphrodites P5.p and P7.p adopt the nonvulval (3°) fate but P6.p often adopts the 1° fate (Ferguson et al. 1987). In double-mutant hermaphrodites containing both a *lin-25* mutation and a mutation that constitutively activates the inductive signaling pathway two or three VPCs closest to the anchor cell are often induced to adopt vulval fates. In these animals the efficiency of lateral signaling appears to be reduced because two adjacent VPCs sometimes both adopt the 1° fate. In view of the fact that *lin-25*

mutations affect vulval induction, the most likely explanation for this observation is that *lin-25* is required for the expression or activation of some component of the lateral signaling pathway. Dominant mutations in *lin-12* are epistatic to mutations in *lin-25*. Therefore, *lin-25* mutations may reduce the efficiency of lateral signaling by affecting the synthesis of the ligand for Lin-12. In this model, the *lin-25* mutant phenotype, the failure of P5.p and P7.p to adopt the 2° fate, could be the result of two separate defects, both essentially caused by a decrease in the amount of induction. First, P5.p and P7.p are less sensitive to the lateral signal from P6.p, because they have not been adequately induced by the anchor cell, and second, P6.p has a reduced ability to signal.

Materials and methods

Strains and genetic analysis

The methods used for the culturing, handling, mutagenesis, and genetic manipulation of *C. elegans* were those described by Brenner (1974). Experiments were performed at 20°C except as otherwise noted. *C. elegans* var. Bristol strain N2 is the wild-type parent for most of the strains used in this work. The only exceptions are the congenic strains DT127 and GS352 in which part of the right arm of chromosome V is derived from *C. elegans* var. Bergerac (Nigon 1949).

We generated *arDf1* during the course of this work by X-ray mutagenesis of a chromosome marked with *unc-42(e270) sqt-3(sc63)*. *arDf1* fails to complement *vab-8(e1017)*, *sqt-3(sc63ts)*, *lon-3(e2175)*, and *lin-25(n545ts)*. It complements *sma-1(e30)*, *par-1(b274mat)*, *him-5(e1467)*, *itDf2*, *unc-61(e228)*, and *unc-76(e911)*. *arDf1* complemented all visible genetic markers to the right of *lin-25* that we tested. However, by Southern blotting we found that *arDf1* extends to the right of *arP2*, which itself lies ~90 kb to the right of *lin-25* (Fig. 3B,C).

To isolate *lin-25(ar90)*, hermaphrodites of the genotype *unc-42(e270) sqt-3(sc63ts)*, grown at 15°C, were exposed to ethyl methanesulfonate (EMS) (Brenner 1974) and then mated to *lon-3(e2175) lin-25(n545ts)* males (also grown at 15°C). Rol non-Unc F₁ cross progeny hermaphrodites, grown at 25°C, were screened for fertile animals that were unable to lay eggs (the bag-of-worms phenotype). A single new *lin-25* allele, *ar90*, was isolated from a screen of ~20,000 hermaphrodites. For technical reasons, we think that this frequency is an underestimate of the actual frequency with which *lin-25* alleles may be generated. *lin-25(n1063)* was isolated using a similar scheme from a screen of 5,000 hermaphrodites (K. Edwards and H.R. Horvitz, pers. comm.).

lin-25(e1446), *lin-25(n1063)*, and *lin-25(ar90)* were tested for suppression by *sup-7(st5)*, an amber suppressor tRNA (Waterston 1981; Wills et al. 1983). *sup-7(st5)* suppresses the egg-laying defect of *lin-25(ar90)*: 50% of hermaphrodites of the genotype *lin-25(ar90); unc-6(e78)/sup-7(st5)* grown at 20°C were able to lay eggs ($n=30$). One hundred percent of animals of the genotype *lin-25(ar90); sup-7(st5)* grown at 22°C were able to lay eggs.

lin-25 alleles and their phenotypes

The mutations *ar90*, *n1063*, *ku70*, *ku77*, *ku78*, *sy29*, *ga67*, and *n1722* are *lin-25* alleles by the following criteria. All eight mutations fail to complement *lin-25(n545ts)* for the egg-laying defect. *ar90*, *n1063*, *sy29*, and *n1722* map to the interval between

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rol-4 and *par-1* on chromosome V. *ku70*, *ku77*, and *ku78* all map to the right of *unc-42* on chromosome V. *arDf1* fails to complement all eight mutations for the egg-laying defect.

Fertility and viability Hermaphrodites homozygous for strong *lin-25* mutations are sometimes sterile (Ferguson and Horvitz 1985; see Table 1). The sterility defect is associated with heterogeneous gonadal and germ-line anatomy, but in some animals, the germ-line phenotype of *lin-25* mutant hermaphrodites resembles that of *let-23* partial loss-of-function mutants (E. Lambie, pers. comm.). We have found that strong *lin-25* mutations also cause a low penetrance larval lethality (Table 1). Approximately six percent of hermaphrodites homozygous for *ar90*, *e1446*, *n1063*, *ku70*, *ku77*, *ku78*, or *ga67* arrest at early larval stages (Table 1). We do not yet know the cellular basis for this phenotype. Many of the animals arrest during the L1 stage and assume a characteristic rod shape. At low magnification the arrested animals appear similar to those carrying strong mutations in genes required for vulval induction such as *lin-3*, *let-60*, and *let-23* (Ferguson and Horvitz 1985; Beitel et al. 1990; Han et al. 1990). The cause of death of these animals is also not known.

Egg-laying Temperature-shift experiments indicate that *lin-25* is required during the early L4 stage for the development of some component of the egg-laying apparatus in addition to its role in VPC fate determination (Ferguson et al. 1987). There is good evidence to suggest that the high penetrance of the egg-laying defect caused by *lin-25* mutations is mostly a reflection of this later role. In double-mutant hermaphrodites of the genotype *let-60(n1046); lin-25(e1446)* the VPC lineage defects caused by the two mutations are often mutually suppressed (Table 4). Interestingly, however, even when the VPC lineages are wild-type, the animals are still completely unable to lay eggs. Furthermore, although the temperature-sensitive allele, *lin-25(n545ts)*, is a very good suppressor of the Multivulva phenotype caused by *let-60(n1046)* even at the semipermissive temperature (20°C), the reverse is not true: *let-60(n1046)* does not suppress the Egl defect of *lin-25(n545ts)*. This latter result suggests that *let-60* may not be involved in the later event that requires *lin-25*. Similar observations have been made for *mpk-1/sur-1* (Wu and Han 1994). One possible explanation for inability of activated *let-60 ras* to suppress the Egl phenotypes caused by hypomorphic alleles of *lin-25* and *mpk-1/sur-1*, therefore, is that in some later signaling event the receptor transduces a signal via a worm homolog (not yet identified) of MEKK/STE11 (for review, see Mordret 1994) to *mpk-1/sur-1* and thence to *lin-25*.

The egg-laying defect displays partial maternal rescue. For example, ~7% of animals of the genotype *lin-25(e1446)* derived from an hermaphrodite of the genotype *lin-25(e1446)/+* are able to lay some eggs and larvae before turning into bags of worms.

Extra VPCs We note that the production of seven pseudovulvae associated with 2° lineages (Fig. 2) is evidence that the extra VPCs observed in *lin-25* mutants (Ferguson et al. 1987) are indeed VPCs.

Germ-line transformation experiments

Microinjection of DNA into the germ line of *C. elegans* hermaphrodites was performed essentially as described by Mello et al. (1991). For *lin-25* transformation rescue experiments, *lin-25(n545ts)* hermaphrodites (raised at 15°C) were injected with the plasmid pRF4 along with plasmid, cosmid, or λ phage DNA potentially harboring the *lin-25* gene. pRF4 encodes a dominant

allele of the *rol-6* gene, *su1006*, which confers a Rol phenotype on transformed animals (Mello et al. 1991). Injected animals were placed on separate plates at 25°C and allowed to self-fertilize. Their progeny were allowed to develop at this temperature, which is nonpermissive for the *n545ts* mutation. F₁ Roller animals were placed on separate plates and scored for their ability to lay eggs and for the absence of bloating, a phenotype characteristic of egg-laying defective hermaphrodites (Trent et al. 1983). pRF4 plasmid DNA, purified by CsCl equilibrium density gradient centrifugation, was injected at a concentration of 50 μ g/ml. Test plasmid, cosmid, or λ phage DNA was injected at a concentration of ~15 μ g/ml. When the plasmid pSP2 was injected under these conditions, 82 of 100 F₁ Roller animals were non-Egl. From these 100 worms, seven transformed lines were generated in which a significant proportion of the animals at each generation had inherited the extrachromosomal array formed from the injected plasmids. For all seven lines the majority of animals displaying the Rol phenotype were non-Egl. Injection of pSP2 into wild-type animals, even at high concentrations (100 μ g/ml) did not cause the transformants to express any clearly visible phenotypes and did not reduce the number of transformants.

Manipulation of recombinant DNA

All standard molecular biological procedures were performed by use of methods described by Sambrook et al. (1989). DNA sequence was determined by use of the dideoxy chain termination method (Sanger et al. 1977) with reagents supplied by U.S. Biochemical. Except where otherwise noted, all sequencing reactions were performed with single-stranded DNA templates. To sequence *lin-25* genomic DNA, conveniently sized pieces of the 10.1-kb rescuing fragment were subcloned into the pBSII KS(–/+) vectors and the resulting constructs used to generate nested sets of deletions by use of *ExoIII* and *S1* nucleases (Erase-a-base system, Promega). The sequence around sites used to generate subclones was confirmed by use of other clones. Sequence was analyzed with computer programs written by Staden (1986) and by the GCG (v. 7) (Devereux et al. 1984). Some programs from the MacVector software package (IBI) were also used.

cDNA cloning

Two *SalI* fragments, 1.6 and 1.85 kb in size, isolated from the plasmid pSP2 (Fig. 3C) were used to screen a *C. elegans* cDNA library kindly supplied by R. Barstead and R. Waterston (1989). Eight positive plaques were identified in a screen of ~400,000 phage clones. None of these cDNAs appeared to be full length: The largest, SNY4, was only 1.9 kb. Restriction mapping, however, indicated that the two longest clones, SNY2 and SNY4, only partially overlapped. We sequenced all of one strand (the sense strand) of SNY2. This clone starts at position 661 in the *lin-25* cDNA sequence (Fig. 5) and ends at position 2144. The clone resulted from oligo(dT) annealing to a polyadenine stretch in the middle of the *lin-25* mRNA. We also sequenced most of one strand (the antisense strand) of SNY4. The sequence generated from this clone starts at position 2043 (Fig. 5) and ends (at one end of the clone) at position 3566. This clone extends toward the 3' end of the gene, past the translation stop codon at position 3571, but does not contain a poly(A) tail. We therefore sequenced the ends of a number of other clones (by use of double-stranded DNA templates). SNY11 was found to contain a poly(A) tail, starting at position 3569, 16 nucleotides 3' of a potential polyadenylation signal sequence, TATAAA. To isolate clones extending further 5' we generated a probe from the 5' end of SNY2 (position 661 to the *EcoRI* site at position 1553,

Fig. 5) and used this to screen a cDNA library kindly supplied by J. Ahlinger and J. Kimble (University of Wisconsin, Madison). Two, apparently identical, clones were isolated from a screen of ~400,000 phage clones. A 3.6-kb *EcoRI* fragment isolated from one clone, SNY16, was inserted into pBSII KS(+) (Stratagene), and a section of one strand was sequenced. Analysis of the sequence revealed that the insert in SNY16 had arisen from the fusion of two different, unrelated cDNAs. The *lin-25* sequence generated from SNY16 extends from position 245 to position 680 (Fig. 5).

Reverse transcription-PCR

A 72-bp single-stranded DNA primer (complementary to the *lin-25* cDNA between positions 349 and 420) was labeled at the 5' end and hybridized to 5 µg of poly(A)⁺ RNA isolated from a mixed population of worms. Primer extension was performed under standard conditions by use of avian reverse transcriptase (Stratagene). One-fourth of the primer-extension reaction was analyzed by polyacrylamide gel electrophoresis under denaturing conditions to determine the size of the primer extended product(s). One-eighth of the remainder was used as a template for PCR (30 cycles). One primer (5'-GTGGATGACTTGATTC-CAGG-3') was complementary to a section of the *lin-25* cDNA between positions 294 and 312 (Fig. 5) and the other had the sequence of the SL1 *C. elegans* splice leader RNA (5'-GGTT-TAATTACCCAAGTTTGA-3') (Krause and Hirsh 1987). Southern blot analysis of the PCR products indicated that three different *lin-25*-related sequences (~120, 230, and 320 bp in size, respectively) had been amplified. Sections of a low-melting-point gel containing each of these three products were excised, and a small amount used as a template for a second round of PCR (30 cycles). In these reactions, one primer (5'-CGA-CAAGTTTGAGAAGATGG-3') was complementary to a section of the *lin-25* cDNA between positions 258 and 278, and the other was the SL1 primer as before. Each reaction generated one band clearly visible on an ethidium bromide-stained agarose gel. All three fragments were cloned into pBSII KS(+) (Stratagene) and sequenced. Analysis of the sequences revealed that the PCR products resulted from *trans*-splicing of the SL1 splice leader to the 5' ends of exons 1, 2, and 3, respectively. Similar experiments with a primer that has the sequence of the SL2 splice leader (5'-GGTTTAAACCCAGTTACTCAAG-3') (Huang and Hirsh 1989) showed that SL2 is also spliced to each of the first three exons. However, it is possible that the SL2 products are extremely rare and were detected only because of the sensitivity of PCR after 60 cycles of amplification.

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Note added in proof

The nucleotide sequence data reported in this paper have been deposited to the GenBank data library under accession no. U20168.

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