LIN-12 protein expression and localization during vulval development in *C. elegans*

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SUMMARY

We have used a LIN-12::GFP fusion protein to examine LIN-12 accumulation during cell fate decisions important for vulval development. During the naturally variable anchor cell (AC)/ventral uterine precursor cell (VU) decision of the somatic gonad, a transcription-based feedback mechanism biases two equivalent cells so that one becomes the AC while the other becomes a VU. LIN-12::GFP accumulation reflects lin-12 transcription: LIN-12::GFP is initially present in both cells, but disappears from the presumptive AC and becomes restricted to the presumptive VU. During vulval precursor cell (VPC) fate determination, six equipotential cells uniformly transcribe lin-12 and have invariant fates that are specified by multiple cell-cell interactions. The pattern of LIN-12::GFP accumulation in VPCs and in the VPC lineages is dynamic and does not always reflect lin-12 transcription. In particular, LIN-12::GFP is expressed initially in all six VPCs, but appears to be reduced specifically in P6.p as a consequence of the activation of the Ras pathway by an EGF-like inductive signal from the AC. We propose that downregulation of LIN-12 stability or translation in response to inductive signalling helps impose a bias on lateral signalling and contributes to the invariant pattern of VPC fates.

Key words: LIN-12, Notch, Cell-cell interactions, *C. elegans*, Vulva, EGF, Ras

INTRODUCTION

The LIN-12 protein is a member of the LIN-12/Notch family of receptors, which mediate cell-cell interactions that specify cell fate during animal development. Genetic studies have established that the lin-12 gene is involved in many different cell fate decisions during *C. elegans* development (Greenwald et al., 1983; Lambie and Kimble, 1991; Newman et al., 1995). In this manuscript, we focus on two decisions that are important for vulval development. One of these is the ‘Anchor Cell (AC)/Ventral Uterine precursor cell (VU)’ decision, which is a simple decision involving cell-cell interactions between two cells. The other is ‘Vulval Precursor Cell (VPC) specification’, which is a more complicated decision involving at least three different cell-cell interactions among multiple tissues.

Much of our understanding of the role of lin-12 in cell fate decisions has come from studies of the AC/VU decision. Two cells of the hermaphrodite gonad, named Z1.ppp and Z4.aaa, are initially equivalent in their developmental potential, in that each has an equal chance of becoming the AC or a VU. However, in any given hermaphrodite, only one of these cells will become the AC, while the other becomes a VU (Kimble and Hirsh, 1979). The AC is a differentiated cell that is required for vulval development (see below). The VU gives rise to descendant cells that form part of the ventral uterus.

The AC/VU decision depends on lin-12-mediated interactions between Z1.ppp and Z4.aaa (Kimble, 1981; Greenwald et al., 1983; Seydoux and Greenwald, 1989). Elevating lin-12 activity causes both Z1.ppp and Z4.aaa to become VUs (the ‘0 AC defect’), while reducing lin-12 activity causes both Z1.ppp and Z4.aaa to become ACs (the ‘2 AC defect’). Initially both Z1.ppp and Z4.aaa express lin-12 and lag-2, which encodes a ligand for LIN-12 (Henderson et al., 1994; Tax et al., 1994). The AC/VU decision is determined after a stochastic small variation in ligand and/or receptor activity becomes amplified by a feedback mechanism that influences lin-12 and lag-2 transcription (Seydoux and Greenwald, 1989; Wilkinson et al., 1994).

lin-12 also plays a critical role in the specification of the fates of the vulvar precursor cells (VPCs). VPC specification involves at least three different intercellular signalling events, and thus offers the opportunity to study how other signalling events are integrated with lin-12 signalling.

The VPCs are six cells, consecutively numbered P3.p-P8.p. Each of these cells has the potential to adopt one of three fates, termed 1°, 2° and 3° (Sulston and White, 1980; Sternberg and Horvitz, 1986). Each of these fates is a discrete cell lineage, and an important distinction among these lineages is the...
terminal fates of cells: the 1° and 2° fates lead to the production of vulval cells, while the 3° fate leads to the production of two cells that join the hyp7 syncytium (Sulston and Horvitz, 1977). Normally, P6.p adopts the 1° fate, P5.p and P7.p adopt the 2° fate, and P3.p, P4.p and P8.p adopt the 3° fate.

The pattern of VPC fates reflects at least three different signalling events, and genetic studies have identified many components that mediate these different interactions (reviewed by Greenwald, 1997). One such event is an inductive signal produced by the anchor cell (AC) of the somatic gonad. This signal, LIN-3, is an EGF-like ligand that activates LET-23, a receptor tyrosine kinase related to the EGF receptor, and a Ras-mediated signal transduction cascade (Aroian et al., 1990; Han and Sternberg, 1990; Hill and Sternberg, 1992). During normal development, the main outcome of the inductive signalling event is for P6.p to adopt the 1° fate. The principle focus of let-23 activity appears to be in P6.p (Koga and Ohshima, 1995; Simske and Kim, 1995), although it has been proposed that LIN-3 is produced in a spatially graded manner and hence may also activate LET-23 in other VPCs, particularly P5.p and P7.p (Katz et al., 1995).

Another signalling event is mediated by LIN-12, a receptor of the LIN-12/Notch family. lin-12 activity is required for a VPC to adopt the 2° fate (Greenwald et al., 1983). One of the consequences of LET-23 activation appears to be the expression or activation of a ligand that activates LIN-12 (Koga and Ohshima, 1995; Simske and Kim, 1995). However, while normally LET-23 must be activated in P6.p to enable P5.p and P7.p to adopt the 2° fate, the constitutive activation of LIN-12 can bypass this requirement for inductive signalling (Greenwald et al., 1983; Levitan and Greenwald, 1995). lin-12 appears to be expressed uniformly in all six VPCs, suggesting that a feedback mechanism involving transcriptional regulation (as described above for the AC/VU decision) may not occur during VPC specification (Wilkinson and Greenwald, 1995).

The third signalling event has been inferred from genetic mosaic analysis: certain genes involved in VPC specification appear to have a focus in hyp7, the major body wall epidermis (Herman and Hedgecock, 1990; Hedgecock and Herman, 1995). This observation, along with the phenotype of this group of mutants, has led to the inference that a signal from hyp7 normally inhibits all VPCs from adopting vulval fates (which is formally equivalent to promoting the 3° fate). Thus, the LET-23-mediated inductive signalling and LIN-12-mediated lateral signalling events may be viewed as overcoming this general inhibition.

Here, we study the patterns of LIN-12 protein accumulation during the AC/VU decision and VPC specification. For the AC/VU decision, we find that the pattern of LIN-12 protein accumulation follows the pattern of lin-12 mRNA expression. However, the patterns of lin-12 mRNA expression and LIN-12 protein accumulation in the VPCs suggest that LIN-12 protein accumulation is negatively regulated by the inductive signalling pathway. This new aspect of lin-12 regulation during vulval development suggests a mechanism by which some LIN-12/Notch signalling events may be biased and how signalling inputs from receptor tyrosine kinase pathways and LIN-12/Notch pathways might be integrated.

**MATERIALS AND METHODS**

**Genetics**

Standard methods for handling and genetic manipulation of *C. elegans* are described by Brenner (1974). Unless otherwise indicated, experiments were performed at 20°C. Mutations used in this study are listed below, with the exception of balancing marker mutations used during constructions.

- LGH: lin-7(e1413) (Ferguson and Horvitz, 1985).
- LGH: unc-52(e189) (Brenner, 1974), lin-12(n137) and lin-12(n137/n720) (Greenwald et al., 1983), qC1 (Austin and Kimble, 1989).
- LGH: let-60(n1046) (Beitel et al., 1990; Han et al., 1990), lin-3(n378) (Hill and Sternberg, 1992).
- LGH: him-5(e1490) (Hodgkin et al., 1979).
- LGX: lin-2(e1309) (Ferguson and Horvitz, 1985).

**Construction of LIN-12::GFP**

A plasmid encoding LIN-12::GFP was constructed as follows. The starting plasmid was pLB30, a plasmid containing the entire lin-12 genomic region with a deletion of a small Xhol fragment (from nucleotides 13640-13694) (G. Struhl, personal communication). pLB30 fully rescues a lin-12(null) mutant (A. Meléndez and I. G., unpublished data). GFP65ST was PCR amplified from plasmid KS-GFP65ST with primers SalGFPr 5¢ TGCTAAGCGCTCGACC-CATGATAAAGGAAAGACTTTC 3¢ and SalGFPr 5¢ GATCCTCGTCACTTTTGTATAGTTCATCCATGCA 3¢. The PCR product was cut with Sall and ligated to Xhol digested pLin12-Eco109, which contains pLB30 sequences lacking an Eco109I fragment comprising nucleotides 5270-1176. The resulting plasmid is called pLin12-Eco109::GFP and contains GFP inserted at the Xhol site at position 13640. This plasmid was digested with Eco109I and ligated to the Eco109I fragment comprising nucleotides 5270-1176 from pLB30 to generate the final plasmid pLin12::GFP, which encodes LIN-12::GFP.

pLin12::GFP was injected at 5 µg/ml along with pRF4 [rol-6(su1006)] (Mello et al., 1991) at 100 µg/ml into the strain unc-32(e189) lin-12(n137 n720)/qC1; him-5(e1490). F1 Rollers were picked and three stable lines established. To assay for rescue of the lin-12(null) phenotype, Rol Unc hermaphrodites were picked and examined for fertility and vulva defects. All three transgenic lines exhibit significant phenotypes associated with gain-of-function lin-12 alleles, suggesting these arrays do not result in overly high levels of lin-12 activity (data not shown).

Only one line resulted in visible GFP expression in live animals. The extrachromosomal array from this line (arEx151) was crossed into a wild-type background and then integrated by treatment with X-rays (Mello and Fire, 1995) to generate the strain arls41[lin-12::GFP], arls41 was used for all experiments described in this paper. Most arls41 hermaphrodites are wild-type: a small proportion of hermaphrodites contain arrays expressing LIN-12(+) (Fitzgerald et al., 1993).
Hirsh, 1986) diluted 1:1000 in PTB (1× PBS, 1% BSA, 0.5% Triton X-100), overnight at 4°C. Worms were washed with 1× PBS, 0.1% BSA, 0.5% Triton X-100 with several changes of buffer for 4 hours at room temperature. Cy3-conjugated goat anti-rabbit secondary antibody and FITC-conjugated goat anti-mouse secondary antibody (both from Jackson Immunoresearch) were diluted 1:300 in PTB and incubated with the fixed worms overnight at 4°C. Worms were washed as before, for 4 hours at room temperature. DAPI was added to the last wash at a final concentration of 1 μg/ml. Worms were mounted on a 2% agarose pad with 3 μl of 10% N-propyl gallate and viewed with a Zeiss LSM 410 laser scanning confocal attachment on a Zeiss Axiovert 100 microscope.

RESULTS

Visualization of the LIN-12 protein during cell fate decisions

In order to visualize LIN-12 protein, we tagged LIN-12 with green fluorescent protein (GFP) (Chalfie et al., 1994). We inserted GFP in frame into the intracellular domain of LIN-12 (Fig. 1) and generated transgenic lines containing extrachromosomal arrays and integrated arrays composed of the lin-12::GFP reporter construct and a transformation marker (see Materials and Methods). All transgenic arrays provided sufficient activity to rescue a lin-12(0) mutation, and transgenes encoding LIN-12::GFP behaved similarly to transgenes encoding LIN-12(+) (see Materials and Methods). However, in some cases the amount of fluorescence from GFP was too low to visualize in living animals; even when GFP fluorescence was visible, however, we found that using an anti-GFP antibody improved the reliability of scoring (see Materials and Methods). We therefore used the arIs41[lin-12::GFP] integrated array and an anti-GFP antibody to heighten the sensitivity of the assay in the experiments described below.

LIN-12::GFP accumulation during the AC/VU decision

A lin-12::lacZ reporter gene for lin-12 transcription is initially expressed in both Z1.ppp and Z4.aaa, and during the AC/VU decision, expression of the reporter gene becomes restricted to either Z1.ppp or Z4.aaa, with expression present in the presumptive VU and absent from the presumptive AC (Wilkinson et al., 1994). We have found that the LIN-12::GFP protein is initially detectable in both Z1.ppp and Z4.aaa, and later in development becomes detectable in the presumptive VU but not in the presumptive AC (Fig. 2). (We could not attempt to determine if the level of LIN-12::GFP accumulation is greater in the presumptive VU than it is initially in Z1.ppp or Z4.aaa.) This pattern of protein accumulation thus reflects the pattern of gene expression (Fig. 3), and is evidence that LIN-12 protein is turned over relatively rapidly during the AC/VU decision.

LIN-12::GFP accumulation during VPC specification

The level of lin-12::lacZ expression from the early L2 stage until the VPCs divide in the L3 stage appears to be uniform in all six VPCs (Wilkinson and Greenwald, 1995). In several different cell types, the modified β-gal reporter protein appears to be unstable and hence not to perdure to a large extent (e.g., Way and Chalfie, 1989; Evans et al., 1994; Wilkinson et al., 1994). Thus, the pattern of lin-12::lacZ expression suggests that lin-12 is continuously transcribed in all six VPCs (Wilkinson and Greenwald, 1995).

In contrast, we have found that the pattern of LIN-12::GFP protein accumulation is not coincident with the pattern of lin-12::lacZ expression during VPC specification. The LIN-12::GFP protein is initially detectable in all six VPCs. However, in the mid-L3 stage, at the time of VPC specification, LIN-12::GFP is reduced in P6.p relative to the other VPCs,
notably with respect to P5.p and P7.p (Fig. 4): 38/41 (93%) arIs41 hermaphrodites displayed reduced fluorescence in P6.p. The proportion of hermaphrodites displaying reduced fluorescence in P6.p is similar to the proportion (91%) of arIs41 hermaphrodites that have an AC (a small proportion of arIs41 hermaphrodites appear to lack an AC; see Materials and Methods).

Because transgenes expressing LIN-12::GFP behave similarly to transgenes expressing LIN-12(+) in rescue assays, it is likely that LIN-12::GFP is regulated like the endogenous LIN-12 protein, so that LIN-12 levels are reduced in P6.p. As described above, the uniform expression of the lin-12::lacZ reporter is likely to reflect uniform lin-12 transcription in the VPCs. Taken together, these results suggest that the level of LIN-12 protein is specifically downregulated in P6.p in response to the inductive signal from the AC. The observation that there is less LIN-12 protein in P6.p than in other VPCs at the time of lateral signalling may be important for VPC fate patterning, as described in the Discussion.

LIN-12::GFP accumulation in the VPC lineages

The patterns of lin-12::lacZ and LIN-12 expression in the VPC lineages is summarized in Fig. 5. lin-12::lacZ expression is evident in all twelve daughters of the VPCs (Pn.px stage), and then becomes restricted to P5.ppa, P5.ppp, P7.paa and P7.pap (Wilkinson and Greenwald, 1995; see Fig. 5). High LIN-12::GFP accumulation is always seen in the daughters of P5.p and P7.p, but LIN-12::GFP accumulation is not seen in the daughters of P6.p, again contrasting with the pattern of lin-12::lacZ expression. There is variable accumulation of LIN-12::GFP in the daughters of P3.p, P4.p and P8.p. We think this variability reflects the absence of a strict regulation of the level of LIN-12 accumulation in P3.p, P4.p and P8.p, because we have also observed hermaphrodites in which P3.p, P4.p and P8.p also exhibit reduced LIN-12::GFP accumulation relative to P3.p and P7.p (although the level is never as low as in P6.p).

At the next step in the lineage, in the VPC granddaughters, the pattern of protein accumulation and gene expression become coincident in all hermaphrodites, with LIN-12::GFP accumulation detectable only in P5.ppa, P5.ppp, P7.paa and P7.pap.

The execution of the VPC lineages is not known to require additional signalling events, in that ablation of selected VPC daughter cells does not affect the fate of other VPC daughter cells (e.g., Sternberg and Horvitz, 1986). It is therefore curious that the patterns of lin-12 gene and protein expression appear to be dynamic in the VPC lineages, with continued expression restricted to certain cells generated from VPCs that adopt the 2° fate. In this context, it is interesting to note that the pattern of expression of EGL-17, an FGF-like ligand (Burdine et al., 1997), also appears to be dynamic in the VPC lineages (Burdine et al., 1998). Perhaps these signalling proteins are involved in cell-cell interactions that refine subtle aspects of terminal differentiation of vulval cells or coordinate events of vulval morphogenesis.

LIN-12::GFP downregulation in P6.p depends on inductive signalling

The inductive signal is encoded by lin-3, and reducing lin-3 activity causes a vulvaless phenotype (Hill and Sternberg, 1992). LIN-3 activates a receptor tyrosine kinase and Ras-mediated signal transduction cascade (reviewed by Greenwald, 1997). If LIN-12 is downregulated in response to the inductive signalling pathway, then in a lin-3 mutant, LIN-12::GFP should persist in P6.p. Indeed, this prediction is borne out (Fig. 6): in 19/21 (91%) of lin-3(n378) hermaphrodites, LIN-12::GFP accumulation was not downregulated in P6.p, a proportion which is consistent with the 90% penetrance of Egl defect caused by this allele (Ferguson and Horvitz, 1985). This result suggests that inductive signalling is necessary for a decrease in the synthesis or stability of LIN-12 protein in P6.p.

We also obtained evidence that activating the inductive signalling pathway is sufficient to downregulate LIN-12::GFP accumulation. In principle, if this cascade is activated in all VPCs, an alternating pattern of 1° and 2° fates would be predicted, based on a combination of inductive and lateral signalling (see Sternberg, 1988). In practice, however, mutations that activate the inductive signalling pathway, either directly or through reducing inhibitory signalling, have variable phenotypes suggesting that VPCs often adopt fates with hybrid character (Ferguson et al., 1987). When we examined LIN-12::GFP accumulation in let-60(n1046), a weakly activating Ras gain-of-function mutation (Beitel et al., 1990; Han et al., 1990), we observed that about 10% of hermaphrodites (5/50) display an alternating pattern of staining and non-staining VPCs (Fig. 6). The proportion of hermaphrodites displaying this phenotype is consistent with available lineage and reporter gene information: in many let-60(n1046) hermaphrodites, P3.p and P8.p are not induced to undergo vulval fates and many of the ectopic vulval divisions are hybrid in nature, so that most hermaphrodites do not exhibit the alternating pattern of 1° and 2° fates expected if the inductive signalling pathway were fully induced (Ferguson et al., 1987; Tuck and Greenwald, 1995; Burdine et al., 1998; D. L. and I. G., unpublished observations). We therefore interpret these results as supporting the hypothesis that ectopic activation of the inductive signalling pathway may be sufficient to cause reduced LIN-12::GFP accumulation.
LIN-12::GFP accumulation in a lin-12(d) mutant

In lin-12(n137), LIN-12 is constitutively active, and all six VPCS adopt 2° fates (Greenwald et al., 1983; Greenwald and Seydoux, 1990). We examined the pattern of LIN-12::GFP accumulation in lin-12(n137) in order to verify that LIN-12::GFP accumulates whenever a VPC adopts the 2° fate. The results we obtained were consistent with this expectation: all six VPCs express LIN-12::GFP throughout the L3 and continue to express LIN-12::GFP after one round of cell division (at the Pn.px stage), just as P5.p and P7.p do in a lin-12(+) background (Fig. 6). We were unable to perform the reciprocal experiment, examining LIN-12::GFP in the absence of lin-12 activity, because the LIN-12::GFP protein has rescuing activity.

We did not see any evidence that the intensity of LIN-12::GFP was increased in the VPCs either in living or stained hermaphrodites (Fig. 6 and data not shown). Furthermore, in a lin-12(+) background, P5.p and P7.p, the cells in which LIN-12 is activated, do not accumulate more LIN-12::GFP than P3.p, P4.p and P8.p, cells in which LIN-12 is likely not to be

**Fig. 4.** Confocal images of the VPCs and their descendants from L3 wild-type hermaphrodites. At the L3 stage, animals were fixed and stained with anti-GFP antibody to visualize LIN-12::GFP and with MH27, to visualize tight junctions which outline the VPCs and counterstained with DAPI, to visualize all nuclei (data not shown). (A,C,E,G,I,K) Anti-GFP staining pattern. (B,D,F,H,J,L) MH27 staining pattern. All images are ventral views except E and F which are lateral views; in all images anterior is to the left. (A,B) Pn.p stage, early L3. White arrows point to P6.p. (C,D) Pn.p stage, mid L3. White arrows point to P6.p. (E) Lateral view, Pn.p stage, mid L3. White arrows point to P6.p. In E, g is above the gonadal staining. (G,H) Pn.p stage, mid L3. 3x zoom. Left arrow points to P6.p, right arrow points to P7.p. (I,J) Pn.px stage. All Pn.ps have divided once; P3.p has already joined the hypodermal syncytium hyp7 so it is no longer outlined by the MH27 antibody. Outlined arrows point to progeny of P5.p and P7.p. (K,L) Pn.pxx stage. P5.p-P7.p have divided twice. P3.p, P4.p and P8.p descendants have joined the hypodermal syncytium hyp7 and are no longer outlined by the MH27 antibody. Arrowheads point to the progeny of P5.p and P7.p.
variable in P3.px, P4.px and P8.px (see text). Greenwald (1995). Note that accumulation of LIN-12::GFP is lin-12::lacZ hermaphrodites. Both expression patterns are dynamic but are not 12::GFP expression patterns in the VPCs and VPC lineages in L3 contrasts with the basolateral localization of LET-23::GFP (Hoskins et al., 1991; Gho et al., 1996; Sherwood and McClay, 1997; Zhong et al., 1997). In cells where LIN-12/Notch proteins have a Notch has been described as either apical, basolateral or reticulum/Golgi, and at the ventral (apical) surface and at the junctions between VPCs (Figs 4, 6). In a variety of systems, Notch has been described as either apical, basolateral or distributed around the entire membrane (e.g., Fehon et al., 1991; Gho et al., 1996; Sherwood and McClay, 1997; Zhong et al., 1997). In cells where LIN-12/Notch proteins have a restricted subcellular distribution, it has not been determined if the specific subcellular locale is important for function.

The apical localization of LIN-12::GFP in the VPCs contrasts with the basolateral localization of LET-23::GFP (Simsek et al., 1996). Mutations in lin-2 and lin-7, which encode members of the MAGUK family of cell junction proteins, appear to reduce inductive signalling and to disrupt the basolateral localization of LET-23::GFP (Hoskins et al., 1996; Simsek et al., 1996). However, mutations in lin-2 and lin-7 appear to have no effect on the apical localization of LIN-12::GFP (data not shown).

**DISCUSSION**

In this paper, we have examined the patterns of LIN-12::GFP protein accumulation during two cell fate decisions, the AC/VU decision and VPC specification. We have found that the pattern of LIN-12::GFP accumulation reflects the pattern of lin-12 reporter gene transcription during the AC/VU decision. In contrast, the pattern of LIN-12::GFP accumulation during VPC specification does not reflect the pattern of lin-12 reporter gene expression. Instead, LIN-12::GFP accumulation appears to be reduced in P6.p response to an inductive signalling event. The difference in the behavior of the patterns of LIN-12::GFP accumulation during these two cell fate decisions is interesting in terms of strategies used to ensure the outcomes of LIN-12/Notch-mediated decisions.

**The AC/VU decision**

In the AC/VU decision, two gonadal cells, Z1.ppp and Z4.aaa, interact with each other so that one becomes an AC and the other becomes a VU (Kimble, 1981, Seydoux and Greenwald, 1989). Both cells initially express lin-12 and lag-2, a gene encoding a LIN-12 ligand. During the AC/VU decision, a feedback mechanism amplifies a small stochastic initial difference in lin-12 activity between Z1.ppp and Z4.aaa; this feedback mechanism involves differential transcription of lin-12 (in the presumptive VU) and lag-2 (in the presumptive AC) (Seydoux and Greenwald, 1989; Wilkinson et al., 1994). The pattern of protein accumulation we see with LIN-12::GFP during the AC/VU decision appears to reflect this transcriptional regulation: LIN-12::GFP accumulates in the presumptive VU, and is absent from the presumptive AC.

The pattern of LIN-12::GFP accumulation also indicates that LIN-12 is relatively unstable during the AC/VU decision, since otherwise we might have observed perdurance of LIN-12::GFP from the initial expression of lin-12 in both Z1.ppp and Z4.aaa. It has been argued elsewhere that rapid turnover of LIN-12/Notch proteins may be important for cell-cell interactions between equipotential cells (Grant and Greenwald, 1997; Hubbard et al., 1997). If LIN-12 were not rapidly turned over, perdurance of signalling from the activated receptor would have the effect of desensitizing the system to differences that arise during the decision-making process. For the AC/VU decision, perdurance of signalling would mask the effects of differential transcription and effectively undermine the mechanism that ensures that only one cell becomes an AC.

**VPC specification**

VPC specification is much more complicated than the AC/VU decision. It involves at least three separate signalling events so that the VPCs are appropriately patterned [P3.p (3°)-P4.p (3°)-P5.p (2°)-P6.p (1°)-P7.p (2°)-P8.p (3°)]. The three signalling events have been termed inductive signalling, lateral signalling, and inhibitory signalling (reviewed by Greenwald, 1997). Most of the work on VPC specification has focused on inductive signalling from the AC, which produces an EGF-like ligand, LIN-3, that activates the LET-23 receptor tyrosine kinase/LET-60 Ras pathway (Han and Sternberg, 1990; Aroian et al., 1990; Hill and Sternberg, 1992). It is not clear if the major patterning influence of LIN-3 depends on its spatial gradation, so that P5.p, P6.p and P7.p all receive some input (Sternberg and Horvitz, 1986; Katz et al., 1995), or if LIN-3 principally activates LET-23 only in P6.p (Koga and Ohshima, 1995; Simsek and Kim, 1995). Inhibitory signalling appears to emanate from the hyp7 hypodermal syncytium (Herman and Hedgecock, 1990; Hedgecock and Herman, 1995), and is believed to result in a general inhibition of VPCs from adopting vulval fates. One role of the inductive signal may be to overcome the effect of inhibitory signalling.

In this study, we have provided evidence that one output of the LET-60 Ras pathway is to influence LIN-12 accumulation. In wild-type hermaphrodites, LIN-12::GFP accumulation is reduced in P6.p at the time of vulval induction. In a lin-3 mutant, which has a reduced a level of the inductive signal (Hill...

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**Fig. 5.** Schematic representation of the lin-12::lacZ and LIN-12::GFP expression patterns in the VPCs and VPC lineages in L3 hermaphrodites. Both expression patterns are dynamic but are not identical. The lin-12::lacZ expression pattern is from Wilkinson and Greenwald (1995). Note that accumulation of LIN-12::GFP is variable in P3.px, P4.px and P8.px (see text).
and Sternberg, 1992), LIN-12::GFP accumulates to the same extent in P6.p as it does in other VPCs. Furthermore, when let-60 Ras is activated, and VPCs other than P6.p are induced (Beitel et al., 1990; Han et al., 1990), we observe reduced LIN-12::GFP in VPCs other than P6.p. As lin-12 transcription appears to be uniform in all six VPCs (Wilkinson and Greenwald, 1995), these observations suggest that one output of the LET-23/Ras pathway is to decrease translation or stability of LIN-12.

Assessing LIN-12::GFP accumulation may be a useful marker for the 1° fate. Currently, the only other marker for the 1° fate is expression of egl-17::GFP, a transcriptional fusion that is expressed in P6.p and its descendants until the end of the L3 stage (Burdine et al., 1998). LIN-12::GFP accumulation and egl-17::GFP expression appear to display somewhat different kinetics and perhaps different sensitivities to activation of the inductive signalling pathway (V. Ambros, personal communication). It is useful to have many different markers to assess cell fate transformations in different vulval mutants, since interpreting mutant lineages, and particularly the 1° fate, is sometimes difficult.

**Downregulation as a mechanism to bias LIN-12/Notch signalling**

The observation that LIN-12 appears to be downregulated in P6.p in response to inductive signalling is relevant to a consideration of how lateral signalling contributes to the patterning of VPC fates. Lateral signalling is believed to involve a ligand from one VPC that activates LIN-12 in a neighboring VPC (Sternberg, 1988; reviewed by Greenwald, 1997). VPC specification differs strikingly from the AC/VU decision in that, in wild-type hermaphrodites, there is no randomness: it is not only that one VPC becomes 1° and two become 2°, but it is always P6.p that becomes 1° and P5.p and P7.p that become 2°. Thus, if lateral signalling involves lin-12-mediated ligand-receptor interactions among VPCs, then something appears to be biasing the outcome.

In *Drosophila*, two mechanisms have been identified that bias LIN-12/Notch-mediated decisions. In one mechanism, asymmetric segregation of the modulating factor Numb to one of two daughters appears to bias some Notch-mediated decisions: in certain neural lineages, two cells are developmentally equivalent, but one cell inherits Numb and adopts the fate associated with the absence of Notch activity (Jan and Jan, 1995; Spana and Doe, 1995). Association with Numb may reduce the ability of Notch to signal (Guo et al., 1996). In another mechanism, differential expression of the modulating factor fringe influences the response of cells to a Notch ligand: during formation of the wing margin, cells of the dorsal compartment express fringe and have a greater response to a particular Notch ligand than cells of the ventral compartment, which do not express fringe (reviewed by Irvine and Vogt, 1997).

The models for numb-mediated and fringe-mediated biases are not simply applicable to VPC specification. The VPCs are not sister cells, so a simple asymmetric segregation of a Numb-like factor is not a possible mechanism. Furthermore, the VPCs are equivalent (Sulston and White, 1980; Sternberg and Horvitz, 1986), and hence do not appear to have an intrinsic...
difference comparable to the compartment distinction that governs fringe expression. While there might be more complicated ways that proteins such as Numb or Fringe might be regulated or utilized, it seems likely that the mechanism for imposing a bias on lin-12 activity during VPC specification will be different.

We propose that downregulation of LIN-12::GFP translation or stability in P6.p is at least one component of the mechanism by which inductive signalling from the AC imposes a bias on lateral signalling among the VPCs. We have shown that LIN-12 accumulation in the VPCs is modulated by inductive signalling: downregulation of LIN-12::GFP does not occur in a mutant that lacks the inductive signal, and appears to occur ectopically in a mutant in which the inductive signalling pathway is constitutively active. V. Ambros (personal communication) has shown that the response to inductive signal occurs in the G1 phase of the VPC cell cycle, whereas commitment to the 2° fate in response to the lateral signal occurs in the G2 phase of the VPC cell cycle. Thus, at the time when lateral signalling is relevant, LIN-12 is likely to be absent from P6.p, or at least greatly reduced.

Whether or not the inductive signal is spatially graded, it is clear that reception of the inductive signal is an important influence on lin-12-mediated lateral signalling. In genetic mosaics for let-23, the receptor for the inductive signal, a let-23(–) VPC adopts the 2° fate when adjacent to a let-23(+) VPC (Koga and Ohshima, 1995; Simske and Kim, 1995). This observation suggests that inductive signalling is likely to govern the expression or activity of the ligand for LIN-12. However, the mechanism by which this effect is achieved is unknown.

The observation that LIN-12 downregulation occurs suggests possible roles in VPC patterning. For example, if the graded inductive signal causes the expression or activation of a ligand for LIN-12 in P5.p, P6.p and P7.p, then downregulation of LIN-12 in P6.p might help impose a bias by preventing P6.p from being able to respond to the ligand produced by P5.p and P7.p. If, however, the inductive signal causes expression or activation of a ligand for LIN-12 only in P6.p, then downregulation of LIN-12 in P6.p might help prevent autocrine activation of LIN-12 in P6.p.

An alternative role for downregulation of LIN-12 in P6.p is as a potential mechanism for activation of the ligand for LIN-12. It has been suggested, based on the analysis of genetic mosaics of ligand-dependent gain-of-function Notch alleles, that Delta-Notch interactions within the same cell reduce the availability of Delta to interact with Notch molecules of a neighboring cell, thereby lowering signalling capacity (HeitzeIzler and Simpson, 1993). This same explanation may account for the behavior of certain gain-of-function alleles of lag-2 (Tax et al., 1994, 1997). Furthermore, Delta and Notch have been found to colocalize within transfected tissue culture cells (Fehon et al., 1990). Thus, if interactions between LIN-12 and its ligand in the same cell reduce the ability of the ligand to activate LIN-12 on the surface of a neighboring cell, then destruction of LIN-12 in P6.p might be the mechanism for releasing the ligand from such inhibition, thereby enabling activation of LIN-12 in P5.p and P7.p, causing them to adopt the 2° fate.

There are numerous unknowns that must be illuminated before we achieve an understanding of VPC fate patterning. We do not yet know if LIN-12 downregulation is essential for proper VPC fate patterning, nor do we know the identity of the ligand for LIN-12 and the cells in which it is expressed. We do not have a clear view of the relative contributions of the various cell signalling events to the final pattern of VPC fates, or how these different signalling inputs are integrated in the VPCs. Nevertheless, the finding that LIN-12 stability or expression is regulated by the LET-23/Ras-mediated inductive signalling pathway suggests a previously underscribed potential level of control that may operate during VPC fate patterning, and perhaps in additional lin-12/Notch-mediated cell fate decisions.

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