Vulval development in *Caenorhabditis elegans*

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Ever since the cell lineage of the nematode Caenorhabditis elegans was shown to be nearly invariant, investigators have tried to understand the mechanisms that control these precise patterns of cell divisions and cell fates. Important insights have come from analyzing the cells that form the hermaphrodite vulva, a specialized region of hypodermis passageway used for egg laying and sperm entry. Early experiments showed that the invariant pattern of vulval cell fates requires highly reproducible intercellular signals. This review describes recent experiments that have begun to characterize molecules that mediate these signals and explore the relationships between different signaling pathways. Many of these molecules and signaling pathways have been conserved during evolution suggesting mechanisms used to establish patterns of cell fates during vulval development have also been conserved.

The nematode *C. elegans* has proved to be extremely useful for investigating how patterns of cell fates are established during animal development because it has a completely defined and largely invariant cell lineage, the development of individual cells can be observed easily in live animals, and it is well suited for genetic and molecular analysis. To understand a particular cell lineage, investigators typically screen for mutations that affect that lineage and then use genetic analysis to explore gene function and molecular analysis to explore mechanisms of gene action. These approaches have been used to investigate the development of the hermaphrodite vulva, a specialized region of hypodermis (the term for *C. elegans* epidermis), and this system has become one of the best-understood examples of how patterns of cell fates are established during animal development. The vulva is well suited for these approaches because it is a relatively simple structure formed by a small number of cells, and the vulva is not essential for viability or fertility of self-fertilizing hermaphrodites, thus, mutant animals with severe defects in vulval development can be identified and propagated.

The vulva itself is formed by descendants of three cells, P5.p, P6.p and P7.p. These three cells are part of a group of six hypodermal precursor cells, P3.p–P8.p, that lie ventrally along the anterior–posterior axis[1] (Fig. 1a). P6.p adopts the primary (1°) vulval cell fate, which is to generate eight descendants that form vulval tissue. P5.p and P7.p each adopt the secondary (2°) vulval cell fate, which is to generate seven descendants that form vulval tissue. The 22 descendants of P5.p, P6.p and P7.p undergo a series of morphogenic movements creating an invagination that connects the gonad interior and the external environment. P3.p, P4.p and P8.p each adopt the tertiary (3°) nonvulval cell fate, which is to generate two descendants that fuse to a multinucleate hypodermal cell named hyp7 (Fig. 1b). Although the fates of P3.p–P8.p are normally invariant, a variety of genetic and cell-ablation experiments revealed that each cell can adopt the 1°, 2° or 3° fate, suggesting these cells have approximately equivalent developmental potential (reviewed in Ref. 2). Thus, these six cells are called the vulval equivalence group or vulval precursor cells (VPCs).

H.R. Horvitz and coworkers conceptually divided vulval development into three temporal phases and identified genes involved in each phase[3]. First, VPCs are generated and positioned along the anterior–posterior axis. Second, each VPC is determined to adopt the 1°, 2° or 3° fate. Third, VPCs express these fates by undergoing a characteristic series of divisions that generate descendants that differentiate as vulval or hypodermal tissue. The first and third phases are complex, multistep processes that remain largely unexplored and will not be considered further. Studies of the second phase have begun to reveal how intercellular signals establish patterns of cell fates during development. Three intercellular signals are known to affect the fates of VPCs (reviewed in Ref. 2). A cell in the somatic gonad, called the anchor cell, signals P6.p to adopt the 1° fate by activating a receptor tyrosine kinase-RAS–mitogen activated protein (MAP) kinase pathway; this signal overcomes inhibitory signals from hyp7. P6.p then signals P5.p and P7.p to adopt the 2° fate by activating a receptor similar to Notch. Each signal is reviewed below.

The anchor cell signal promotes the 1° vulval cell fate

The anchor cell of the somatic gonad signals P6.p to adopt a 1° vulval cell fate; if the anchor cell is ablated in a live animal, then P5.p, P6.p and P7.p adopt the nonvulval 3° fate, which causes a vulvaless phenotype[2] (Fig. 1c). The primary effect of the anchor cell on P5.p and P7.p appears to be indirect, via a signal mediated by P6.p (discussed below). Thus, an inductive signaling mechanism is used to coordinate the position of the egg-containing gonad, an internal organ, and the hypodermal specialization that transmits those eggs into the environment. Many genes involved in the anchor cell signaling process have been identified and analyzed. These genes can be divided into core molecules of a receptor tyrosine kinase–RAS–MAP kinase signal transduction pathway, transcription factors that might be regulated by this pathway, positive modulators that function upstream or downstream of RAS, and negative modulators (Table 1).

Eight genes, *lin-3, let-23, sem-5, let-341, let-60, lin-45, mek-2/let-537* and *mpk-1/sur-1*, appear to encode core signaling molecules[3–11]. These genes appear to function together in vulval development, as well as early in development, because strong loss-of-function mutations cause larval lethality. Their role in vulval development is revealed by partial loss-of-function mutations; the effects of such mutations vary from a minor defect in the vulval lineages to a completely penetrant vulvaless phenotype like that caused by ablating the anchor cell[3–11]. These observations suggest each of these genes is important for induction of vulval cell fates. Furthermore, overexpression of wild-type *lin-3* or *let-60*, or a mutation that constitutively activates *let-23, let-60*, or *mek-2* can be sufficient to cause all six VPCs to adopt vulval fates[6,9,12,13]; this phenotype is called multivulva (Fig. 1c). Thus, activation of these genes is sufficient to cause a VPC to adopt a vulval fate.

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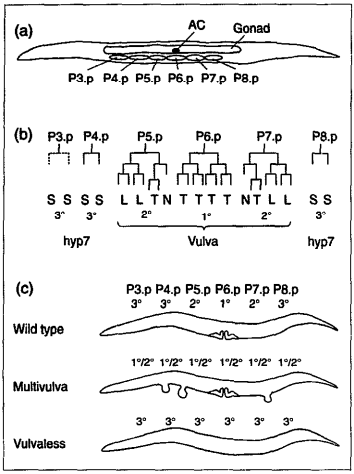

FIGURE 1. The vulval equivalence group. (a) Lateral view of a third larval stage hermaphrodite; anterior is left and ventral below. The anchor cell (AC), part of the somatic gonad, is dorsal to P6.p. (b) Lineages of P3.p–P8.p. Vertical lines represent cells and horizontal lines represent cell divisions. Fates of vulval precursor cells are determined by direct observation of cell divisions in live animals. The 1°, 2° and 3° fates are characterized by the number and properties of descendant cells: S, fuse with hyp7; L, divide longitudinally and adhere to ventral cuticle; T, divide transversely and detach from ventral cuticle; N, no division. Dotted lines indicate P3.p often does not divide. (Adapted from Ref. 1.) (c) Fates of P3.p–P8.p are shown above, and left lateral views of adult hermaphrodites are shown below. The wild-type vulva is depicted as a ventral, mid-body invagination. In multivulva mutants the descendants of P3.p, P4.p and P8.p form protruding 'pseudovulvae' (three are shown); 1° (primary) and 2° (secondary) cell fates typically alternate, and adjacent 1° cells are rarely observed[11]. Vulvaless mutants lack vulval tissue.

Seven of these genes have been cloned, and each is predicted to encode a protein that is highly similar to vertebrate and *Drosophila* proteins that function in receptor tyrosine kinase RAS–MAP kinase signal transduction pathways (reviewed in Ref. 14). The hypothesis that these *C. elegans* proteins have the same mechanism of action as homologous vertebrate proteins is supported by the observations that vertebrate homologs of *sem-5* and *mpk-1* can functionally substitute for the *C. elegans* genes, and SEM-5 and MEK-2 proteins display the predicted biochemical activities[9,11,15]. In addition, the order of gene action predicted by genetic epistasis tests is consistent with the predicted biochemical interactions (see Fig. 2 legend). It is important to note that mutations in each of these eight genes were identified in genetic screens for animals with defects in vulval development and not by targeted approaches based on DNA sequence. The genetic evidence that the molecules encoded by these genes function together *in vivo* has provided a powerful complement to the biochemical evidence that these molecules can interact *in vitro*. Considered together, the evidence from genetic and biochemical experiments provides compelling support for the relevance and mechanism of action of these signaling molecules.

LIN-3, which is similar to epidermal growth factor, is expressed in the anchor cell and is likely to be the anchor cell signal[12,16]. LET-23 is similar to transmembrane receptor tyrosine kinases and is likely to be the receptor for the anchor cell signal[6]. Upon binding LIN-3, LET-23 is likely to dimerize and autophosphorylate. SEM-5 contains one SH2 (SRC homology) domain and two SH3 domains[4]; the SH2 domain is likely to bind phosphorylated LET-23 receptor, and the SH3 domains are likely to bind a guanine-nucleotide exchange factor. A *C. elegans* guanine-nucleotide exchange factor has not been identified. *let-341*, which has not been analyzed molecularly, is a candidate because it appears to function downstream of *lin-3* and upstream of *let-60 ras* (Ref. 5). LET-60 is similar to RAS (Ref. 6). RAS proteins are converted from an inactive, GDP-bound form to an active, GTP-bound form by guanine-nucleotide exchange factors. Activated LET-60 is likely to initiate a cascade of protein kinase activation by binding RAF, a protein serine/threonine kinase that can phosphorylate and, thereby, activate MEK (MAP kinase kinase or ERK kinase), a protein serine/threonine and tyrosine kinase that can phosphorylate and, thereby, activate MAP kinase, a protein serine/threonine kinase (reviewed in Ref. 17). LIN-45 is similar to RAF (Ref. 7), MEK-2 is similar to MEK (Refs 8, 9) and MPK-1 is similar to MAP kinase[10,11]. Thus, the anchor cell signal is mediated by an ancient signaling pathway composed of a series of genes that probably functioned together in the common ancestor of nematodes, vertebrates and insects (Fig. 2).

Activated vertebrate MAP kinase can enter the nucleus, suggesting it can phosphorylate nuclear localized proteins and, *in vitro*, it can phosphorylate a wide variety of proteins, including transcription factors[17]. *lin-1* and *lin-31* appear to function downstream of *mpk-1* and encode proteins similar to transcription factors, suggesting they might be phosphorylated and regulated by MPK-1. A loss-of-function mutation in *lin-1* causes a strong multivulva phenotype, and genetic epistasis experiments suggest *lin-1* functions downstream of the RAS signaling pathway[3,10,11]. Thus, *lin-1* negatively regulates vulval cell fates, and the RAS pathway is likely to promote the 1° fate by negatively regulating *lin-1* (Fig. 2). A loss-of-function *lin-31* mutation causes a multivulva phenotype and also causes P5.p, P6.p and P7.p sometimes to adopt the nonvulval 3° fate, suggesting *lin-31* can promote and repress vulval cell fates[3,18]. LIN-1 protein contains an ETS-domain and LIN-31 is similar to the HNF-3/fork head family; similar vertebrate proteins can bind DNA and regulate transcription[18,19]. Genes regulated by *lin-1* or *lin-31* have not been identified; such genes are likely to reveal how programs of transcription change in response to signals during development.

Strong loss-of-function mutations in *lin-2*, *lin-7* and *lin-10* cause a partially penetrant vulvaless phenotype suggesting these genes positively modulate the

TABLE 1. Genes involved in vulval development

Source of signal	Role	Genes involved	Predicted protein
Anchor cell	Ligand	*lin-3*	Epidermal growth factor
	Receptor	*let-23*	Receptor tyrosine kinase
	Core transducers	*sem-5*	SH3–SH2–SH3 adapter
		let-341	–[a]
		let-60	RAS
		lin-45	RAF
		mek-2	MEK
		mpk-1/sur-1	MAP kinase
	Transcription factors	*lin-1*	ETS
		lin-31	HNF3/Fork head
	Positive modulators	*lin-2*	MAGUK
		lin-7	PDZ
		lin-10	Pioneer[b]
	Negative modulators	*unc-101*	AP47
		sli-1	CBL
	Downstream of RAS	*ksr-1*	Novel protein kinase
		lin-25	Pioneer
		sur-2	Pioneer
hyp7	Class A synthetic multivulva	*lin-15A*	Pioneer
		lin-8	–
		lin-38	–
	Class B synthetic multivulva	*lin-15B*	Pioneer
		lin-9	–
		lin-35	–
		lin-36	–
		lin-37	–
		n770	–
		n771	–
		n883	–
P6.p	Receptor	*lin-12*	Notch

[a]The '–' symbol indicates that the gene has not been cloned.
[b]Pioneer proteins are not significantly similar to proteins in current databases.

signaling pathway[3]. Recent results suggest two of these genes, *lin-2* and *lin-7*, are involved in the subcellular localization of the LET-23 receptor. The VPCs are polarized epithelial cells that have apical and basal surfaces separated by cell junctions. The membrane-associated guanylate kinase (MAGUK) family of proteins associate with tight junctions (reviewed in Ref. 20), and it is known that LIN-2 is similar to MAGUK proteins[21]. LIN-7 has a PDZ (PSD-95/*discs-large*/ZO-1) domain similar to those found in MAGUK proteins, and LIN-7 associates with cell junctions[22]. The LET-23 receptor also associates with cell junctions in wild-type animals, but not in *lin-2* or *lin-7* mutants[22]. Thus, *lin-2* and *lin-7* are required for LET-23 localization. A possible mechanism is suggested by the observation that LIN-7 can bind LET-23 protein[22]. These data suggest LIN-2 and LIN-7 localize LET-23 to cell junctions, and this localization is important for LET-23 function. This hypothesis is consistent with genetic analyses that suggested *lin-2*, *lin-7* and *lin-10* function upstream of *let-23* (Ref. 22). The similarities between the *lin-2*, *lin-7* and *lin-10* phenotypes suggest *lin-10* functions in concert with *lin-2* and *lin-7* (Fig. 2). *lin-10* encodes a pioneer protein that is not significantly similar to known proteins[23].

Loss-of-function mutations in *unc-101* and *sli-1* were identified as suppressors of the vulvaless phenotype caused by partial loss-of-function *let-23* mutations[24]; these genes appear to be weak negative regulators of vulval cell fates (Table 1). Genetic analyses have not yet defined how these genes connect to the core signaling pathway. UNC-101 is similar to AP47, a subunit of clathrin-associated protein complexes typical of the *trans*-Golgi (Ref. 25). Thus, UNC-101 might be involved in trafficking a transmembrane or secreted protein involved in signaling. *sli-1* is similar to CBL, a mammalian proto-oncoprotein with an unknown mechanism of action[26].

By screening for mutations that suppress the multivulva phenotype caused by constitutively active *let-60 ras*, many genes have been identified that appear to function downstream of *let-60* and positively mediate RAS signaling[8–11,27–29]. Six such genes have been analyzed molecularly: the core signaling genes *lin-45 raf*, *mek-2* and *mpk-1*, and modulating genes *ksr-1*, *sur-2* and *lin-25* (Fig. 2). Many additional suppressor genes have not yet been analyzed. *sur-2* and *lin-25* have similar genetic properties: strong loss-of-function mutations cause a partially penetrant vulvaless phenotype, and

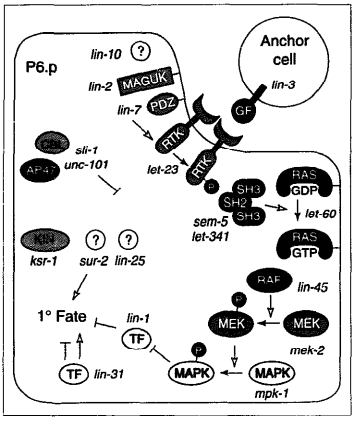

FIGURE 2. Genes involved in the anchor cell signal. Genetic epistasis experiments suggest the core signaling molecules act in the following order: *lin-3*, *let-23*/*let-341*, *sem-5*/*let-341*, *let-60*, *lin-45*/*mek-2*/*mpk-1*, *lin-1*/*lin-31* (genes separated by diagonals have not been ordered)[8–13,18,19]. *lin-2*, *lin-7* and *lin-10* are likely to act upstream of *let-23* (Ref. 22). *sli-1* might act upstream of *lin-45* (Ref. 24). The position of *unc-101* in the pathway is uncertain. *ksr-1*, *sur-2* and *lin-25* are likely to act downstream of *let-60* (Refs 27–30). Mosaic analyses suggest *lin-2*, *lin-7*, *let-23*, *mpk-1* and *lin-31* function in VPCs (Refs 10, 21, 22, 43–45). *lin-3* is expressed in the anchor cell, and *let-23* and *sur-2* are expressed in vulval precursor cells (Refs 12, 22, 27). The site of action or expression of other genes has not been demonstrated. Open arrows signify positive effects, bars signify negative effects, and closed arrows indicate a protein transition. Abbreviations: AP47, clathrin associated protein; CBL, *c-CBL* proto-oncogene product; GF, growth factor; KIN, protein kinase; MAGUK, membrane-associated guanylate kinase; MAPK, MAP kinase; MEK, MAP kinase kinase or ERK kinase; PDZ, PSD-95/*discs-large*/ZO-1; P in a black circle, phosphorylation; RTK, receptor tyrosine kinase; SH, SRC homology; TF, transcription factor; (?), pioneer protein not significantly similar to proteins in current databases.

their epistatic relationships with other downstream genes are complex[27,30]. Both genes encode pioneer proteins. Strong loss-of-function *ksr-1* mutations cause a weak vulvaless phenotype suggesting *ksr-1* might function on a branch that feeds into or out of the main signaling pathway[28,29]. *ksr-1* encodes a protein kinase that is distantly related to RAF and similar to *Drosophila* KSR, which also positively mediates RAS signaling. *Drosophila ksr* appears to function downstream of RAS and upstream of RAF, suggesting KSR might be involved in activating RAF (Ref. 31). The identification of many genes that function downstream of RAS suggests this portion of the pathway is likely to be complex and involves redundant and/or parallel pathways.

Genetic screens for animals with vulval defects have identified genes that encode proteins previously known to be involved in signal transduction, such as the core signaling molecules, genes that encode familiar proteins not previously known to be involved in signal transduction, such as *lin-2*, *lin-7*, *sli-1* and *unc-101*, and genes that encode novel proteins such as *ksr-1*, *sur-2* and *lin-25*. The first class complements biochemical experiments and provides a framework for understanding the signal transduction pathway, and the second two classes promise to provide new insights into signaling mechanisms and illustrate the ability of sensitive genetic screens to identify components of signaling systems that are not readily identified using biochemical approaches.

Signals from hyp7 inhibit vulval cell fates

In addition to receiving a signal from its dorsal neighbor, the anchor cell, P6.p appears to receive signals from its lateral neighbor hyp7. hyp7 is a large hypodermal syncytium that envelops most of the animal and contacts the lateral surfaces of VPCs. The hyp7 signals appear to inhibit all six VPCs from adopting vulval fates. Ultimately, signals promoting vulval cell fates overcome the hyp7 signals in P5.p, P6.p and P7.p, but not in P3.p, P4.p and P8.p. The existence of signals from hyp7 was inferred from genetic analyses of the synthetic multivulva genes.

The synthetic multivulva genes are divided into class A (*lin-8*, *lin-15A*, *lin-38*) and class B (*lin-9*, *lin-15B*, *lin-35*, *lin-36*, *lin-37*, *n770*, *n771*, *n833*) based on the following criteria[32]: (1) single mutants containing a loss-of-function class A or class B mutation are not multivulva suggesting the two classes have redundant functions; (2) double mutants containing two class A or two class B mutations are not multivulva, suggesting each class consists of genes that mediate one pathway. However, double mutants containing any class A mutation and any class B mutation are multivulva. These results suggest class A genes and class B genes represent two pathways that inhibit VPCs from adopting vulval cell fates (Table 1). Although genes that have redundant functions are notoriously difficult to identify in genetic screens, the synthetic multivulva genes were actually discovered twice: a multivulva strain identified following standard mutagenesis turned out to have independent mutations in the class A gene *lin-8* on chromosome II and the class B gene *lin-9* on chromosome III. In addition, multivulva strains were identified that had a single mutation that disrupted the *lin-15A* and *lin-15B* genes, both of which are adjacent on chromosome X.

Although a comprehensive picture of how these pathways function has yet to emerge, several important observations have been made. Molecular analysis of *lin-15* reveals that it is a complex locus containing adjacent class A and class B transcripts[33,34]. Both transcripts encode pioneer proteins suggesting these pathways have not been identified in other organisms, perhaps because they also function redundantly in other organisms. Mosaic analysis suggests that *lin-15* functions in hyp7 (Ref. 35). Thus, *lin-15A* and *lin-15B* might be signaling molecules or important for the production or activity of a signaling molecule(s). No other data have addressed the hypothesis that the synthetic multivulva genes mediate intercellular signaling pathways, and it

will be exciting to learn the site of action of other synthetic multivulva genes.

How does P6.p integrate the anchor cell signal that promotes a 1° vulval fate and the inhibitory hyp7 signals? A fascinating clue has come from the observation that the synthetic multivulva phenotype is not suppressed by a loss-of-function mutation in *lin-3*, which encodes the anchor cell signal, or by ablation of the anchor cell. In contrast, the synthetic multivulva phenotype is suppressed by loss-of-function mutations in *let-23*, which encodes the receptor for the anchor cell signal, or in genes encoding more-downstream signaling molecules[3,4,34]. Because vulval cell fates in synthetic multivulva mutants require LET-23 receptor, but not LIN-3 ligand, it appears that LET-23 has a significant basal activity in the absence of ligand. The synthetic multivulva genes seem to regulate this basal activity negatively, and the LIN-3 ligand appears to overcome this negative regulation (Fig. 3). It is not clear why it is advantageous for LET-23 to have high basal activity that is negatively regulated by a widely dispersed signal and positively regulated by a localized ligand, as opposed to having low basal activity that requires activating ligand. Perhaps dual signals reduce the chance that cells distant from the ligand source will respond to low levels of signal and, thereby, enhance the reproducibility of the pattern of cell fates.

A lateral signal from P6.p promotes 2° cell fates

P6.p appears to signal P5.p and P7.p to adopt 2° fates. This signal requires P6.p to be stimulated by the anchor cell to adopt a 1° fate, and it requires *lin-12* that appears to encode the receptor for the signal from P6.p. *lin-12* activity is necessary and sufficient for cells to adopt the 2° fate; in *lin-12* loss-of-function mutants none of the VPCs adopt the 2° fate, whereas a gain-of-function mutation that constitutively activates *lin-12* causes all six VPCs to adopt the 2° fate[2]. LIN-12 is similar to transmembrane receptor proteins in the Notch family[36], and these proteins act in a variety of lateral signaling events in *Drosophila* and at other times during *C. elegans* development[36]. Early in development, the *lin-12* signaling pathway includes LAG-2, which is similar to *Drosophila* Delta and likely to be a ligand for LIN-12 (Refs 37, 38) and LAG-1, which is similar to the *Drosophila* transcription factor Suppressor of Hairless [Su(H)] and likely to be a downstream effector of LIN-12 (Ref. 39). However, *lag-1* and *lag-2* have not been shown to function in VPCs.

lin-12 function has been analyzed most thoroughly in the cell fate decisions of Z1.ppp and Z4.aaa, and this provides useful insights into how *lin-12* is likely to function in VPCs (Ref. 36). The fates of Z1.ppp and Z4.aaa are variable: either Z1.ppp or Z4.aaa becomes the anchor cell, and the other cell becomes the ventral uterine precursor cell (VU)[36]. Both cells initially express *lin-12* and *lag-2* (Ref. 40). *lin-12* is required in the VU cell (Ref. 36), and as development proceeds, the presumptive VU cell loses expression of *lag-2* and increases expression of *lin-12*, whereas the presumptive anchor cell loses expression of *lin-12* and increases expression of *lag-2* (Ref. 40). It appears that Z1.ppp and Z4.aaa compete, and slight differences in the initial expression of *lin-12* and *lag-2* are amplified by feedback loops that ensure that ultimately only one cell signals and one cell receives.

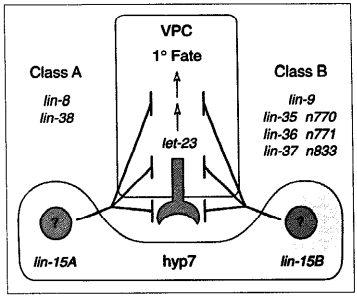

FIGURE 3. Genes involved in the hyp7 signals. If the synthetic multivulva genes and *let-23* function in a linear signaling pathway, then genetic epistasis experiments suggest the synthetic multivulva genes act upstream to regulate *let-23* negatively; they might act on the extracellular or intracellular portion of *let-23*. Alternatively, the synthetic multivulva genes and *let-23* might act in parallel, in which case the synthetic multivulva genes might inhibit the effects of *let-23* activity by negatively regulating downstream signaling molecules. Branching bars indicate these possibilities. Mosaic analysis showed *lin-15A* and *lin-15B* are not required in VPCs, but are required in descendants of two cells that both contribute nuclei to hyp7 (Ref. 35). These data are consistent with the hypothesis that both *lin-15A* and *lin-15B* function cell non-autonomously in hyp7. The site of action of other synthetic multivulva genes has not been determined. Mosaic analysis showed *let-23* functions in VPCs (Refs 43, 44). Symbols are defined in Fig. 2 legend.

VPCs with an activated anchor-cell signaling pathway can engage in a similar competition. In *lin-15* multivulva mutants, the six VPCs adopt an alternating pattern of 1° and 2° fates, suggesting 1° cells signal neighboring cells to adopt 2° cell fates[41]. Furthermore, if the anchor cell and four VPCs are ablated leaving two adjacent VPCs in a *lin-15* mutant, either the anterior or posterior VPC adopts the 1° fate, and the other cell always adopts the 2° fate[41]. These results are strikingly similar to the results seen with Z1.ppp and Z4.aaa, and suggest lateral signaling can play a similar role in both equivalence groups. VPCs with an inactive anchor cell signaling pathway do not signal neighboring cells to adopt 2° fates, but do express *lin-12*, and can adopt a 2° fate suggesting they can respond to *lin-12* ligand[42–44]. These observations suggest activation of the anchor cell signaling pathway leads to activation of the *lin-12* ligand.

Unlike the anchor cell/VU fate decision which has a variable outcome, P5.p–P7.p always adopt the 2°–1°–2° pattern of fates. The most likely explanation for this invariant pattern is that the anchor cell, which is adjacent to P6.p, biases the competition by signaling P6.p exclusively or more strongly than P5.p and P7.p. In the former case, only P6.p would be capable of lateral signaling whereas in the latter case P5.p and P7.p would compete with P6.p but invariably lose. This is likely to

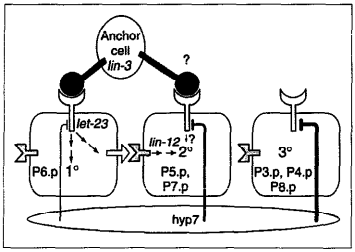

FIGURE 4. Model of cell fate determination. Unique cell borders and spatially restricted ligands appear to be crucial elements that establish the characteristic 3°–3°–2°–1°–2°–3° pattern of cell fates. The anchor cell signal (LIN-3) causes P6.p to adopt the 1° fate by activating the LET-23 receptor (Fig. 2) and overcoming the inhibitory signals from hyp7 (Fig. 3). P6.p then signals P5.p and P7.p to adopt the 2° fate using an unidentified ligand (open arrowhead) to activate the LIN-12 receptor. It is not yet clear whether the anchor cell signals P5.p and P7.p because the *in vivo* distribution of LIN-3 has not been demonstrated (indicated by ?).

Although the predicted LIN-3 protein is a transmembrane molecule[12], LIN-3 can function as a secreted protein[16], and some evidence suggests LIN-3 can diffuse considerably distances *in vivo*[2]. Thus, P5.p and P7.p might be exposed to LIN-3 in wild-type animals. However, P6.p might sequester LIN-3 and, thus, prevent it from diffusing to P5.p and P7.p. Furthermore, the role of the anchor cell signal in promoting the 2° cell fate is unclear (indicated by ?). P3.p, P4.p and P8.p receive inhibitory signals from hyp7, but do not appear to receive the ligand for either LET-23 or LIN-12. Abbreviations: 1°, primary; 2°, secondary; 3°, tertiary.

be a robust mechanism that insures P6.p will become the lateral signaling cell while P5.p and P7.p will become the receiving cells over a wide range of anchor cell signal intensity (Fig. 4). Furthermore, if the anchor cell is positioned between two VPCs, this mechanism ensures only one VPC will adopt the 1° fate and a normal pattern will be generated.

It has been proposed that LIN-3, the anchor cell signal, acts as a graded morphogen such that high levels cause VPCs to adopt the 1° cell fate and low levels cause VPCs to adopt the 2° cell fate[2,16]. This model is supported primarily by analyses of isolated VPCs that lack neighboring VPCs and, thus, are not adjacent to a 1° cell that could provide the lateral signal. When exposed to low levels of LIN-3, these cells sometimes adopt 2° fates[16]. In principle, a low level of LIN-3 could be the sole mechanism that establishes 2° cell fates, a low level of LIN-3 might act in concert with a lateral signal from P6.p, or a lateral signal from P6.p could be the sole mechanism that establishes 2° cell fates. An elegant test of the first possibility was recently reported; a mosaic analysis revealed that *let-23*, which encodes the receptor for the anchor cell signal, is not required in P5.p and P7.p for these cells to adopt 2° fates[43,44]. Thus, a low level of the anchor cell signal does not appear to be required for the 2° fate and is unlikely to be the sole patterning mechanism. Furthermore, these experiments suggested a low level of anchor cell signal is not sufficient to induce a 2° fate reproducibly; in mosaic animals where P6.p was *let-23*(–) and P7.p was *let-23*(+), P7.p was never observed to adopt the 2° fate[43,44]. This result is difficult to reconcile with the observation that a low level of anchor cell signal can be sufficient to induce a 2° fate in an isolated VPC, but suggests the behavior of isolated VPCs may not reflect the behavior of VPCs in wild-type animals. In contrast, the mosaic analyses confirmed the importance of the lateral signal; a 1° cell was necessary and frequently sufficient to induce a 2° fate in a neighboring VPC (Refs 43, 44). These data suggest a lateral signal from P6.p is either the sole mechanism that establishes 2° cell fates, or the primary mechanism that functions in conjunction with a low level of the anchor cell signal. To distinguish between these possibilities, it will be necessary to demonstrate a mechanism whereby a low level of LIN-3 causes a cell to adopt the 2° fate.

Cell borders and patterns of cell fates

An important principle emerging from the analysis of vulval development is that borders between non-equivalent cells are a crucial source of patterning information. The cells P3.p–P8.p initially have equivalent potential. However, they do not have equivalent neighboring cells. Only P6.p borders the anchor cell. This unique border is used to break the symmetry of the vulval equivalence group and make P6.p different from the other five cells. After this occurs, two additional novel borders are created because P6.p contacts P5.p and P7.p, but not the other three cells. These novel borders are exploited to cause P5.p and P7.p to adopt the 2° cell fate. This is a powerful mechanism for generating patterns of cell fates because each novel border that is exploited creates new novel borders. Although the pattern of fates in the vulval equivalence group is rather simple, there is no theoretical limit to the complexity of patterns that can be achieved by successively exploiting novel borders.

An important aspect of the border mechanism of patterning is that signaling ligands must be spatially restricted. In contrast, receptors can be expressed broadly. This appears to be the case for the LIN-3 ligand, which is expressed specifically in the anchor cell[12], and for the LET-23 and LIN-12 receptors, which are expressed in each cell in the equivalence group[22,42].

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Production of transgenic mice with yeast artificial chromosomes

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Techniques are now available that allow the transfer of intact yeast artificial chromosome (YAC) DNA into transgenic mice. Coupled with the ability to perform mutagenesis on YAC sequences by homologous recombination in yeast, they enable the analysis of large genes or multigenic loci in vivo. This system has been used to study the developmental regulation of the human β-globin locus.

Mammalian genetics has been revolutionized by the implementation of molecular techniques that permit the stable integration of recombinant DNA into mice. Ideally, genomic DNA sequences containing introns and essential regulatory sequences should be used as transgenes, because they are generally expressed correctly *in vivo* compared with cDNA constructs. However, the use of intact loci as transgenes suffers from several limitations: (1) the maximum size DNA molecule that can be introduced into mice is 40–50 kb, due to constraints on the sequence length that can be cloned and stably maintained in phage and/or cosmids; (2) mechanical shear of large DNAs during *in vitro* manipulations cannot be avoided; and (3) 'nonessential' DNA sequences are omitted in the design of the constructs to be transferred because of the size limitation. Decisions have to be made about the regulatory relevance of the omitted sequences and the distances between *cis* control elements. These limitations have been overcome by using yeast artificial chromosomes (YACs) in the generation of transgenic mice[1–21]. The maximum insert size that can be contained in YACs is approximately 2 Mb, allowing the study of complete genes, multigene loci, distant regulatory sequences and higher-order genomic structure in the context of their native sequence environment. Additionally, precise site-specific mutations, such as nucleotide substitutions, deletions and insertions can be readily introduced into YAC insert sequences, using the inherent homologous recombination system of yeast. This provides the opportunity to generate murine models of human genetic disease, and to produce mice that can be utilized to express and to purify foreign proteins. Here, we outline the current state of YAC transgenic

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