Caenorhabditis elegans lin-25: cellular focus, protein expression and requirement for *sur-2* during induction of vulval fates

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SUMMARY

Induction of vulval fates in the *C. elegans* hermaphrodite is mediated by a signal transduction pathway involving Ras and MAP kinase. Previous genetic analysis has suggested that two potential targets of this pathway in the vulva precursor cells are two novel proteins, LIN-25 and SUR-2. In this report, we describe further studies of *lin-25*. The results of a genetic mosaic analysis together with those of experiments in which *lin-25* was expressed under the control of an heterologous promoter suggest that the major focus of *lin-25* during vulva induction is the vulva precursor cells themselves. We have generated antisera to LIN-25 and used these to analyse the pattern of protein expression. LIN-25 is present in all six precursor cells prior to and during vulva induction but later becomes restricted to cells of the vulval lineages. Mutations in genes in the Ras/MAP kinase pathway do not affect the pattern of expression but the accumulation of LIN-25 is reduced in the absence of *sur-2*. Overexpression of LIN-25 does not rescue *sur-2* mutant defects suggesting that LIN-25 and SUR-2 may function together. LIN-25 is also expressed in the lateral hypodermis. Overexpression of LIN-25 disrupts lateral hypodermal cell fusion, suggesting that *lin-25* may play a role in regulating cell fusions in *C. elegans*.

Key words: *Caenorhabditis elegans*, Vulva, *lin-25*, *sur-2*, Cell signalling, *let-60*, *ras*

INTRODUCTION

The fates of cells giving rise to the vulva in the *Caenorhabditis elegans* hermaphrodite are influenced by three distinct cell signaling events (Greenwald, 1997). One event, the induction of vulva fates by the anchor cell (AC), is mediated by a signal transduction pathway involving Ras and MAP kinase. Two targets of this pathway in *C. elegans* are the transcription factors, LIN-1 and LIN-31 (Jacobs et al., 1998; Tan et al., 1998). It is not known in detail, however, how these proteins function to regulate induction, nor have the other targets of the pathway been identified.

The vulva in *C. elegans* is formed from the descendants of three hypodermal (epidermal) cells, P5.p, P6.p and P7.p (Sulston and Horvitz, 1977). These cells are part of a group of six cells, P3.p-P8.p, named collectively the vulva precursor cells (VPCs). Initially each cell has the potential to adopt one of three different fates, 1°, 2° or 3° (Fig. 1) (Sulston and White, 1980). In wild-type worms P6.p adopts the 1° fate, P5.p and P7.p the 2° fate, and P3.p, P4.p and P8.p the 3° fate. The fate that each cell adopts is thought to be influenced by three separate cell signaling events (Greenwald, 1997). A signal from hyp7, a large syncytial hypodermal cell, is thought to inhibit the VPCs from adopting vulval (1° or 2°) fates. A signal

from a cell in the somatic gonad, the anchor cell (AC), allows P6.p to overcome this inhibition and adopt the 1° fate. Signalling also occurs between adjacent VPCs. In particular a lateral signal from P6.p is believed to induce P5.p and P7.p to adopt the 2° fate.

The signal that the AC cell sends to P6.p, LIN-3 (Hill and Sternberg, 1992), appears to function by activating the receptor-type tyrosine kinase, LET-23 (Aroian et al., 1990), and in turn a signal transduction pathway involving Ras (LET-60) (Beitel et al., 1990; Han and Sternberg, 1990), Raf (LIN-45) (Han et al., 1993), MEK (MEK-2) (Kornfeld et al., 1995; Wu et al., 1995) and MAP kinase (MPK-1/SUR-1) (Lackner et al., 1994; Wu and Han, 1994). One target of the pathway is the putative ETS domain transcription factor, LIN-1, which in the absence of signaling functions as a negative regulator of vulval fates (Beitel et al., 1995; Jacobs et al., 1998). Another target of the pathway is LIN-31, a member of the HNF-3/*fork head* family of transcription factors (Miller et al., 1993; Tan et al., 1998). Analysis of VPC fates in hermaphrodites homozygous for null mutations in both *lin-1* and *lin-31*, however, indicate that these two proteins cannot be the only targets of the pathway (Beitel et al. 1995 and S. T., G. J. Beitel, H. R. Horvitz and I. G., unpublished observations).

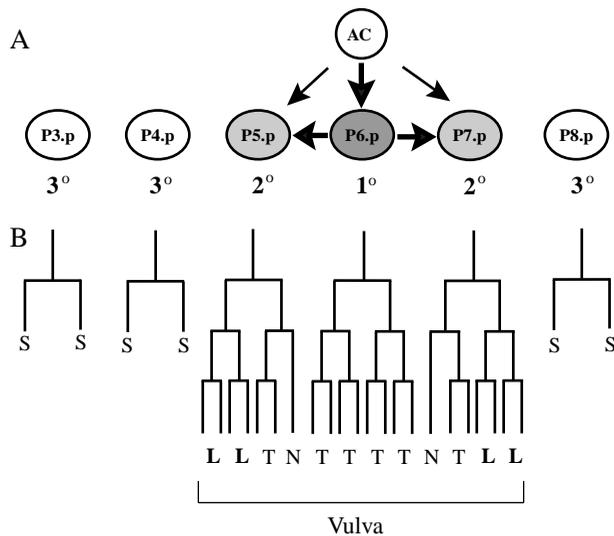Genetic epistasis experiments indicate that *lin-25* and *sur-2*

A

B

L L T N T T T T N T L L

Vulva

Fig. 1. (A) Six multipotent hypodermal (epidermal) cells, P3.p, P4.p, P5.p, P6.p, P7.p and P8.p (the VPCs) are located just ventral to the gonad. The anchor cell (AC) of the gonad induces P5.p, P6.p and P7.p to adopt vulval fates. A VPC adopting the primary fate appears to signal its neighbours to adopt the secondary fate. (B) Each VPC fate is a characteristic lineage, which can be distinguished by planes of cell division and terminal cell types. Vulval lineages are characterized by three rounds of cell division. T and L designate the orientation of the last division: T indicates a transverse (left-right) division (with respect to the symmetry of the entire animal), L a longitudinal (anterior-posterior) division. N indicates that the cell does not divide, but does not fuse with hyp7. If the cells produced by a longitudinal division adhere to the ventral cuticle this is denoted by bold face type (i.e. L). Nonvulval lineages are characterized by progeny that fuse with hyp7; S designates fusion.

function downstream of *let-60 ras* in the genetic pathway for VPC fate specification (Singh and Han, 1995; Tuck and Greenwald, 1995). Mutations in *lin-25* or *sur-2* suppress phenotypes associated with a dominant, hypermorphic mutation in *let-60*. In an otherwise wild-type genetic background, null mutations in *lin-25* or *sur-2* cause highly penetrant defects in VPC fate specification similar to those seen in hermaphrodites homozygous for certain reduction of function mutations in *let-60 ras* (Ferguson et al., 1987; Singh and Han, 1995; Tuck and Greenwald, 1995). *lin-25* and *sur-2* are predicted to encode proteins of molecular masses 130 kDa and 180 kDa, respectively, neither of which are significantly similar in sequence to any other proteins in the public databases (Singh and Han, 1995; Tuck and Greenwald, 1995).

In this paper, we report experiments to characterize further the role of *lin-25* in vulva development. The results of a genetic mosaic analysis and of experiments in which we expressed LIN-25 under the control of a tissue-specific promoter/ enhancer show that *lin-25* has a complex focus for VPC fate determination and that one major focus is likely to be the VPCs. We have also generated antisera to LIN-25 and used them to examine the pattern of LIN-25 protein expression during vulva development. The analysis of *sur-2; lin-25* double mutant strains and of LIN-25 expression in a *sur-2* mutant background suggest that LIN-25 and SUR-2 may function together during VPC fate specification.

MATERIALS AND METHODS

Genetic mosaic analysis

Hermaphrodites lacking *lin-25* activity in AB (and its descendants) or ABp (and its descendants) were identified as Unc-36 non-Dpy-17 animals segregating from the strain GS534 [*dpy-17(e164)ncl-1(e1865)unc-36(e251); unc-42(e270)lin-25(e1446); ctDp11*]. Hermaphrodites lacking *lin-25* activity in ABa and its descendants were identified by randomly screening animals segregating from GS534 by Nomarski differential interference contrast microscopy (Nomarski DIC) for those containing clones of cells displaying the Ncl-1 phenotype (Hedgecock and Herman, 1995). Hermaphrodites lacking *lin-25* activity in P_1 and its descendants were identified as non-Sup-10 animals segregating from the strain GS930 [*sup-18(ar30) ncl-1(e1865) unc-36(e251); unc-42(e270) lin-25(e1446); ctDp11; sup-10(n983)*]. *sup-18(ar30)* is a recessive suppressor of the Unc phenotype conferred by *sup-10(n983)* (M. Postner and I. G., unpublished observations). The focus of *sup-10(n983)* is P_1 (Herman and Hedgecock, 1990). All mosaic animals were examined by Nomarski DIC microscopy to determine which cells had lost the duplication.

Expression of LIN-25 under the control of *lin-12* regulatory sequences

We generated a full-length *lin-25* cDNA clone by ligating together several different partial *lin-25* cDNA clones described previously (Tuck and Greenwald, 1995). Details of the cloning strategy are available upon request. The full-length composite cDNA was sequenced and the sequence found to be that of the wild-type gene. To generate worms expressing *lin-25* under the control of *lin-12* regulatory sequences, we subcloned a *Not*I fragment containing the *lin-25* cDNA into the expression vector pLEX (Struhl et al., 1994). The resulting plasmid is named pVB31LN. pVB31LN was injected at a concentration of 50 μg/ml into hermaphrodites of the genotype, *unc-54(r293) smg-1(r861); unc-4(e120); lin-25(ar90)* together with 50 μg/ml of the plasmid, pNC4-21 (Miller and Neimeyer, 1995) which encodes *unc-4(+)*. Four transformed lines were obtained in which exogenous DNA was maintained extrachromosomally. We examined 8-10 early L4 hermaphrodites from each of the lines and found that most (31/35) carrying the array appeared to be rescued for the VPC lineage defect. Direct analysis of the lineages generated by P3.p-P8.p in six hermaphrodites from one of the lines revealed that they were wild-type. Vulva lineages of worms from the same line but lacking the array were mutant in all cases indicating that the genetic markers in the strain had no effect on the *lin-25* mutant phenotype.

Preparation of anti-LIN-25 antisera

lin-25 cDNA sequences from 421 to 1476 (Tuck and Greenwald, 1995) and from 1230 to 2151 were subcloned into the pQE-31 bacterial expression vector (Qiagen). The resulting plasmids encode LIN-25 polypeptides containing 6XHis affinity tags fused to their N termini. The tagged proteins were expressed in *E. coli* strain M15(pREP4), purified under denaturing conditions and then sent to East Acres Biologicals, Massachusetts, USA for injection into rabbits. Positive antisera were affinity purified by standard procedures (Gu et al., 1994).

Generation of worms containing multiple copies of *lin-25* or *sur-2*

pSP1, a plasmid containing the complete *lin-25* genomic sequence and 2.5 kb of upstream flanking DNA (S. T. and I. G., unpublished) was injected at a concentration of 100 μg/ml together with pRF4, which confers a dominant roller phenotype on transformed progeny (Mello et al., 1991). Stable lines containing arrays that rescued the *lin-25* egg-laying defect were established and the array in one line integrated into the genome by γ-ray mutagenesis. Two lines, *svIs1* and *svIs2*, in which the array had been integrated were found to express

high levels of LIN-25. The pattern of expression of LIN-25 in worms containing non-integrated (extrachromosomal) arrays was subsequently found to be the same as that in worms harbouring an integrated array. Thus the expression pattern is not dependent upon insertion site flanking sequences. Worms containing multiple copies of *lin-25* on extrachromosomal arrays also displayed the defect in hypodermal cell fusion, albeit with lower expressivity.

Indirect immunofluorescence microscopy

Worms were fixed and stained according to the protocol of Bettinger et al. (1996). Affinity-purified anti-LIN-25 antibody was used at a concentration of 1:1600. The secondary antibody was cy2-conjugated anti-rabbit antibody (Amersham) diluted 1:200.

RESULTS

lin-25 shows maternal effects with respect to VPC fate specification

In homozygous *lin-25(e1446)* mutant hermaphrodites segregating from homozygous parents, P5.p and P7.p usually adopt the 3° fate and the lineage of P6.p is frequently abnormal (Ferguson et al., 1987). We found, however, that in homozygous *lin-25(e1446)* progeny segregating from a heterozygous *lin-25/+* parent P5.p is usually induced (and in some cases adopts a normal 2° fate) (Table 1) and that the P6.p lineage is always wild-type. This implies that there is partial maternal rescue of *lin-25* in P5.p and P6.p. Since the fates of the VPCs are determined during the L3 stage (Sternberg and Horvitz, 1989), the maternal rescue effect implies that *lin-25* mRNA or protein deposited into the egg may perdure. Curiously there does not seem to be any maternal rescue of *lin-25* in P7.p: in *lin-25(e1446)* progeny segregating from a heterozygous *lin-25/+* parent, P7.p adopts the 3° fate (Table 1). This might indicate that the requirement for *lin-25* activity is higher in P7.p than in P5.p or P6.p.

Analysis of VPC lineages in homozygous mutant hermaphrodites derived from heterozygous parents also revealed that two different defects in Pn.p cell development seen in *lin-25* mutants are separable. In approximately 75% of homozygous *lin-25* mutant hermaphrodites derived from homozygous parents one (or sometimes two) of the Pn.ps divides during the L1 stage to generate two cells that both subsequently become VPCs (Ferguson et al., 1987; Tuck and Greenwald, 1995). We found that this precocious division defect was completely rescued by maternal *lin-25* gene product (Table 1). Despite the absence of the earlier defect, however, all animals showed VPC lineage defects during the L3 stage. This suggests that the VPC lineage defects reflect a role for *lin-25* in VPC fate determination and is not the indirect result of the earlier defect during the L1 stage. This finding is supported by the results of experiments with a heat-shock-*lin-25* transgene (this paper) and temperature-shift experiments (Ferguson et al., 1987) that suggest that *lin-25* acts in the L2-early L3 stage, the time at which vulva induction occurs.

lin-25 appears to function within the VPCs

We have previously suggested that *lin-25* gene activity may be required within the VPCs for their ability to respond appropriately to LIN-3 (Tuck and Greenwald, 1995). In order to test this model, we analysed VPC fates in hermaphrodites mosaic for *lin-25*. To carry out mosaic analysis, we used a
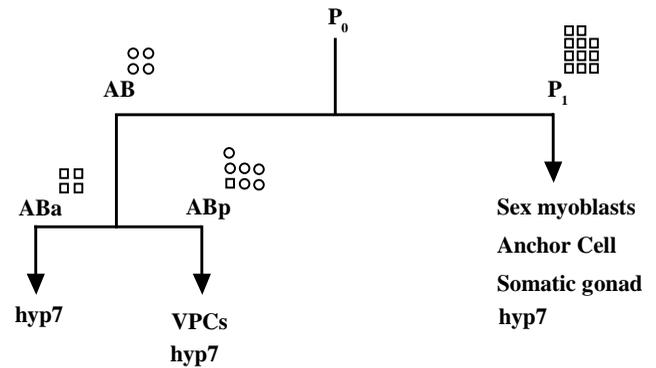

Fig. 2. Summary of mosaic analysis of *lin-25*. A square indicates a mosaic animal with wild-type vulval lineages; a circle indicates an animal in which the VPC lineages were abnormal.

chromosomal duplication, *ctDp11*, rather than an extrachromosomal array since arrays that rescue *lin-25* mutant defects display excessive perdurance of *lin-25* activity (data not shown). Since *ctDp11* is lost relatively rarely during mitosis (Herman, 1995), we confined the analysis to establishing in which broad areas of the *C. elegans* lineage *lin-25* is required for proper VPC fate specification rather than attempting to identify mosaic worms that had lost *lin-25* gene activity specifically in the VPCs or their immediate progenitors. We generated genetic mosaics by loss of the free duplication (which carries a wild-type *lin-25* allele) in strains in which both chromosomal copies of *lin-25* were mutant. Using appropriate genetic markers, we were able to identify losses in different embryonic founder cells and their descendants (see Table 2, Fig. 2 and Materials and Methods).

One aspect of the mosaic analysis supports the hypothesis that *lin-25* has a focus in the VPCs. Specifically we found that one focus for *lin-25* with respect to VPC fate specification is in cells descended from ABp. Most (6/7) individuals in which the duplication (and hence *lin-25* activity) had been lost in ABp showed abnormalities in the VPC lineages. In contrast VPC lineages were normal in hermaphrodites in which the duplication had been lost in ABa (4/4) or P1 (11/11).

Besides being the progenitor of the VPCs, ABp also gives rise to many other cells. In order to delimit further the possible focus of *lin-25* activity with respect to VPC fate specification, we expressed a *lin-25* cDNA in the VPCs prior to and during vulva induction using an heterologous promoter. We placed a *lin-25* cDNA into the expression vector, pLEX, which contains regulatory sequences from the *lin-12* gene (Struhl et al., 1993), and examined the ability of the transgene to rescue the vulva defect in *lin-25* mutant hermaphrodites. In hermaphrodites containing a *lacZ* reporter gene in the pLEX vector, after the L1 stage the only cells descended from AB expressing *lacZ* are the VPCs (and certain VPC descendants) and the only cells descended from P1 are selected cells in the somatic gonad and the sex-muscle lineages (Wilkinson and Greenwald, 1995). Examination of hermaphrodites homozygous for *lin-25(ar90)* but carrying the *lin-12::lin-25* transgene on an extrachromosomal array revealed that 90% (37/41) were completely rescued for the vulva lineage defect. This result suggests that expression of *lin-25* in the VPCs is sufficient for wild-type VPC fate specification.

Table 1. VPC fates in *lin-25* and *sur-2* mutant hermaphrodites

Genotype	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	Pn.p*
lin-25(e1446)‡ (From heterozygous parent)	3°	3°	2°/ind	1°/ind	3°	3°	
	S S	S S	**LL**TN	TTTO	S S	S S	
	S S	S S	**LL**TN	TOOT	SS S	S S	
	S S	S S	UUTN	TTTO	S S	S S	
	S S	S S	LLOT	TOTO	S SS	S S	
	S S	S S	S TT	TTTT	S S	S S	
	S	S S	LLOT	TTOO	S S	S S	
lin-25(e1446) (From homozygous parent)§	3°	3°	3°	1°/ind	3°	3°	3°
sur-2(ku9)	3°	3°	3°/ind	1°/ind	3°	3°	3°
	S S	S S	S S	DD S	S S	S	S S
	S S	S S	LLOT	LTTT	S S	S S	
	S S	S S	S S	LLOO	S S	S S	S S
	S S	S S	S S	LTOT	LL S	S S	
	S S	S S	S S	LDDD	S S	S S	S S
	S S	S S	**LL**TU	OTOL	S	S S	
	S S	S S	**LL**OO	OTOO	S S	S S	
	S S	S S	S S	LTOO	N**TLL**	S S	S S
sur-2(ku9); lin-25(ar90)	3°	3°	3°	1°/ind	3°	3°	3°
	S	S	S S	S S	S	S	S
	S S	S S	**L**OTT	TOOU	S S	S S	
	S	S	**LL**TT	TOOO	S S	S S	
	S S	S	S S	LOTO	OUO**L**	S S	
	S	S S	OOTT	TT S	S S	S S	
	S S	S S	S UU	UUUU	S S	S S	
	S S	S S	UUUU	S S	LTTT	S	S S
	S S	S S	S	OOTT	O**L** S	S S	S S
mpk-1(oz140)	3°	3°	2°/ind	1°/ind	2°	3°	
	S S	S S	UUON	TOTO	N**LLL**	S S	
	S S	S S	**L**OTN	TTOT	N**TLL**	S S	
	S S	S S	U**O**TN	S TT	NO**LL**	S S	
	S S	S S	**LL**ON	TTTT	N**TLL**	S S	
	S S	S S	**LL**ON	TTTT	N**TLL**	S S	
mpk-1(oz140); lin-25(ar90)	3°	3°	3°	3°/ind	3°	3°	3°
	S S	S S	S S	S S	S S	S S	S S
	S S	S S	S S	UU S	S S	S S	S S
	S S	S S	S S	S UU	S S	S	S S
	S S	S S	S	S OO	S S	S S	S S
	S S	S S	S S	TT S	S S	S S	S S
	S S	S S	S S	S S	S S	S S	S S
sur-2(ku9); svIs1	3°	3°	3°/ind	ind	3°/ind	3°	3°
	S S	S S	**LL**ON	OTOT	S S	S	S S
	S	S S	S S	S S	TOOO	N**TLL**	S S
	S S	S S	UU**L**T	OTOT	S S	S S	
	S S	S	S S	**LL**TT	S S	S S	S S
	S	S S	S	S TL	S S	S	S S
	S S	S S	S S	**LL**T**L**	OO S	S S	S S

See the legend to Fig. 1 for the meaning of S, N, T, L. O indicates oblique division; U indicates cell did not divide; D indicates that cell divided but division was not observed.

lin-25(ar90) is a transition in codon 197 that results in the creation of an amber stop codon (Tuck and Greenwald, 1995). *sur-2(ku9)* is predicted to encode a truncated protein lacking the C-terminal 25% of full length SUR-2 and is thought to strongly reduce or eliminate *sur-2* activity (Singh and Han, 1995).

*In animals carrying strong *lin-25* or *sur-2* mutations P3.p, or another of the cells that later become part of the vulva equivalence group, often divides during the L1 stage to produce 2 cells that subsequently become VPCs (Ferguson et al., 1987). *lin-25* or *sur-2* mutant hermaphrodites, threfore, often have seven rather than 6 VPCs. We did not determine which Pn.p cell had divided precociously in these animals: Cells are therefore numbered arbitrarily starting at P3.p, the most anterior VPC.

‡Animals derived from heterozygous mutant hermaphrodites. Complete genotype *unc-42(e270)lin-25(e1446)*.

§Data from Ferguson et al. (1987) and Tuck and Greenwald (1995).

Inspection of the lineage defects in mosaic hermaphrodites lacking *lin-25* activity in ABp suggests, however, that the VPCs may not be the sole focus of *lin-25* with respect to VPC fate specification. Specifically, comparison of VPC lineages in homozygous mutant hermaphrodites derived from heterozygous parents with VPC lineages seen in mosaic hermaphrodites lacking *lin-25* in ABp revealed that the *lin-25* genotype of cells descended from ABa or P₁ can influence the fates of the VPCs when the VPCs lack *lin-25* activity. In genetic mosaics lacking *lin-25* activity in ABp and its descendants, the vulval lineage defects are consistently weaker than those seen in homozygous mutant hermaphrodites derived from heterozygotes (Tables 1, 2). In particular P7.p is always induced in ABp mosaics whereas in

Table 2. Vulval fates in hermaphrodites mosaic for *lin-25*

Pt. of Loss*	Egg-laying‡	No.	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
ABp			3°	3°	ind	1°	ind	3°
	−	1	S S	S S	S S	TOTO	OOUU	S S
	+/−	1	S	S S	S LD	TTTT	NTLL	S S
	+/−	1	S S	S S	**LLTN**	OTOT	UO S	S S
	+	1	S S	S S	UULN	TTTO	**NTLL**	S S
	+	1	S S	S S	**LLTN**	TTTT	**NTLL**	S S
	+/−	1	S S	S S	S OT	TOOT	NTUU	S S
	−	1	S S	S S	ULTN	TOTT	TT S	S S
ABa			3°	3°	2°	1°	2°	3°
	+	1	S S	S S	**LLTN**	TTTT	**NTLL**	S S
	+	1	S S	S S	**LLTN**	OTTT	**NTLL**	S S
	+	1	S S	S S	**LLTN**	TTTO	**NTLL**	S S
	+	1	S S	S S	**LLTN**	TTTT	**NTLL**	S S
AB			3°	3°	ind	1°	ind	3°
	+	1	S S	S S	**LLTN**	TTTT	NTOL	S S
	−	1	S S	S S	S UU	TTOO	S S	S S
	+	1	S S	S S	**LLTN**	TTTT	NTUU	S S
	−	1	S S	S S	ULUT	TTOT	NLLL	S S
P$_1$			3°	3°	2°	1°	2°	3°
	−	8	S S	S S	**LLTN**	TTTT	**NTLL**	S S
	+	3	S S	S S	**LLTN**	TTTT	**NTLL**	S S

See the legend to Fig. 1 or the footnote to Table 1 for the meaning of S, N, T, L and O. Each line showing lineages represents the vulval lineages of the number of animals indicated under 'No.'

*Indicates the cell in which the duplication was lost.

‡Indicates egg-laying ability. (−) Animal was completely egg-laying defective. (+/−) Animal laid some eggs and larvae before turning into a 'bag-of-worms'.

homozygous mutant hermaphrodites segregating from heterozygotes, P7.p always adopts the 3° fate. Thus the focus for the specification of P7.p is complex with one component in ABp and a second in either ABa, P$_1$ or both. (Another possibility, that zygotic *lin-25* activity expressed in AB perdures, is unlikely since most zygotic genes are not transcribed at this time.) The behaviour of P7.p in turn suggests that the focus of *lin-25* activity for the specification of the other VPCs may also be complex.

We conclude that the main focus appears to be in the VPCs but that *lin-25* activity elsewhere can also influence VPC fate.

Detection of LIN-25 in protein extracts

To help elucidate the biochemical function of LIN-25, we raised antibodies to bacterially expressed LIN-25 polypeptides (Materials and Methods). The results of western blot analysis of total worm protein using the first of these antisera is shown in Fig. 3A and B. The antiserum reacts with several different

Fig. 3. Western blots of total worm proteins probed with affinity purified anti-LIN-25 antibodies. *svIs1* and *svIs2* denote strains containing multiple copies of the *lin-25* gene under the control of its own promoter. *lin-25(ar90)* (W197STOP) and *lin-25(ga67)* (F284T) are null alleles (Tuck and Greenwald, 1995). *lin-25(n1722)* is a weak, temperature sensitive allele, grown here at the restrictive temperature, 25°C. *sur-2(ku9)* is predicted to encode a truncated protein lacking the C-terminal 25% of full length SUR-2 (Singh and Han, 1995). (A,B) Western blots probed with antibody 4452. The same blot was exposed for 4 minutes (A) or 30 minutes (B). The large arrow indicates the most slowly migrating band, which corresponds to the predicted size of the full-length LIN-25 protein (~130 kDa). The smaller arrows indicate the doublet of proteins of ~90 kDa; the circular marker indicates a non-specific cross-reacting product. Although the pattern of bands seen in the lane containing protein from *sur-2* mutant worms is largely the same as that from wild-type worms, we have consistently observed subtle differences. In particular in the doublet of bands of approximately 90 kDa, the lower of the two is stronger in extracts from wild-type worms but the upper is stronger in protein extracts from *sur-2* mutants. (C,D). Western blots probed with antibody 4478. (D) The most-prominent LIN-25 species are indicated with arrows. The band seen in extracts from *lin-25(ar90)* mutant worms (which is also weakly visible in extracts from *sur-2* mutant worms) appears to be an antigen with which 4478 reacts that is specifically induced in the absence of *lin-25* or *sur-2*. It is probably not derived from the *lin-25* gene since the same band is not seen on blots probed with 4452. (C,D) 50 μg of total protein were loaded into each track.
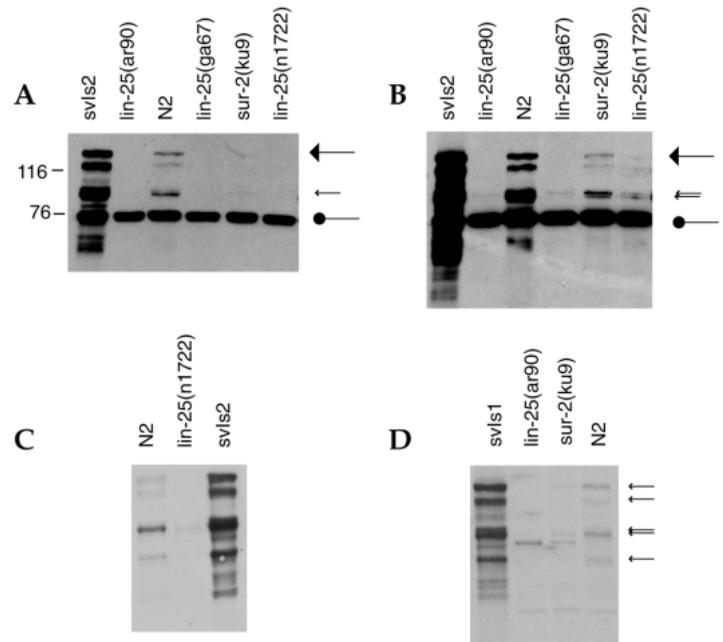
species present in extracts from wild-type worms (and in worms containing multiple copies of the *lin-25* gene) that are absent from worms homozygous for *lin-25* null mutations. (The antiserum also reacts with one prominent species present in all cell extracts which apparently is not a product of the *lin-25* gene.) While a product of the predicted molecular mass, 130 kDa, is observed, the most abundant species have considerably lower apparent molecular weights. We do not know at the present time whether these species represent degradation products or result from the processing of the primary translation product to a mature form.

Western blot analysis of protein extracts from wild-type worms of different developmental stages showed that LIN-25 is expressed throughout development. Expression is robust at all stages but is highest during the L1, L2 and L3 stages (Fig. 4A).

Expression pattern of LIN-25 in the VPCs and their descendants

In order to analyse LIN-25 protein expression in whole worms by immunofluorescence microscopy, we purified a second antiserum, 4478, (Fig. 3C,D). Using this antiserum we were able to detect LIN-25 by immunofluorescence microscopy in worms containing multiple copies of the *lin-25* gene (under the control of its own promoter) but not in wild-type worms (Materials and Methods). These worms display an apparent gain-of-function defect within the seam cell lineages (see below) but have wild-type induction of vulva fates and are able to lay eggs.

Strong expression of the LIN-25 protein is detected in all six VPCs (Fig. 5D). Expression begins in the L1 stage and remains strong up until the time the VPCs divide. Shortly after division, staining becomes restricted to the six descendants of P5.p, P6.p and P7.p (Fig. 5F). Thereafter LIN-25 expression is detected
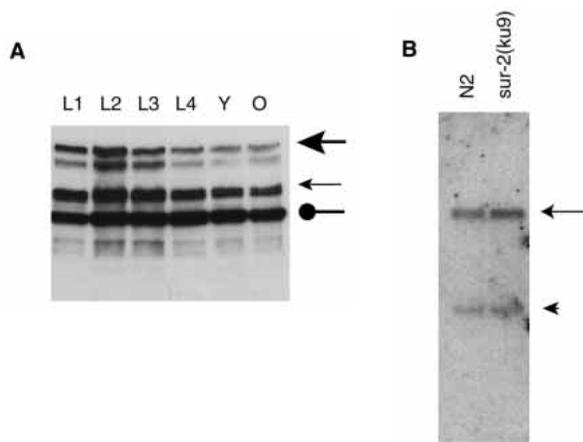

Fig. 4. (A) Western blot of total worm protein from wild-type (N2) worms at different developmental stages. Y denotes young adult; O, old adult; L1, L2, L3 and L4 denote protein extracts from the different larval stages. The blot was probed with the affinity purified anti-LIN-25 antiserum, 4452. Markers to the right indicate the species described in Fig. 3A. (B) Northern blot of poly(A)+ RNA from wild-type and *sur-2(ku9) C. elegans* hermaphrodites. The blot was probed with a full-length *lin-25* cDNA and a cDNA encoding EIF-4A, which functions as a control for loading (Roussell and Bennett, 1992). Arrow indicates the *lin-25* message, the arrowhead, the EIF-4A mRNA. 10 μg of RNA were loaded into each lane.

in all descendants of P5.p, P6.p and P7.p through both subsequent rounds of division (Fig. 5H and data not shown). Staining is also detected in all descendants of P5.p, P6.p and P7.p during the early L4 stage, after morphogenesis has occurred (data not shown). Throughout all stages LIN-25 protein is detected in the nuclei of positive cells but appreciable cytoplasmic staining is also observed.

Elsewhere in the worm strong LIN-25 expression is seen in a number of cells, most notably the seam cells and many cells in the somatic gonad (Fig. 5D,F,H). Although many other cells are also stained weakly with the antiserum (such as the distal tip cells, the coelomocytes and the excretory cell), many cells fail to stain. Staining was not detected for example in nuclei in the hyp7 syncytium or in the hyp7 cytoplasm.

lin-25 and *sur-2* appear to function together in vulva induction

Mutations in *lin-25* and *sur-2* give rise to strikingly similar phenotypes (Ferguson and Horvitz, 1985; Ferguson et al., 1987; Singh and Han, 1995; Tuck and Greenwald, 1995). Null mutations in either gene reduce but do not eliminate the induction of vulval fates (Ferguson et al., 1987; Singh and Han, 1995; Tuck and Greenwald, 1995). A small proportion (~6%) of hermaphrodites homozygous for null mutations in either gene die as larvae; of the animals surviving to adulthood approximately 15% are sterile (Ferguson and Horvitz, 1985; Singh and Han, 1995; Tuck and Greenwald, 1995). The egg-laying defect in fertile hermaphrodites homozygous for null mutations in either gene shows 100% penetrance and expressivity; no eggs are ever observed on plates containing homozygous mutant strains.

To characterise further the relationship between *lin-25* and *sur-2*, we constructed double mutant strains homozygous for null mutations in both genes. Analysis of the VPC lineages in hermaphrodites of the genotype *sur-2(ku9); lin-25(ar90)* revealed that the defects in the double mutant animals were no greater than that seen in either single mutant (Table 1). In particular P6.p, the cell closest to the anchor cell, often generated a 1°-like lineage in the double mutant as it does in most single mutant animals (Singh and Han, 1995; Tuck and Greenwald, 1995). In addition, the penetrances of the other phenotypes associated with *lin-25* and *sur-2* (larval lethality, sterility and precocious Pn.p cell divisions) were not greater in the double mutant (not shown). These results suggest that the incomplete expressivity of the vulval lineage defect seen in *lin-25* and *sur-2* mutant hermaphrodites is not the result of partial redundancy between the two genes. Instead the results suggest that *lin-25* and *sur-2* probably function on the same branch of the pathway downstream of *let-60*.

The failure of *sur-2* mutations to increase the penetrance or expressivity of phenotypes caused by *lin-25* stands in contrast to the effect of a mutation in *mpk-1* on the same *lin-25* alleles. The *mpk-1* mutation, *oz140* (Church et al., 1995), although functioning as a potent suppressor of the multivulva phenotype caused by *let-60(n1046gf)*, causes only mild vulval lineage defects (Table 1). However, in *mpk-1(oz140); lin-25(ar90)* double mutant hermaphrodites, the vulval lineage defect is consistently stronger than that seen in *lin-25(ar90)* single mutant worms (Table 1).

lin-25 mutations also show strong synergy with partial reduction-of-function mutations in *let-23*. Mutants lacking *let-*

23 activity arrest growth during the L1 stage and the dead larvae have a characteristic rod shape (Aroian et al, 1990). While a significant proportion of hermaphrodites homozygous for the partial reduction-of-function alleles, *let-23(n1045)* or *let-23(sy97)*, survive to adulthood (42% and 11% respectively), *let-23(n1045)* or *let-23(sy97)* mutant hermaphrodites that also lack *lin-25* activity invariably die as larvae.

Animals homozygous for *sur-2* mutations accumulate less LIN-25 protein

Western blot analysis of protein extracts from *sur-2* mutant worms revealed that LIN-25 protein levels are approximately 10-fold lower than wild-type in worms lacking *sur-2* activity (Fig. 3A,B,D). Since *sur-2* mutations cause a vulvaless phenotype and LIN-25 appears to be expressed in VPC descendants one explanation for this observation could have

been simply that *sur-2* mutant worms lack cells in which LIN-25 is expressed. LIN-25 levels were normal, however, in hermaphrodites lacking a vulva because of the mutation, *lin-3(n378)*. Moreover vulvaless worms of the genotype *lin-2(e1309)* or *lin-10(e1439)* also showed wild-type LIN-25 protein levels judged by western blot analysis as did worms containing excess vulval tissue resulting from the multivulva mutations, *lin-15(n309)*, *let-60(n1046)* and *lin-12(n137)* (data not shown). Protein extracts from late L2/early L3-staged populations of *sur-2* mutant worms (*i.e.* from worms in which the VPCs had yet to divide) also contained approximately 10-fold less LIN-25 than extracts from equivalent populations of wild-type worms again suggesting that the low LIN-25 levels observed in extracts from *sur-2* mutant worms are not the result of the absence of the VPC descendants.

We crossed *svIs1* (an integrated array giving high levels of LIN-25 expression) into *sur-2*, *lin-10*, *lin-3* and *let-60(gf)* mutant backgrounds and analysed the expression of LIN-25 in the VPCs in the resulting strains by indirect immunofluorescence microscopy. VPCs in hermaphrodites homozygous for *lin-3(n378)* or *lin-10(e1439)* (which cause a vulvaless phenotype) or *let-60(n1046gf)* (which causes a multivulva phenotype) expressed the same levels of LIN-25 as those in animals carrying the same array in an otherwise wild-type genetic background (Fig. 6). VPCs in hermaphrodites homozygous for *sur-2(ku9)* in contrast expressed lower (but still detectable) levels of LIN-25 (Fig. 6G). It is possible, therefore, that in wild-type hermaphrodites the expression and accumulation of LIN-25 in the VPCs is independent of activation of the inductive signalling pathway. The results are also consistent with the possibility that either the expression or the accumulation of LIN-25 in the VPCs requires *sur-2* activity.

Overexpression of LIN-25 does not bypass the requirement for SUR-2

One possible explanation for the results described above concerning *sur-2* and *lin-25* is that the sole function of *sur-2* is to allow efficient transcription of *lin-25*. Northern blot
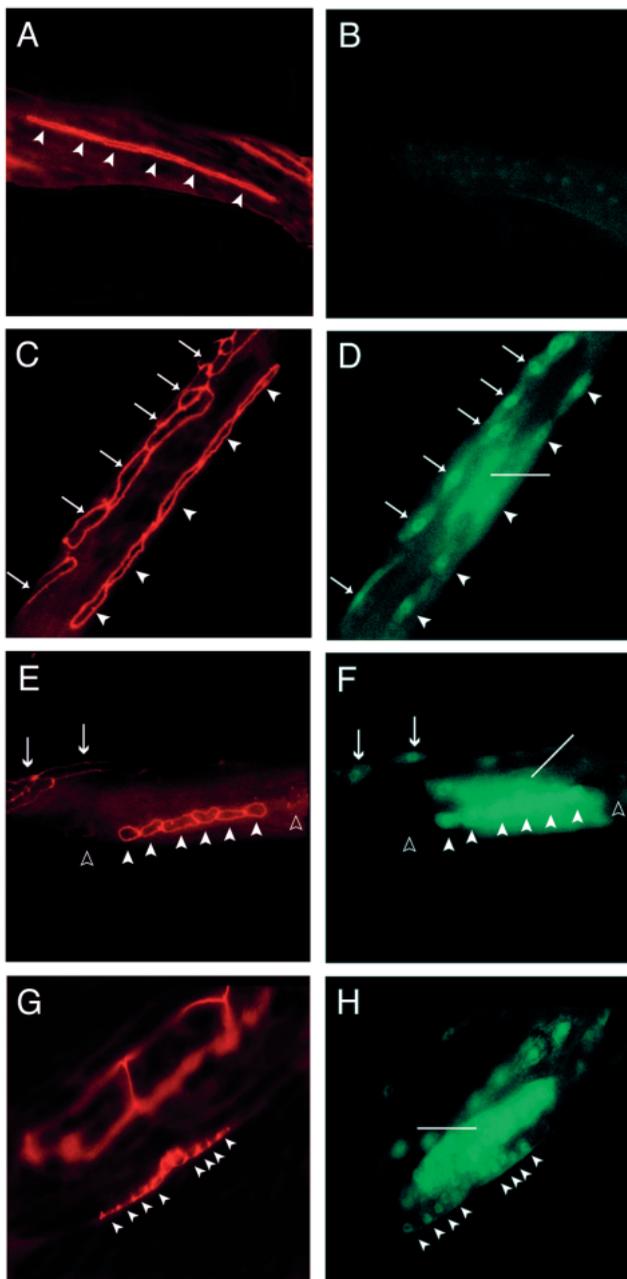
Fig. 5. Cellular expression and sub-cellular distribution of the LIN-25 protein. Hermaphrodites in the left column (A,C,E,G) were stained with MH27 antibody (which stains adherens junctions (Francis and Waterston, 1991) and hermaphrodites in the right column were stained with anti-LIN-25 antiserum 4478 (B,D,F,H). (A,B) *lin-25(ar90)* mutant worms. (A) Arrowheads indicate the positions of the VPCs. (C-H) Worms that overexpress LIN-25 from the integrated array, *svIs1*. (C,D) Arrowheads indicate the positions of the VPCs, arrows indicate the positions of the seam cells and other lateral hypodermal cells. The line in D indicates the position of the central part of the somatic gonad. Note that in C there are far more lateral hypodermal cells than seen in wild-type worms (not shown). (E,F) Absence of staining in the descendents of P4.p and P8.p. Anterior is to the left. Open arrowheads point to P4.pa and P4.pp (anterior arrowhead) and P8.pa and P8.pp (posterior arrowhead). The MH27 staining of the P4.p and P8.p descendents is still weakly visible in E but no LIN-25 staining of these cells is visible in F. Expression of LIN-25 in P5.ap is clearly visible in F. Expression of LIN-25 by the other descendents of P5.p, P6.p and P7.p is largely obscured by the staining of the underlining somatic gonad (indicated by a line). Arrows point to seam cells. (G,H) Staining of P5.p, P6.p and P7.p descendents after two rounds of division. Line in H indicates staining of the somatic gonad.
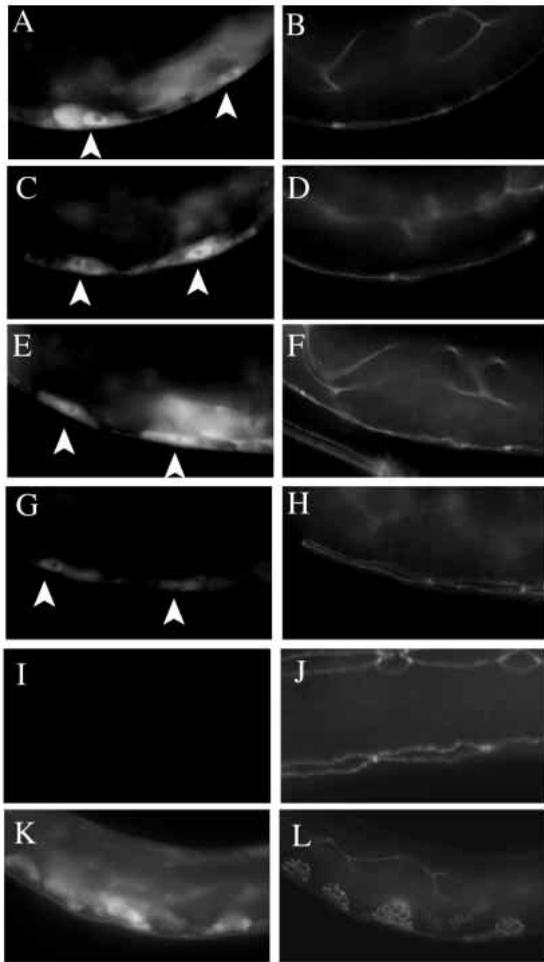
Fig. 6. Staining of the VPCs in worms harbouring mutations affecting VPC fate specification. All worms carry the *svIs1* integrated array. (A,C,E,G,I,K) Worms were stained with anti-LIN-25 antiserum, 4478; (B,D,F, H,J,L) the same worms stained with MH27 antibody (Francis and Waterston, 1991). (A,B) *svIs1* in an otherwise wild-type genetic background. (C,D) *let-60(n1046gf); svIs1*. (E,F) *lin-3(n378); svIs1*. (G,H) *sur-2(ku9) svIs1*. (I,J) Wild-type (N2). Arrowheads in A, C, E and G indicate the positions of VPCs. (K,L) *let-60(n1046gf); svIs1* hermaphrodites after three rounds of division of the VPCs. Although activation of *let-60* does not appreciably increase the LIN-25 levels in the VPCs themselves, LIN-25 protein is expressed in the ectopic vulval tissue generated by P3.p, P4.p and P8.p in *let-60(n1046gf)* mutant hermaphrodites. Conversely, while *lin-3* mutations do not reduce the amount of LIN-25 protein in the VPCs, in *lin-3* mutants VPCs adopt the tertiary fate and their descendents fail to express LIN-25 (data not shown).

analysis, however, revealed that *lin-25* mRNA levels are not lower in *sur-2* mutant worms than in wild-type (Fig. 4B). Furthermore, we found that a heat-shock-*lin-25* transgene that rescues *lin-25* mutant defects failed to rescue those caused by *sur-2(ku9)* (Table 3). This result implies that *sur-2* is unlikely to function principally in facilitating the transcription of *lin-25*.

An alternative possibility is that the LIN-25 and SUR-2 proteins function together in the same process perhaps as part of a protein complex. In this case, high levels of LIN-25 might not be able to bypass the need for SUR-2. To test this

Table 3. Heat-shock-induced expression of *lin-25* fails to rescue the *sur-2* vulva defect

Genotype	No. of animals	Rescue of vulva defect§	Rescue of egg-laying defect¶
*lin-25(ar90); svEx29**	10	+	+
*lin-25(ar90); svEx29**	2	+/−	−
*lin-25(ar90); svEx29**	1	−	
sur-2(ku9); svEx29‡	12	−	−

*Complete genotype *unc-36(e251); lin-25(ar90); svEx29[pVB25LN-R1p16]*.

‡Complete genotype *sur-2(ku9); unc-36(e251); svEx29*.

§(+) indicates that the vulva lineages were WT. (+/−) indicates that the vulva lineage defect was partially rescued.

¶(+) indicates that the animals were able to lay eggs.

pVB25LN is a plasmid containing a full-length WT *lin-25* cDNA cloned into the heat-shock vector, p49.78 (Mello and Fire, 1995). Details of its construction are available upon request. R1p16 encodes *unc-36(+)* (Herman et al., 1995). To generate *svEx29*, pVB25LN and R1p16 were both injected at a concentration of 50 μg/ml. To assay for rescue of the vulva defect early L2 hermaphrodites carrying the extrachromosomal array were placed at 33°C for 2 hours and then left to develop for 12 hours at 20°C. The animals were then mouted for Nomarski DIC microscopy and the vulva lineages followed until the early L4 stage. After the third and final round of divisions had occurred the animals were transferred to plates and scored for their ability to lay eggs.

possibility, we analysed the vulva lineages in hermaphrodites of the genotype, *sur-2(ku9); svIs1*. Despite the fact that these worms express higher levels of LIN-25 than wild type (Fig. 6G), the vulva lineages are mutant and resemble those of *sur-2(ku9)* (Table 1).

We also tested the ability of high concentrations of DNA encoding *sur-2* to rescue *lin-25* mutant defects. We generated strains homozygous for *lin-25(n545ts)* that contained multiple extrachromosomal copies of DNA encoding *sur-2*. In no case was the *lin-25* mutant defect rescued. It was not possible, however, to determine whether these lines contained higher than wild-type levels of SUR-2 protein since existing antibodies against SUR-2 do not detect the protein in worms (Q. Sun and M. Han, personal communication).

The results suggest that LIN-25 might function in the same process in the cell. Using the yeast 2-hybrid system (Fields and Song, 1989), we tested the possibility that LIN-25 and SUR-2 could bind to one another. Fragments spanning the *lin-25* and *sur-2* cDNAs were cloned into the appropriate yeast vectors and tested in all possible pairwise combinations but no evidence for a physical interaction was found. We also failed to find evidence for binding in coimmunoprecipitation analyses. Failure to detect binding in these assays, however, does not demonstrate that the proteins do not in fact bind in the cell. Further biochemical experiments will be necessary to address this issue.

lin-25 and cell fusion

In wild-type hermaphrodites, Pn.p cells outside the vulva equivalence group fuse directly with hyp7 without dividing. This is referred to as the quarternary fate (4°). In wild-type hermaphrodites P3.p occasionally adopts the 4° fate but P4.p and P8.p invariably adopt the 3° fate. In *lin-25* mutant hermaphrodites, however, with the exception of P6.p (or the Pn.p cell closest to the AC – see note in legend to Table 1), all VPCs occasionally adopt the 4° fate (Tuck and Greenwald, 1995). We found that the same defect is seen in *sur-2* mutant

worms and in *sur-2; lin-25* double mutants (Table 1). VPCs also adopt the 4° fate in hermaphrodites expressing intermediate levels of LIN-39, a HOX protein expressed in nuclei of cells in the mid-body region, including the VPCs, that is required in both the L1 and L2/L3 stages for proper vulva development (Maloof and Kenyon, 1998). However, we have not observed any differences between the levels of expression of LIN-39 in *lin-25* and wild-type hermaphrodites (data not shown) suggesting that the *lin-25* vulval defects do not result from effects on LIN-39 expression in P3.p-P8.p.

One possible role for *lin-25* during vulva development may be to keep the VPCs separate from hyp7 by directly or indirectly regulating fusion (Herman and Hedgecock, 1990). To explore this possibility, we examined the consequences of overexpressing LIN-25 on cell fusion. Overexpression of LIN-25 did not appear to prevent ventral hypodermal cells (VPCs and their descendants) from fusing to the hyp7. However we saw a dramatic effect on the fusion of cells of the lateral hypodermis.

At hatching, the major hypodermal (epidermal) syncytium, hyp7, contains 23 nuclei. During larval development 110 cells fuse to hyp7. Some of these cells are derived from the Pn.p cells that do not give rise to vulval tissue while a number of others are derived from lineages generated by a group of lateral hypodermal cells, called the seam cells (Sulston and Horvitz, 1977). In L4 larvae, the seam cells themselves form a separate syncytium by fusing with one another. *lin-25* mutants do not show defects in seam cell development. We found that in hermaphrodites overexpressing LIN-25, however, many hypodermal cells in the vicinity of the seam cells remain unfused to hyp7 (Figs 5C, 7). In addition, the seam cell syncytia fail to form properly (Fig. 7). Thus overexpressing
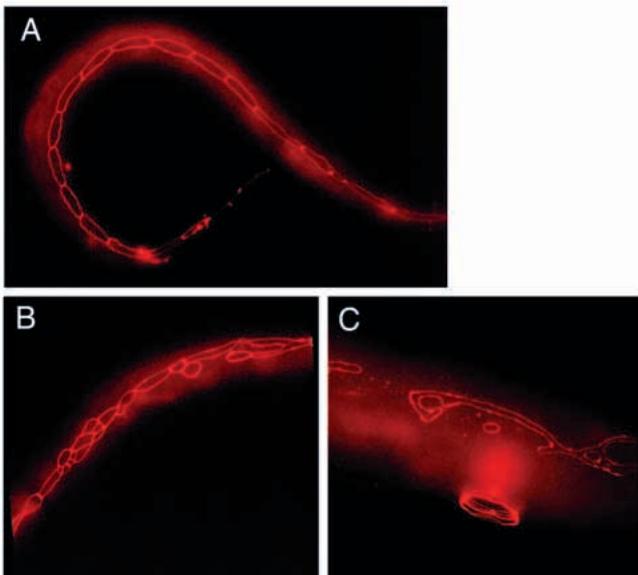

Fig. 7. Overexpression of LIN-25 disrupts fusions of lateral hypodermal cells. Hermaphrodites were stained with the monoclonal antibody, MH27, which recognizes adherens junctions (Francis and Waterston, 1991). (A) A wild-type (N2) L2 hermaphrodite. The antibody stains adherens junctions surrounding the seam cells. (B) An L2 hermaphrodite overexpressing LIN-25. Ectopic MH27 positive cells are present. (C) An adult hermaphrodite overexpressing LIN-25, stained with MH27. The seam cell syncytium is incomplete.

LIN-25 appears to prevent some cells that would normally fuse with hyp7 or with one another from doing so.

DISCUSSION

LIN-25 expression and function in the VPC lineages

LIN-25 is a novel protein that we proposed may serve as a target of the *let-60* Ras-mediated signalling pathway during vulva induction (Tuck and Greenwald, 1995). We have provided support for this hypothesis by analyzing the pattern of LIN-25 protein accumulation and the cellular focus of *lin-25* activity.

We made antibodies to LIN-25 polypeptides, and, although we were able to detect staining in situ only when LIN-25 was highly expressed (under the control of its own promoter) from a transgene, the expression pattern is consistent with a role in the VPCs. In otherwise wild-type hermaphrodites, we detected LIN-25 in P3.p-P8.p from the L1 stage up until the time that the cells divide in the mid-L3 stage. After division of the VPCs, LIN-25 is detected only in vulval cell lineages, *i.e.* in the descendants of P5.p, P6.p and P7.p. It is not detected in cells descended from P3.p, P4.p or P8.p. At all stages, LIN-25 is most clearly visible in the nuclei of positively stained cells but appreciable cytoplasmic staining is also present. Given the fact that the worms stained expressed appreciably higher levels of LIN-25 than wild-type worms, however, it is possible that the subcellular localization of the protein seen in our experiments does not accurately reflect the situation in wild type.

We have also obtained evidence that the VPCs are a major focus for *lin-25* activity. We have shown that *lin-25* is required in the descendants of the embryonic blastomere ABp for correct VPC fate specification and that vulval development occurs normally in mosaic hermaphrodites lacking *lin-25* activity in the descendants of the early blastomere ABa or P$_1$. Furthermore, we have shown that expression of LIN-25 under the control of *lin-12* regulatory sequences is sufficient to rescue the *lin-25* vulval lineage defect. These results together suggest a major focus for *lin-25* activity in vulval induction within the VPCs.

It also appears that *lin-25* activity in other cells can influence VPC fate. Specifically when *lin-25* activity is absent only from the descendants of ABp the VPC lineages were less frequently aberrant than those in homozygous *lin-25* mutant hermaphrodites derived from heterozygous parents. This observation implies that genotypically wild-type cells descended from ABa or P$_1$ (or both) can influence the fates of VPCs that lack *lin-25* activity. However, this secondary focus seems likely to be relatively inconsequential under normal circumstances, since the VPC lineages are always normal when *lin-25* activity is present in ABp descendants.

LIN-25 and SUR-2 may function together in VPC fate specification

The *sur-2* gene, which encodes another novel protein, also appears to function downstream of *let-60* Ras, and the *lin-25* and *sur-2* mutant phenotypes are strikingly similar (Singh and Han, 1995). These similarities led us to investigate the relationship between the two genes. We found that the penetrance and expressivity of the phenotype of hermaphrodites homozygous for null mutations in both genes

were the same as those of hermaphrodites mutant for either gene alone. Furthermore we found that overexpressing LIN-25 does not bypass the need for *sur-2*. These observations imply that *lin-25* and *sur-2* are not partially redundant with one another but suggest instead that *sur-2* and *lin-25* function in the same process. Combined with previous genetic studies (Singh and Han, 1995; Tuck and Greenwald, 1995), the results suggest that *lin-25* and *sur-2* function on the same branch of the genetic pathway downstream of *let-60 ras*.

We have also found that SUR-2 appears to be required for the translation or stability of LIN-25. One possibility is that LIN-25 and SUR-2 function together in a physical complex and that the instability of LIN-25 in a *sur-2* mutant background results from the absence of a component of the complex. Attempts to date, however, to demonstrate binding between LIN-25 and SUR-2 have not been successful.

Since both LIN-25 and SUR-2 are novel proteins with no recognizable amino acid sequence motifs, it is a challenge to elucidate the molecular mechanism by which they act during VPC fate specification. Ras-MAP kinase pathways have been studied in several different systems. In many cases, downstream genes have been found to function as transcription factors that are required for expression of specific genes in response to the signal (O'Neill et al., 1994). It is possible that LIN-25 and SUR-2 function in this way. Another possibility is that LIN-25 and SUR-2 function as scaffold proteins. In the signal transduction pathway mediating pheromone response in *Saccaromyces cerevisiae*, STE5 is required to sequester several components of the pathway together into one complex (Choi et al., 1994).

LIN-25 and cell fusion

We have found that overexpression of LIN-25 prevents cell fusion during differentiation of the lateral hypodermis. Although we did not detect defects in the ventral hypodermis (Pn.p cells and their descendants), the lateral hypodermal cell fusion defects may be a valuable clue in determining how *lin-25* might function during vulval development.

One possibility suggested by the lateral hypodermal cell fusion defect is that LIN-25 is required in the VPCs to counteract the inhibitory signal from hyp7. Very little is known at the present time about how hyp7 signals to the VPCs yet one proposal is that hyp7 acts by promoting fusion with the VPCs and their descendants (Herman and Hedgecock, 1990). We have found that high levels of LIN-25 appear to cause some lateral hypodermal cells that normally fuse with hyp7 to remain unfused and, later in development, to prevent the fusion of the seam cells with one another. These observations suggest that *lin-25* might play a role in preventing hypodermal cell fusions during development and that the defect in VPC fate specification seen in *lin-25* mutant animals might be the result of the failure of VPCs and their descendants to resist fusion. Overexpression of LIN-25 does not cause the VPCs or their descendants to remain separate from hyp7. It is possible, however, that in VPC fate specification only cells that have been activated by the inductive or lateral signalling pathways have all the necessary factors to resist fusion.

It is noteworthy that, in hermaphrodites lacking *lin-25* or *sur-2*, the VPCs sometimes fuse directly with hyp7 without dividing. Premature fusion of the VPCs also occurs in hermaphrodites expressing reduced levels of the HOX protein, LIN-39. LIN-39 protein levels are normal, however, in *lin-25* mutants suggesting that premature fusion of the VPCs in *lin-25* mutants is not a result of a failure to express wild-type levels of LIN-39.

Premature fusion is not seen in hermaphrodites homozygous for partial reduction of function mutations in *let-60 ras* or in other genes encoding the components of the upstream signalling pathway. However, it is not known whether premature fusion occurs in hermaphrodites entirely lacking *let-60 ras* in the VPCs. A hypermorphic allele of *let-60 ras*, *n1046*, partially suppresses the premature fusion defect in *lin-25* mutants (Tuck and Greenwald, 1995) as do null alleles of *lin-1*, which encodes a target of the induction pathway (Jacobs et al, 1998; S. T. and I. G. unpublished). These results suggest a role for the Ras pathway in preventing cell fusion and it will be interesting to learn more about this as yet unexplored role.

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