
Morphogenesis of the vulva and the vulval-uterine connection*

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Table of Contents

1. Introduction	2
2. Patterning of vulval cell types	3
2.1. Specification of cell fates	3
2.2. Transcriptional program	6
3. Morphogenetic changes	8
3.1. Polarity of the 2° lineage cells	8
3.2. Vulval cell migration and invagination	9
3.3. Formation of vulval toroids and control of cell fusion events	10
3.4. Organization of neurons and muscles	10
4. The vulval-uterine connection	10
4.1. Induction of π cell fate	11
4.2. Uterine seam cell differentiation	11
4.3. uv1 cell development	12
4.4. AC invasion and vulF lumen formation	13
4.5. AC-utse fusion	13
4.6. AC transcriptional program	14
5. Concluding remarks	14
6. Acknowledgements	15
7. References	15

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Abstract

The *C. elegans* hermaphrodite vulva is an established model system to study mechanisms of cell fate specification and tissue morphogenesis. The adult vulva is a tubular shaped organ composed of seven concentric toroids that arise from selective fusion between differentiated vulval progeny. The dorsal end of the vulval tubule is connected to the uterus via a multinucleate syncytium utse (uterine-seam) cell. The vulval tubule and utse are formed as a result of changes in morphogenetic processes such as cell polarity, adhesion, and invagination. A number of genes controlling these processes are conserved all the way up to human and function in similar developmental contexts. This makes it possible to extend the findings to other metazoan systems. Gene expression studies in the vulval and uterine cells have revealed the presence of regulatory networks specifying distinct cell fates. Thus, these two cell types serve as a good system to understand how gene networks confer unique cell identities both experimentally and computationally. This chapter focuses on morphogenetic processes during the formation of the vulva and its connection to uterus.

1. Introduction

The *Caenorhabditis elegans* hermaphrodite vulva is an epidermal-derived tube that connects the uterus to the external environment. Development of the vulva involves a broad range of cell biological processes that are shared among all eukaryotes. The model has been adopted for the study of signal transduction and signal integration and the subsequent transcriptional networks that direct cell differentiation. Vulva development is also a successful model for studying tissue remodeling during organ morphogenesis, including common morphogenetic processes such as invagination, lumen formation and cell fusion. The vulva provides a powerful paradigm because it has simple anatomy and rapid development and because it is easily manipulated via genetics and transgenics. Investigations with this system have revealed the function of a large number of genes and their network of interactions, including important transcription factors such as **LIN-11** (LIM domain family), **EGL-38** (Pax2/5/8 family of proto-oncogene), **LIN-29** (zinc finger family), and **COG-1** (Nkx6.1/6.2 family) (see [Ririe et al., 2008](#) and references therein). These findings demonstrate that the vulva is a powerful system to identify and study the function of conserved genes in important cellular processes, allowing us to formulate a coherent picture of formation of a single organ.

During vulval development a subset of twelve Pn.p cells acquire competence and become vulval precursor cells (VPCs). Three VPCs adopt 1° and 2° fates in response to conserved signaling pathways mediated by Ras, Wnt and Notch and divide to generate 22 progeny ([Sternberg, 2005](#)). The vulval progeny differentiate and undergo morphogenetic changes to form the adult vulva, composed of a stack of toroid-shaped cells surrounding a central lumen ([Figure 1](#)) ([Sharma-Kishore et al., 1999](#)). A specialized cell from the gonad, the anchor cell (AC), plays multiple roles in vulval development. The AC first induces VPCs to adopt 1° (P6.p) or 2° (P5.p and P7.p) fates ([Sternberg, 2005](#)). Subsequently, it controls differentiation of 1° lineage cells. The AC also patterns nearby uterine cells, via production of the DSL ligand **LAG-2**, which acts on **LIN-12** to generate the uterine seam (utse) cell and the four uv1 cells that form the tight connection with the vulva ([Newman et al., 2000](#); [Newman et al., 1995](#)). A connection between the uterine and vulval lumens is also formed by the anchor cell, which invades between the 1° lineage cells in a process analogous to invasive behavior of metastatic tumor cells ([Sherwood and Sternberg, 2003](#)). During this invasion process, the basement membranes between the gonad and body wall are degraded. Thus the AC patterns two neighboring tissues and ensures that the vulva and uterus connect with each other to generate a functional egg-laying system. In this chapter we summarize cellular events in the vulva and uterus that occur following VPC induction. We also present the current understanding of the roles of genes and signaling pathways that mediate the underlying processes.

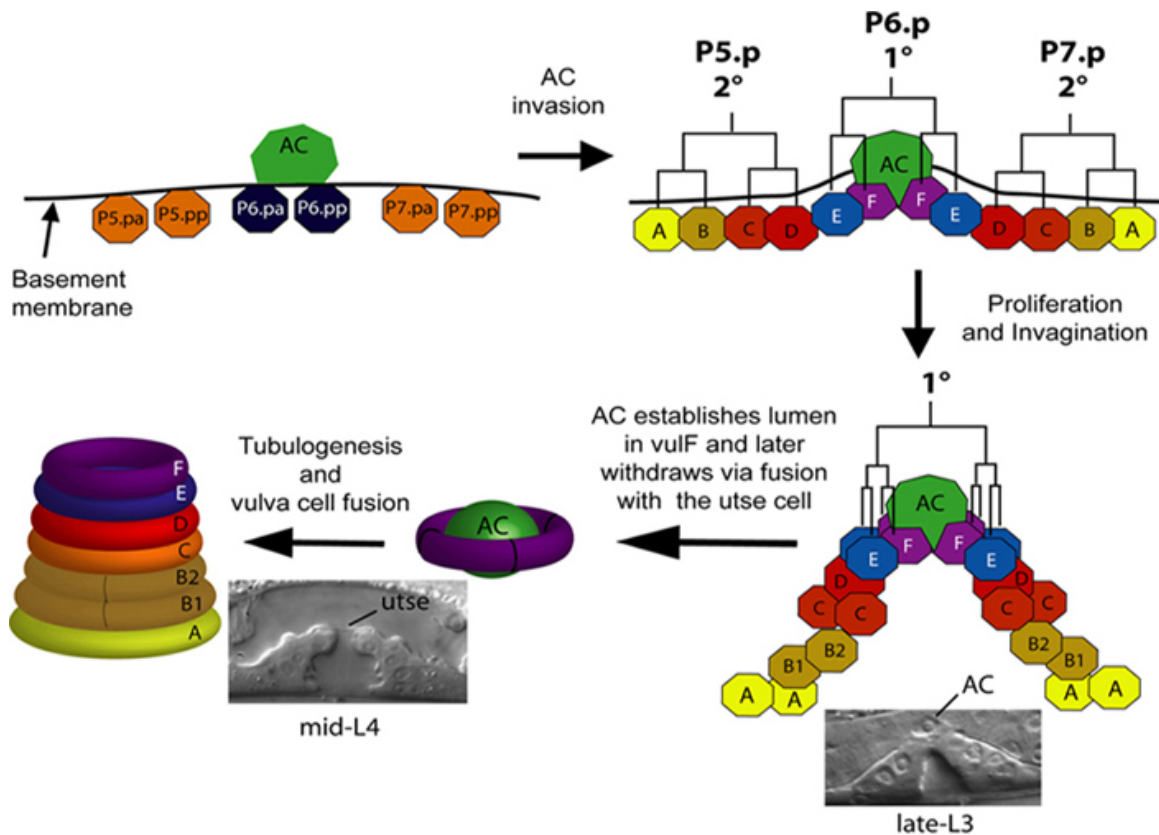


Figure 1. Vulval cells and toroids. The P5.p, P6.p and P7.p VPCs divide three times to produce 22 progeny. The vulval progeny differentiate to give rise to 7 different cell types (vulA to vulF). The homologous cells from P5.p and P7.p lineages fuse selectively to form 7 concentric toroids that are interconnected via AJM-1-containing apical junctions. During the mid-to-late-L3 stage the AC initiates invasion into the vulval epithelium. At the early-L4 stage it can be seen establishing the lumen in vulF thereby promoting a connection between vulva and uterine lumens. By the mid-L4 stage AC has fused with π progeny to form the utse cell and its nucleus has migrated (either anteriorly or posteriorly) away from the connection between the two lumens.

2. Patterning of vulval cell types

2.1. Specification of cell fates

The seven vulval cell types (vulA, vulB1, vulB2, vulC, vulD, vulE and vulF) are formed by the fusion of defined vulval progeny. While vulA to vulD are generated by the 2° lineage VPCs P5.p and P7.p in mirror symmetric polar patterns (ABCD and DCBA), the remaining cell types (vulE and vulF) are the progeny of the 1° lineage VPC P6.p (Figure 1). Each of these cell types has a distinct pattern of gene expression (Table 1, Figure 2) and a specialized function. For example, the vulval muscles vm1 insert between the vulE and vulF cells, while the vm2 muscles insert between the vulC and vulD cells. Additionally, the vulF cell forms the main contact with the anchor cell and induces the uv1 fate in the ventral uterine π lineage (Chang et al., 1999; Newman et al., 1996; Sherwood and Sternberg, 2003).

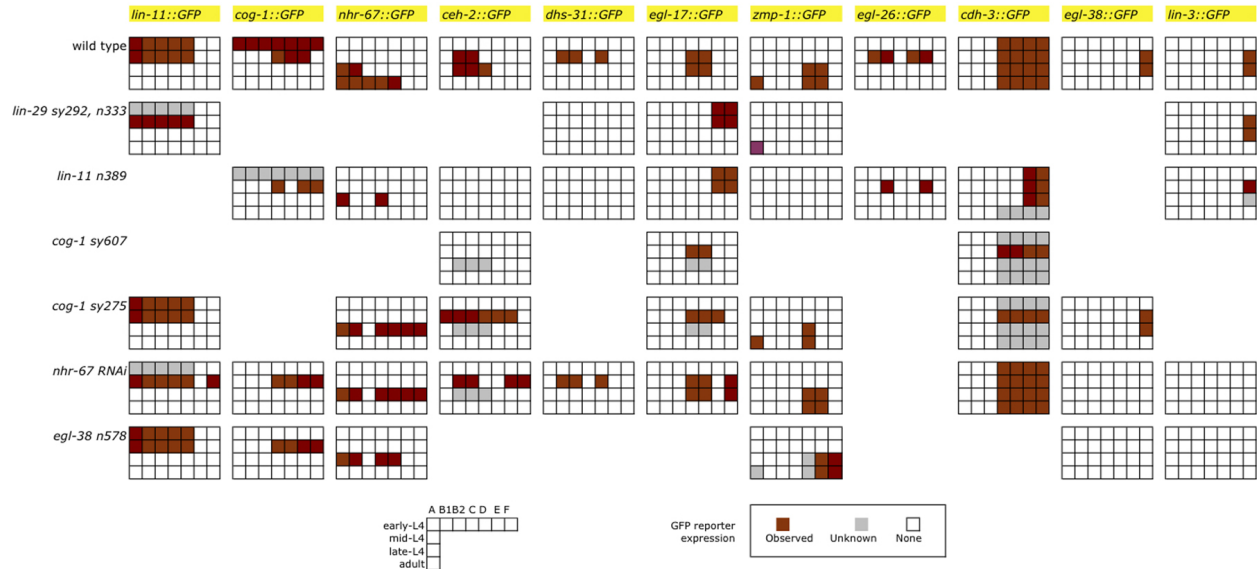


Figure 2. Gene expression patterns in vulval cells. Overview of the expression profile of 11 genes in vulval cells in wild type and mutant animals as described in literature. Expression of each gene is plotted in a matrix form where X-axis represents the vulval cell type and Y-axis represents the stage of the animal.

Table 1. Genes expressed in vulval cells between late-L3 and L4 stage (transcription factors are in bold).

Gene	Gene family/Ortholog	Expression domain [#]	Key references
<i>B0034.1</i>	unknown	E, F	(Inoue et al., 2002)
<i>bam-2</i>	Neurexin-related	F	(Colavita and Tessier-Lavigne, 2003)
<i>C55C3.5</i>	unknown	F	(Inoue et al., 2005)
<i>cdh-3</i>	FAT-like cadherin	C, D, E, F	(Inoue et al., 2005; Pettitt et al., 1996)
<i>ceh-2</i>	HOX	B1, B2, C	(Inoue et al., 2002)
<i>cog-1</i>	Nkx6.1/6.2	C, D, E, F, and occasionally in A and B	(Palmer et al., 2002)
<i>daf-6</i>	Patched-related	E, F	(Perens and Shaham, 2005)
<i>dhs-31</i>	Predicted short chain-type dehydrogenase	B1, B2, D	(Inoue et al., 2002)
<i>egl-17</i>	Fibroblast growth factor-like	C, D	(Burdine et al., 1998)
<i>egl-26</i>	Acyltransferase-like	B, D, E	(Hanna-Rose and Han, 2002)
<i>egl-38</i>	Paired-box domain (Pax2/5/8)	F	(Rajakumar and Chamberlin, 2007)
<i>grd-5</i>	Groundhog hedgehog	B1, B2, D	(Hao et al., 2006)
<i>grd-12</i>	Groundhog hedgehog	C	(Hao et al., 2006)
<i>grl-4</i>	Ground-like hedgehog	A, B1, B2, D	(Hao et al., 2006)
<i>grl-10</i>	Ground-like hedgehog	A, B1, B2	(Hao et al., 2006)
<i>grl-15</i>	Ground-like hedgehog	B1, B2, C, D, E	(Hao et al., 2006)
<i>grl-25</i>	Ground-like hedgehog	A	(Hao et al., 2006)
<i>grl-31</i>	Ground-like hedgehog	F	(Hao et al., 2006)
<i>lin-3</i>	Epidermal growth factor-like	F	(Chang et al., 1999)

Gene	Gene family/Ortholog	Expression domain [#]	Key references
<i>lin-11</i>	LIM-HOX	A, B1, B2, C, D	(Gupta and Sternberg, 2002)
<i>lin-29</i>	C2H2-type Zn finger	All cells	(Bettinger et al., 1997)
<i>lin-39</i>	HOX	A	(Wagmaister et al., 2006)
<i>nas-37</i>	Metalloprotease	B	(Ririe et al., 2008)
<i>nhr-67</i>	Zn finger NHR/Tailless	A, B, C	(Fernandes and Sternberg, 2007)
<i>nhr-113</i>	Zn finger NHR	A	(Ririe et al., 2008)
<i>npax-2</i>	Paired-box domain	C, D	(Reece-Hoyes et al., 2007), (Ian Hope, pers. comm.)
<i>pax-2</i>	Paired-box domain (Pax2/5/8)	D	(Fernandes and Sternberg, 2007)
<i>pepm-1</i>	Peptidase	C, D, E, F	(Inoue et al., 2002)
<i>sqv-4</i>	UDP-glucose dehydrogenase	C, D, E, F	(Hwang and Horvitz, 2002)
<i>syg-2</i>	Cell adhesion Ig domain	E, F	(Shen et al., 2004)
<i>unc-53</i>	Nuclear pore membrane and/or filament interacting like (POMFIL)	C	(Stringham et al., 2002)
<i>vrk-1</i>	Protein kinase (vaccinia-related)	All cells	(Klerkx et al., 2009)
<i>zmp-1</i>	Zn metalloproteinase	A, D, E	(Inoue et al., 2002)

[#]refers to expression in vulA to vulF vulval cell types (in short, A to F). In some cases expression in B1 and B2 was not clearly identified. These are shown as B.

2.1.1. 1° lineage vulval cells

The 1° lineage generates two cell types, vulE and vulF, in a mirror symmetric pattern EFFE around the anchor cell (Figures 1 and 3). The anchor cell is necessary for establishing this pattern after it induces the 1° VPC via the LET-23 (Epidermal Growth Factor Receptor, EGFR)-LET-60 (RAS)-mediated pathway (Wang and Sternberg, 2000) (Figure 3). WNT receptor LIN-17 plays a role as well, but the phenotype of a *lin-17* mutation has only been seen in the absence of the anchor cell (Wang and Sternberg, 2000). The relative timing of the signaling involved in these events has not been resolved. The identities of vulE and vulF are specified by the expression of an array of genes including *egl-26* (acyltransferase-like), *egl-38* (PAX2/5/8 family), and *cog-1* (Nkx3.1 family) (Estes et al., 2007; Hanna-Rose and Han, 2002; Inoue et al., 2005; Palmer et al., 2002; Rajakumar and Chamberlin, 2007). Although the precise roles of these genes are not fully resolved, both cell autonomous and non-autonomous mechanisms are likely to play roles.

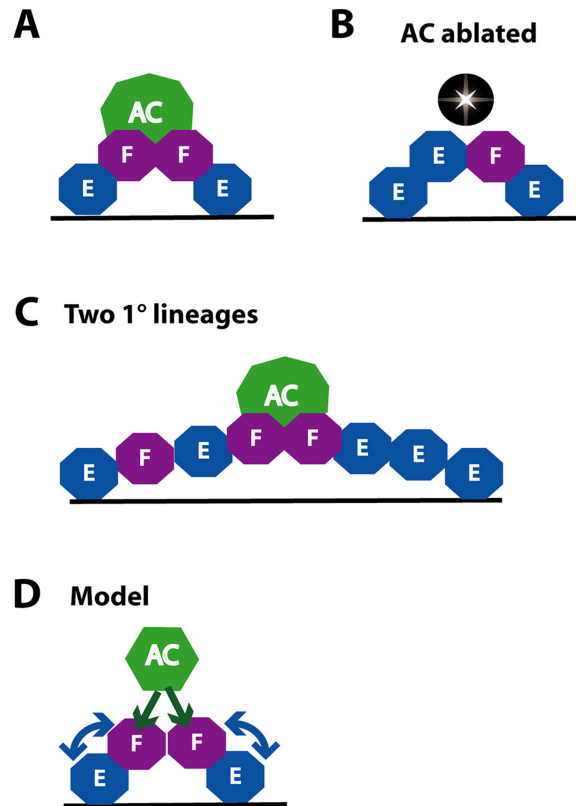


Figure 3. Patterning of the 1° lineage. A. Intact, wild-type 1° lineage has two F cells near the anchor cell and two more distal E cells. B. After AC ablation, the patterns are variable. C. In a *lin-12(lf)* mutant in which all ventral uterine cells but one anchor cell have been ablated, and in which P7.p has been ablated to keep only two 1° lineages, there is a normal pattern at the anchor cells, but a variable distal pattern. D. Model posits an AC-mediated signal via RAS to specify vulF cells near the AC. In addition, vulE and vulF signal each other to ensure that correct fates are specified. Data and model summarized previously (Wang and Sternberg, 2000).

2.1.2. 2° lineage vulval cells

Each of the 2° lineages (P5.p and P7.p) generates seven progeny that produce five vulval cell types, vulA to vulD (see above). The specification of the 2° fate is dependent on the LIN-12-mediated lateral signal (Greenwald, 2005). A MAP kinase phosphatase LIP-1 that acts downstream of LIN-12, antagonizes MAP kinase MPK-1 activity in P5.p and P7.p, allowing them to adopt a 2° fate (Berset et al., 2001). The negative feedback on the kinase cascade downstream of the LET-60 (Ras) - LIN-45 (Raf) signaling module also allows LET-60 to specifically signal via RGL-1 (Ral GEF) and RAL-1 to promote 2° fate in P5.p and P7.p (Zand et al., 2011). Wnt signaling (involving LIN-17/Frizzled and PRY-1/Axin) is also involved in 2° fate determination (Eisenmann, 2005; Seetharaman et al., 2010), in part by interacting with LIN-12 signaling (Seetharaman et al., 2010). In addition to fate specification, Wnt signaling also plays a role in reversing the polarity of P7.p such that a symmetrical vulval invagination can be formed (see below). A Ryk family tyrosine kinase-related protein LIN-18 functions as a co-receptor of LIN-17 in this process (Inoue et al., 2004).

2.2. Transcriptional program

Many genes have been identified that act downstream of the signaling pathways (Ras, Notch, and Wnt) to regulate identities of specific vulval cell types. These include conserved genes encoding transcription factors and other proteins (Table 1). Genetic epistasis and transgene expression experiments have revealed a functional hierarchy in a few cases but the overall transcriptional network underlying vulval differentiation appears to be complex (Figure 2). This is in part due to seven different cell types and their unique gene expression programs (Figure 2). Cis regulatory analysis has also revealed a similar extent of complexity in the specification of the vulval cell types (Cui and Han, 2003; Kirouac and Sternberg, 2003; Marri and Gupta, 2009; Ririe et al., 2008). Below we discuss some of the genes that have been studied in some detail and shown to play major roles in vulval development.

The LIM Hox transcription factor *lin-11* is a key regulator of vulval cell fates (Ferguson et al., 1987; Gupta et al., 2003). It is expressed in both the 1° fate (during mid-L3) and 2° fate lineages (during L4) and promotes fates in a cell autonomous manner (Gupta et al., 2003). During the L4 stage *lin-11* controls differentiation and morphogenesis of all P5.p and P7.p progeny, as judged by expression of markers such as *zmp-1*, *cdh-3* and *egl-17* (Gupta et al., 2003). This is mediated in part by a *lin-11* expression level that is higher in vulC and vulD compared to vulA, vulB1 and vulB2 cells (Freyd, 1991 ; Gupta et al., 2003). Promoter dissection experiments have revealed the presence of Ras, Notch and Wnt response elements in the *lin-11* 5' UTR suggesting that *lin-11* is regulated by all three signaling pathways (Gupta and Sternberg, 2002).

Another transcription factor *lin-29* (Zn finger family) is also expressed in 1° and 2° lineage vulval cells and controls cell fate specification (Bettinger et al., 1997). *lin-29* mutants show reduced *lin-11::GFP* fluorescence in 2° vulval cells suggesting that *lin-29* positively regulates *lin-11* expression (Ririe et al., 2008). However the nature of the interactions between the two genes is complex because they affect target genes differently. As a regulator of developmental timing *lin-29* was earlier thought to act within a temporal window (Inoue et al., 2005; Newman et al., 2000) but this hypothesis has been weakened by analysis of additional targets (Ririe et al., 2008). Specifically, the mid-L4 expression of *lin-3* in vulF cells is not dependent upon LIN-29 while *dhs-31*, expressed later in the adult, does require LIN-29 function (Ririe et al., 2008). *lin-29* is also involved in the development of the uterus and the seam cells and their interconnections (Newman et al., 2000).

Besides *lin-11* and *lin-29*, several other genes have been identified that function in subsets of vulval progeny. *egl-26* encodes an acyltransferase-like protein that is expressed in the membranes of vulE cells and is proposed to promote vulF morphogenesis via an unknown cell-cell interaction mechanism (Hanna-Rose and Han, 2002). EGL-26 is located in the apical membrane region of cells and this localization is necessary for proper function of the protein. Mutations in *egl-26* do not cause defects in vulE toroid formation as judged by the cell junction marker *ajm-1*. Instead they specifically affect vulF morphology (Estes et al., 2007).

Other vulF specificity determinants include the PAX2/5/8-like transcription factor EGL-38 and the EGF family ligand LIN-3 both of which are specifically localized to vulF. Mutations in *egl-38* cause a failure of vulF cells to separate leading to a physical block in the egg-laying passage (Chamberlin et al., 1997; Chang et al., 1999; Rajakumar and Chamberlin, 2007). *egl-38* expression is positively autoregulated (Rajakumar and Chamberlin, 2007). *egl-38* promotes expression of *lin-3* and represses *cog-1* in these same cells (Chang et al., 1999; Inoue et al., 2005). In addition, the vulE marker *zmp-1* is ectopically expressed in vulF in some of the *egl-38* animals, possibly due to an F to E fate transformation (Inoue et al., 2005). However the pattern of another vulF marker *bam-2* remains unchanged suggesting that *egl-38* does not cause a widespread change in the vulF transcriptional program (Rajakumar and Chamberlin, 2007).

The vulF expression of *egl-38* and *lin-3* are promoted by the Zn finger/nuclear hormone receptor (NHR) family member *nhr-67* (Ririe et al., 2008). In addition *nhr-67* also regulates several other targets in vulval cells. It promotes *zmp-1* (in vulA) and inhibits *egl-17* (in vulF), *cog-1* and *ceh-2* (both in vulE and vulF) (Fernandes and Sternberg, 2007). The relationship between *cog-1* and *nhr-67* appears to be complex. The two genes negatively autoregulate each other's expression in vulE and vulF cells. Interestingly, the early expression of *nhr-67* in vulval cells is negatively autoregulated since it can be seen only in the absence of *nhr-67* function (Fernandes and Sternberg, 2007). Thus, genes other than *zmp-1* are likely to be indirect targets of *nhr-67*. The complex genetic interactions of *nhr-67* and other vulval cell type regulators and targets are more easily rationalized with *nhr-67* acting in the vulval lineages, but its strong expression in the anchor cell suggests that it acts in the anchor cell to specify vulval cell fates. Definitive site-of-action experiments are necessary to clear up this key issue.

The Nkx6.1 transcription factor family member *cog-1* is dynamically expressed in vulval cells. It is first observed in vulE and vulF precursors during the L3 stage. At a later stage (L4) expression is detected in 2° lineage cells in a pattern similar to *lin-11*. Thus, vulC and vulD precursors show a higher level of expression compared to others. The *cog-1* locus encodes two transcripts that are thought to function differently in vulval cell fate specification. This is based on the phenotypic analysis of *cog-1* mutations that affect only one (*sy607*) or both (*sy275*) transcripts (Palmer et al., 2002). The two alleles differentially affect vulval cell fates as judged by cell lineage and marker gene (*ceh-2* and *egl-17*) expression studies (Inoue et al., 2005; Palmer et al., 2002). The *cog-1(sy275)* animals show a physical block in the vulval-uterine connection, which is caused by a failure in the separation of vulF cells (Palmer et al., 2002). This phenotype is similar to *egl-26* and *egl-38*, but the relationship among *cog-1*, *egl-26* and *egl-38* is unknown.

Apart from the above genes a few others, such as *lin-26* (C2H2 Zn finger) (Eisenmann and Kim, 2000) and *bed-3* (BED type Zn finger) (Inoue and Sternberg, 2010), also control vulval cell fates but their roles have not been studied in much detail.

3. Morphogenetic changes

The patterned vulval cells ultimately form an epithelial tube with a central lumen that allows passage of eggs. Tubulogenesis begins even before all cell divisions are complete and is not completed until the mid-to late L4 stage, hours after the last cell divisions. During morphogenesis the vulval cells orient and migrate towards the anchor cell, undergo epithelial invagination, and fuse to form seven toroids (see below). After the vulval tube forms, it everts during the L4 molt to form the mature vulva, required for regulated egg laying and mating with males.

3.1. Polarity of the 2° lineage cells

The epithelial vulval cells are highly polarized with their apical edges adjacent to the lumen. While there is no evidence that the *par* genes, which are required for polarizing cells in the embryo and elsewhere, are involved in setting vulval cell polarity, experiments to examine their function during vulval development are complicated by maternal effects and early larval lethality of null alleles. Thus, it remains to be seen if *par* genes function during polarity establishment in the vulva. One of the *par* family members, *par-1*, was earlier found to be expressed in vulval cells and is necessary for morphogenesis (Hurd and Kemphues, 2003), however its cellular role in vulva formation remains unknown.

Wnt signaling is another major regulator of cell polarity decisions in *C. elegans*. In the vulva Wnt signaling controls the orientation of 2° lineage cells (Figure 4). A default “ground state” polarity is initially established by Wnt/EGL-20 in which P5.p and P7.p progeny orient towards the posterior. Subsequently another Wnt signal from the anchor cell reverses the default polarity of the P7.p lineage and reinforces the polarity of the P5.p lineage (Deshpande et al., 2005; Green et al., 2008; Inoue et al., 2004). As a result, the cell division pattern and cell fates of the P5.p and P7.p lineages are mirror images, ABCD and DCBA respectively (Figure 4). The asymmetry within each of these lineages is independent of the gonad, as for example, a single VPC in a gonad-ablated *let-23(gf)* mutant has asymmetry (Katz et al., 1996). Three WNT signaling pathways act together to specify polarity (Green et al., 2008; Inoue et al., 2004). (1) The WNT protein EGL-20, likely due to its expression in the tail, acts as a long-range signal and orients P5.p and P7.p to the posterior (Green et al., 2008). This pathway requires WNT receptor CAM-1 and membrane protein VANG-1, although the detailed mechanism is not known. (2) WNT protein MOM-2, which is expressed in the anchor cell, helps to reverse P7.p polarity and orient P5.p and P7.p towards the anchor cell. MOM-2 acts via the Ryk-type receptor LIN-18. (3) WNT protein LIN-44, which is also expressed in the anchor cell, cooperates with MOM-2, but acts via the Frizzled-type receptor LIN-17 (Ferguson et al., 1987; Inoue et al., 2004; Sawa et al., 1996; Sternberg and Horvitz, 1988). This signal antagonizes the action of EGL-20 for P7.p, and reinforces the action of EGL-20 for P5.p. In addition, *lin-44* transcript is also observed in the anchor cell region based on *in situ* hybridization (Y. Kohara, personal communication).

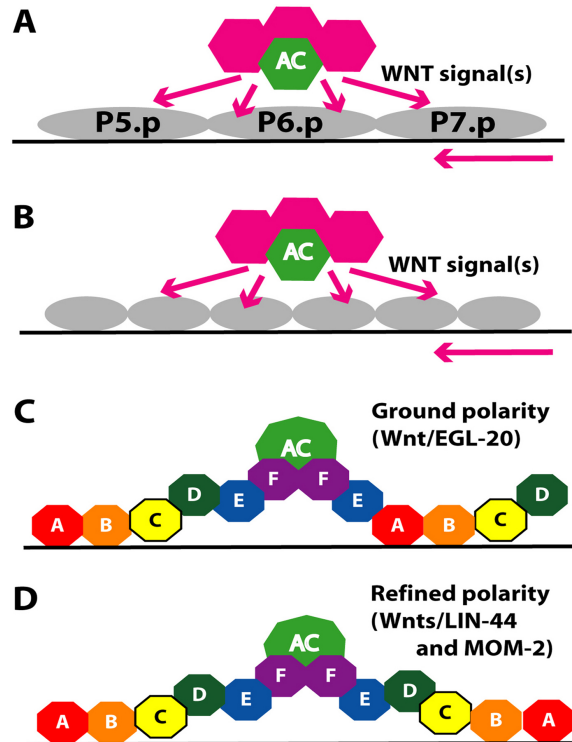


Figure 4. Polarity of $P7.p$ 2° lineage. Multiple WNT signals from the tail region (not shown), anchor cell (green) and other gonadal cells (pink) help establish the correct polarity of the $P5.p$ and $P7.p$ lineage cells. Wnt/EGL-20 originating from the tail region orients cells towards the posterior (ground polarity). This pattern is reversed in the $P7.p$ lineage by Wnts/LIN-44 and MOM-2 (refined polarity) such that it has an DCBA pattern rather than the default ABCD pattern as does $P5.p$. A. early-L3. B. mid-L3. C and D. late-L3. Model is based on published studies (Deshpande et al., 2005; Green et al., 2008; Inoue et al., 2004).

The levels of nuclear POP-1 (TCF/LEF family), a WNT target, correlate with WNT signaling: a High-Low (HL) pattern is observed in $P5.p$ daughters and HLHL pattern in $P5.p$ granddaughters, while a LH then a LHLH pattern is seen in the case of $P7.p$ (Deshpande et al., 2005). Thus, the pattern of POP-1 protein localization in the $P7.p$ lineage is reversed from the default by WNT signaling through LIN-17 and LIN-18. This ultimately leads to reversal of $P7.p$ polarity and giving rise to a vulva with single invagination where the two halves are mirror symmetric. A reporter for POP-1 activation of transcription, POPTOP (Green et al., 2008), indicates that $P7.p$ polarity reversal is largely independent of POP-1-mediated transcriptional activity. Reduction of function in the SP1 family protein SPTF-3 causes a similar $P7.p$ polarity reversal, leading to the hypothesis that SPTF-3 interacts with Wnt signaling in some way. However, this remains to be investigated (Ulm et al., 2011).

3.2. Vulval cell migration and invagination

At the end of L3 even before vulval cell divisions are complete, the distal vulval cells begin to move, migrating under their proximal neighbors and pushing the proximal cells dorsally. Invagination of these epithelial cells continues during the early L4 larval stage, forming a rudimentary vulva lumen beneath the anchor cell and between the anterior and posterior cells of the vulva. These morphogenetic movements involve cytoskeletal reorganization.

At least two different G protein-mediated signaling pathways facilitate morphogenetic changes in the vulva. A member of the ARL2 family of small GTPases called EVL-20 controls invagination of vulval cells (Antoshechkin and Han, 2002). *evl-20* mutants exhibit defects in microtubule reorganization suggesting that vulval abnormalities are caused by a failure to regulate cytoskeletal dynamics. Two other GTPases of the Rac family, encoded by *mig-2* and *ced-10*, are also necessary for appropriate division and migration of vulval cells (Kishore and Sundaram, 2002). Their activities are likely to be regulated by the Trio-like guanine nucleotide exchange factor UNC-73.

Semaphorin SMP-1 and its Plexin receptor PLX-1 represent yet another signaling pathway that acts downstream of Ras signaling and in parallel to Rac signaling to control vulval cell movements and invaginations (Dalpe et al., 2005; Pellegrino et al., 2011). The expression of SMP-1 in the VulD, E and F cells is regulated by

VAB-23 in response to the inductive signal from the AC. The inductive signal results in activation of the homeobox protein LIN-39, which binds directly to the *vab-23* promoter. VAB-23, in turn, binds the *smp-1* promoter and promotes SMP-1 expression (Pellegrino et al., 2011). Based on mutant phenotypes and expression data it has been proposed that PLX-1 and SMP-1 mediate orderly attraction of vulval cells (starting from vulF) to form toroids.

The invagination of vulval cells results in a space (lumen) (Figure 1) that expands during L3 and L4 and is filled with fluid. Lumen expansion is mediated by proteoglycans. So far eight *sqv* genes (*sqv-1* to *sqv-8*) have been identified that regulate expansion of the vulval lumen (Herman et al., 1999). Hermaphrodites with decreased *sqv* function develop only a rudimentary lumen (SQUASHED VULVA phenotype). The *sqv* genes encode a nucleotide-sugar transporter (*sqv-7*) (Berminson et al., 2001; Herman and Horvitz, 1999) and glycosaminoglycan biosynthetic enzymes that are required for synthesis of chondroitin and heparan (Herman and Horvitz, 1999; Hwang and Horvitz, 2002; Hwang and Horvitz, 2002; Hwang et al., 2003; Hwang et al., 2003). Accumulation of hygroscopic chondroitin sulfate in the vulval lumen is proposed to draw water into the lumen, generating the pressure to hold the lumen open during development (Hwang et al., 2003). The *sqv* genes are expressed broadly in the vulva with the exception of *sqv-4*, which is expressed in vulC, vulD, vulE and vulF (Hwang and Horvitz, 2002).

3.3. Formation of vulval toroids and control of cell fusion events

The mature vulva is composed of seven concentric toroids that form as each vulval cell extends processes laterally around the developing vulval lumen, contacting each other and subsequently fusing in a selective manner (Sharma-Kishore et al., 1999) (Figure 1). The multinucleate vulval toroids vulA, vulC, vulD, vulE and vulF form via homotypic fusion. The vulB1 and vulB2 cells persist as rings composed of two distinct cells (Sharma-Kishore et al., 1999).

Two FF (fusion failure) family genes, *eff-1* and *aff-1*, function independently to control cell-fusion events in the vulva (Mohler et al., 2002; Sapir et al., 2007). Both encode transmembrane proteins with similar domain organization that are conserved in nematodes. *eff-1* is expressed in a subset of vulval cells and is required for fusion of some of the vulval toroids but not vulA and vulD (Sapir et al., 2007). The expression of *eff-1* is regulated by VAB-23 in the vulva. When VAB-23 function is reduced, *eff-1* is ectopically expressed, resulting in ectopic fusions and fewer toroids (Pellegrino et al., 2011). *aff-1* is expressed in the vulD precursor cells and is required for fusion of the vulD cells and possibly the vulA cells (Sapir et al., 2007). *aff-1*, but not *eff-1*, is also involved in the AC-utse cell fusion event (see 4.5). Given that AFF-1 has no apparent role in the primary vulval lineage and that both fusogens mediate homotypic fusion events, it is difficult to envision how *eff-1* alone mediates the vulE to vulE and vulF to vulF specific fusions within the primary lineage. The fusogens may act combinatorially or other factors may regulate their activities. Alternatively strict control in the timing of expression between vulE and vulF could promote specific fusions within the primary lineage.

In addition to the FF genes, components of the vacuolar ATPase complex, *fus-1*, *vha-1* and *vha-12* (e, c and B subunits, respectively), have also been found to control cell fusion in the embryo by repressing *eff-1* activity (Kontani et al., 2005). However their roles in the vulva have not been examined.

3.4. Organization of neurons and muscles

The vulval epithelium organizes the development of the muscles and neurons that mediate egg laying (Li and Chalfie, 1990). This is most obviously seen in *dig-1* mutants in which the gonad is shifted anteriorly and the vulva, vulval muscles and neurons are all similarly shifted anteriorly (Thomas et al., 1990). Specific gene products expressed in subsets of vulval cells direct details of development of individual neurons. *bam-2* is necessary for axonal branch termination of the VC motoneurons, and is expressed in the vulF cells (Colavita and Tessier-Lavigne, 2003). *syg-1* and *syg-2* are required for synapse formation by the HSN neurons (Shen and Bargmann, 2003; Shen et al., 2004). *syg-2* is expressed in vulE cells and *syg-1* in the HSNs.

4. The vulval-uterine connection

The anchor cell is the key organizer of the vulva, the ventral uterus and the vulval-uterine connection (Figures 1 and 5). Once specified during the L2 molt (see WormBook chapter Hermaphrodite cell-fate specification), the anchor cell induces and patterns the vulva. The anchor cell also patterns the ventral uterus, inducing the nearest six of twelve ventral uterine cell (VU) granddaughters to adopt a π fate via production of the LAG-2 signal (Figure 5). Two cell types, the syncytial uterine seam (utse) cell and the four uterine vulva 1 (uv1) cells, are derived from the π lineage. In addition to its role as a signaling center, the anchor cell also plays important roles during morphogenesis of the connection between the vulva and the uterus (see 4.4 and 4.5).

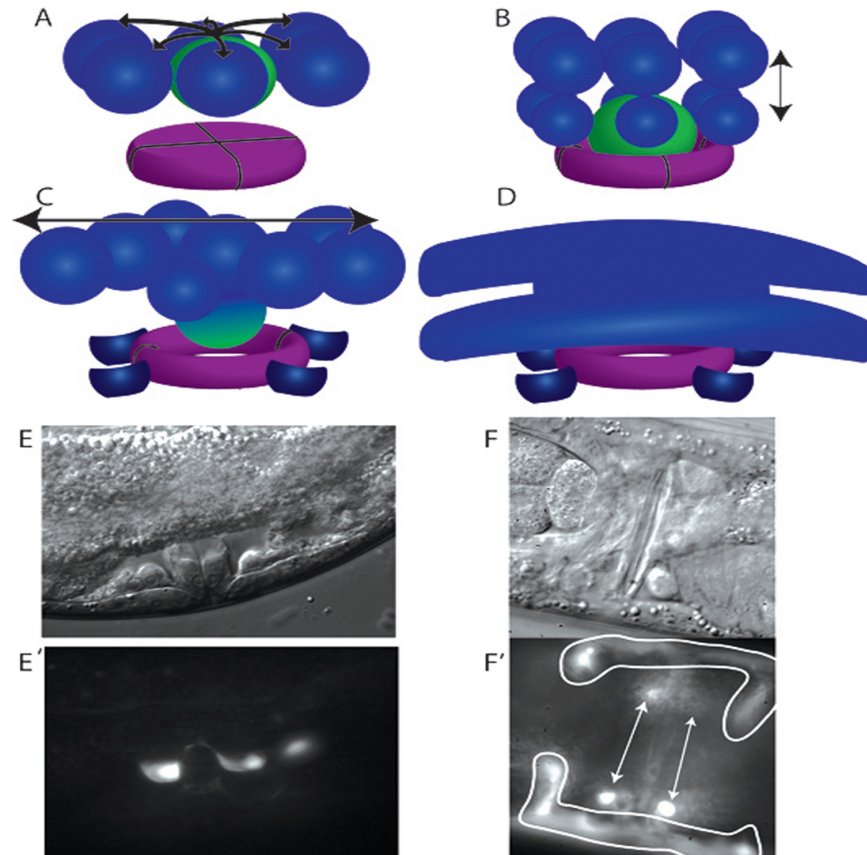


Figure 5. Uterine π cell developmental progression. (A) The six ventral uterine cells (blue) surrounding the AC (green) adopt a π fate in response to signaling (arrows) from the anchor cell. (B) The π cells divide once with a dorsal-ventral orientation (double headed arrow), producing twelve π cells. The AC invades the vulF precursors, forming a lumen in vulF (purple). (C) The ventral, outer π progeny change shape and adopt the uv1 fate (dark blue). The uv1 cells remain in close proximity to vulF. The remaining eight π progeny fuse with each other and the retreating AC to form the uterine seam (utse) cell, which expands in the anterior posterior plane (double headed arrow) to form the mature utse cell. (D) The mature utse cell has a thin cytoplasm that overlies the vulva and the uv1 cells and arms that extend laterally to the anterior and the posterior along the lateral wall of the animal. The arms curve ventrally to flank the vulva. The nine nuclei of the utse cell are found at the ends of the arms (not shown). (E and E') The uv1 cells have adopted a stereotypical morphology with a concave dorsal surface (E') by the time of vulval eversion (E). The uv1 cells are visualized with a *Pida-1::GFP* transgene, *inIs179*. (F and F') Both the uv1 cells (arrows) and utse cell (outlined) are visible in this ventral view of a young adult animal with a mature vulva. Cells are visualized with a *Pexc-9::GFP* transgene.

4.1. Induction of π cell fate

Anchor cell production of the LAG-2 signal is received in the VU cell granddaughters via the LIN-12 receptor (Newman et al., 2000; Newman et al., 1995) (Figure 5). Genetic experiments have shown that while *lag-2* is regulated by the Zn-finger gene *lin-29*, *lin-12* expression depends on the NHR family member *nhr-67* (Newman et al., 2000; Verghese et al., 2011). In epistasis analysis, the *Evi1* homolog *egl-43* is required downstream of or in parallel to *lin-12* to specify π fate (Rimann and Hajnal, 2007), and *egl-43* is likely directly regulated by the *lin-12* coactivator *lag-1* (Hwang et al., 2007). The π cells undergo a single round of dorsal-ventral oriented cell divisions and are easily distinguished from the uninduced VU granddaughter cells, which undergo two rounds of cell division, primarily in the anterior posterior plane (Newman et al., 1996). The twelve resulting π progeny give rise to the single syncytial utse cell and the four uv1 cells, which occupy positions between the vulva and the gonad and surrounding the connection between the vulval and uterine lumens (Figure 5).

4.2. Uterine seam cell differentiation

Eight of the π progeny fuse and undergo morphogenesis to form the uterine seam cell. This cell underlies the uterus and connects to the lateral seam cells, holding the uterus in place. The LIM domain transcription factor LIN-11 and the Sox domain transcription factor EGL-13 (a.k.a. COG-2) are expressed in all π cells and their daughters (Hanna-Rose and Han, 1999; Newman et al., 1999). Although π cells are still generated in either the *egl-13* or the *lin-11* mutant, each of these transcription factors is required for full differentiation of the utse cell

Cinar et al., 2003; Newman et al., 1999). In the absence of differentiation and/or maintenance of the utse cell fate in a *lin-11* or an *egl-13* mutant, the anchor cell and utse do not fuse (Hanna-Rose and Han, 1999; Newman et al., 1999) (see 4.5 AC-utse fusion). The uterine π lineage enhancer of the *lin-11* gene has binding sites for FOS-1, LAG-1 and EGL-43, each of which is required for the expression of *lin-11* in the π lineage cells (Marri and Gupta, 2009). Sumoylation of LIN-11 is also necessary for its function in π lineage specification (Broday et al., 2004). *egl-13* is co-regulated by LAG-1 and FOS-1, which each bind its promoter (Oommen and Newman, 2007). The current model for utse differentiation suggests that activation of LIN-12 leads to expression of EGL-43 (Figure 6). Then LAG-1 and EGL-43 in conjunction with FOS-1 jointly regulate expression of *lin-11* and *egl-13*.

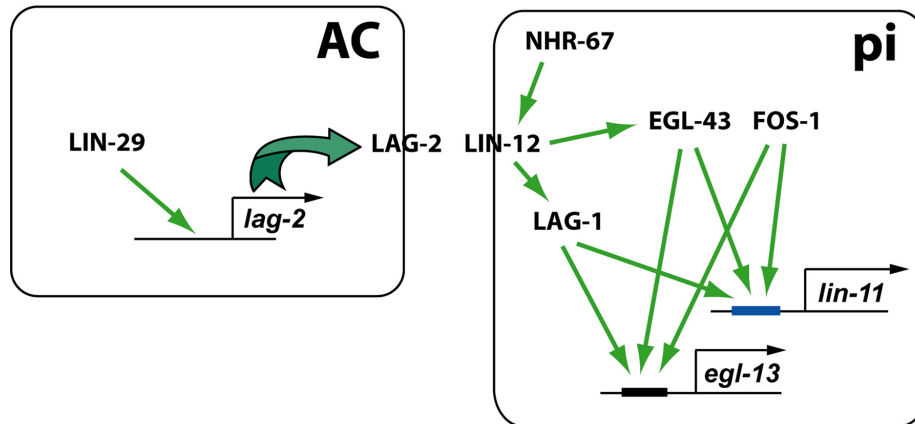


Figure 6. Regulation of uterine π cell induction. The anchor cell expresses DSL-type ligand LAG-2 in a LIN-29-dependent manner and signals presumptive π cells via LIN-12. LIN-12 activation leads to expression of *lin-11* and *egl-13*, two transcription factors necessary for utse development, including fusion of the anchor cell with utse cell. Model adapted from published studies (Cinar et al., 2003; Marri and Gupta, 2009; Newman et al., 1999; Newman et al., 2000; Verghese et al., 2011).

4.3. uv1 cell development

The remaining four π progeny generate the uv1 cells that lie under the uterus in proximity to the side and the top of the vulF cells (Figure 5). Specification of the uv1 cells first requires specification of the π lineage and then a second signaling event. The neighboring vulF cells express LIN-3 in an *egl-38*-dependent manner, and the uv1 cells respond to LIN-3 via the LET-23 receptor (Chang et al., 1999) (Figure 7). The eight π progeny that give rise to the utse do not appear to express LET-23, and a gain-of-function mutation in LET-23 does not cause a utse to uv1 fate transformation (Huang and Hanna-Rose, 2006). However, the mechanism for generating this initial difference in expression pattern between the utse and uv1 precursors is not known and the upregulation of *let-23* in the uv1 cells may be in response to the *lin-3* signal (Chang et al., 1999). It has been suggested that the utse fate is the default fate in the π lineage since uv1 cells adopt the utse fate in certain mutant situations (e.g., in *egl-38* mutants) (Rajakumar and Chamberlin, 2007) whereas no utse to uv1 fate transformation has been observed.

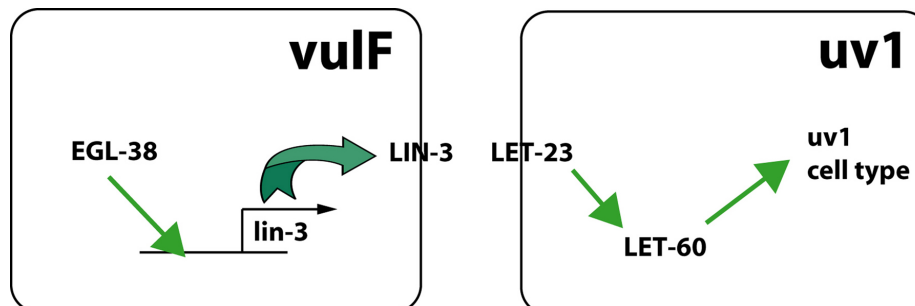


Figure 7. uv1 specification. The vulF cell expresses LIN-3 and signals the presumptive uv1 cells via LET-23 and LET-60. *egl-38* is expressed in vulF and is necessary for *lin-3* expression in the same cell (Chang et al., 1999; Rajakumar and Chamberlin, 2007). Activated LET-23 bypasses this requirement for EGL-38 function.

The uv1 cells are hypothesized to be mechanosensory neurosecretory cells (Alkema et al., 2005; Jose et al., 2007; Kim and Li, 2004). They express TRP channels that negatively regulate egg laying. Dominant negative mutations in *ocr-2*, a TRP channel subunit, have increased rates of egg laying under high osmolarity conditions (Zhang et al., 2010) and a concomitant decrease in number of eggs held in the uterus (Jose et al., 2007). This egg-laying function requires expression of *ocr-2* within the π lineage uv1 and/or utse cells (Jose et al., 2007). TRP channel subunits appear to be co-expressed only in the uv1 cells and not the utse. Nonetheless, animals engineered to have no uv1 cells (by induction of *let-23* RNAi specifically in the π lineage) have no detectable increase in egg laying under high osmolarity conditions and no decrease in egg accumulation in the uterus (Johnson et al., 2009) (W. Hanna-Rose, unpublished). These animals also have no detectable defects in morphogenesis of the vulval-uterine connection (Johnson et al., 2009). Thus, the role of the uv1 cells is not clear.

4.4. AC invasion and vulF lumen formation

In addition to inducing π cell fate, the anchor cell also promotes formation of a direct physical connection between the vulva and the uterus via other cell biological processes. The gonad and the vulval epithelium are originally separated by basement membranes secreted by each tissue. The anchor cell responds to a netrin signal from the ventral nerve cord by assembling an invasive membrane rich in actin and phospholipid PI(4,5)P₂ adjacent to the basement membranes (Ziel et al., 2009). The INA-1-PAT-3 integrin heterodimer is required in the anchor cell for assembly of the invasive membrane and subsequent invasion of the basement membrane (Hagedorn et al., 2009). After the invasive membrane is formed, the anchor cell degrades and removes the basement membranes and invades, coming to lie with its basolateral side embedded in the apex of the vulva (Figure 1) (Sherwood et al., 2005; Sherwood and Sternberg, 2003). Three transcription factors, *fos-1a*, *egl-43* and *nhr-67*, control the invasive behavior of the anchor cell (Hwang et al., 2007; Rimann and Hajnal, 2007; Sherwood et al., 2005; Verghese et al., 2011). *fos-1a*, a component of the AP-1 transcriptional regulator, is strictly required for anchor cell invasion and acts upstream of *egl-43*. Some downstream targets of *fos-1a* and *egl-43* with roles in invasion include the metalloproteinase *zmp-1*, *him-4* hemiceitin and the Fat-like protocadherin *cdh-3*. These genes have partially penetrant invasion defects, suggesting redundant function in invasive processes.

Anchor cell invasion usually occurs only in the presence of the vulva, but the anchor cell can invade in ~20% of vulvaless animals (Sherwood and Sternberg, 2003). During invasion, the anchor cell extends processes towards the developing 1° lineage. The anchor cell apparently responds to cues from vulF (and possibly negative cues from vulE) to locate the vulF cells (Sherwood and Sternberg, 2003). When the anchor cell is not ideally situated above the vulF precursor cells, it can send its process by the vulE cells to locate the vulF cells. The as-yet-unidentified signal from the vulva to the invading anchor cell acts at a distance because a distant 1° VPC can attract extension of the anchor cell. A large-scale screen for pro-invasive genes has identified other genes that play no role in invasive membrane formation and appear to be outside the *fos-1a* pathway, indicating that other aspects of anchor cell invasion in addition to the vulval cue have yet to be elucidated (Matus et al., 2010).

A second function of anchor cell invasion is to contribute to lumen formation in the vulva. The anchor cell, which has breached the basement membranes, sits in a pocket surrounded by the four vulF precursors (Figure 5) (Estes and Hanna-Rose, 2009). One of the anchor cell processes that has extended towards vulF penetrates between the vulF precursors and is followed by a significant portion of the anchor cell body (Figure 1). The basolateral surface of the anchor cell appears to contact the vulval lumen (Sherwood and Sternberg, 2003). While no defects in vulF specification have been detected when the anchor cell fails to occupy this position, the lumen in vulF fails to form (Estes and Hanna-Rose, 2009). Thus, the anchor cell is proposed to be key to opening the dorsal side of the vulva lumen potentiating a connection to the uterine lumen.

4.5. AC-utse fusion

After the anchor cell completes its signaling and invasion functions during morphogenesis, it sits in a position that blocks the connection between the developing uterine and vulval lumens. To clear this opening, the utse cell syncytium fuses to the anchor cell during the L4 stage, leaving a thin process of utse cell cytoplasm overlying the vulva (Figures 1 and 5) (Kimble and Hirsh, 1979; Newman et al., 1996). This hymen is ruptured when the first egg is laid, establishing a direct connection between the uterine and vulval lumens. The fusogen AFF-1 is necessary for AC-utse fusion as well as other cell fusion events in *C. elegans* development (Sapir et al., 2007). *aff-1* is expressed in a FOS-1A-dependent manner in both the AC and the utse, promoting homotypic fusion (Sapir et al., 2007). A missense mutation in the N-ethylmaleimide-sensitive factor NSF-1 specifically abrogates anchor cell-utse cell fusion, but not other cell fusions (Choi et al., 2006), and the *nsf-1L* isoform of the *nsf-1* gene acts in the anchor cell

to promote fusion. Whereas, *AFF-1* is a membrane protein with an extracellular domain that mediates homotypic fusion, the role of the intracellular vesicle trafficking protein *NSF-1* is not clear.

4.6. AC transcriptional program

The regulatory circuitry controlling the anchor cell's many functions has started to be elucidated (Figure 8). Anchor cell expression of *LIN-3*, which induces the vulva, requires *HLH-2* and *NHR-25* or a related protein (Hwang and Sternberg, 2004). Anchor cell expression of *LAG-2*, which induces uterine π cells, requires *LIN-29* and *NHR-67* (Newman et al., 2000; Verghese et al., 2011). However, the regulation of anchor cell expression of the wnt proteins *MOM-2* and *LIN-44*, which orient the polarity of the *P5.p* and *P7.p* lineages toward the anchor cell, is unknown.

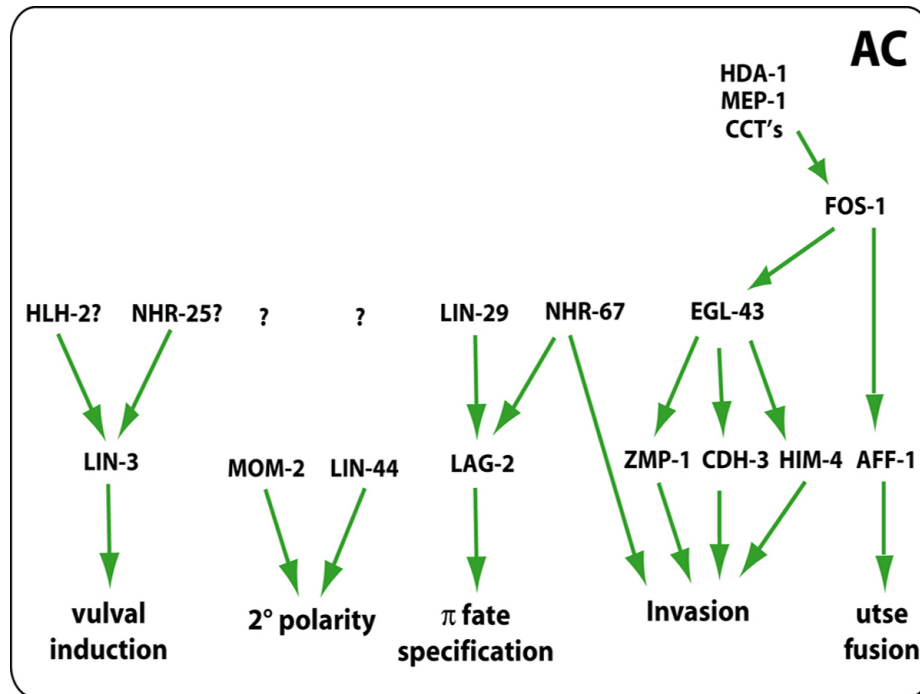


Figure 8. Signaling network in the AC. The AC coordinates multiple processes in the egg-laying system by activating expression of several genes during L3 and L4 stages. In addition to producing *LIN-3*/EGF ligand that activates Ras signaling in VPCs, the AC is also the source of two Wnt ligands, *MOM-2* and *LIN-44*, that control 2° lineage polarity. The invasion of AC into vulval epithelium is controlled by *nhr-67*, *fos-1*, and *egl-43*, and their downstream targets. In the case of utse fusion *fos-1* controls *aff-1* function. The upstream regulators of *fos-1* include genes such as *hda-1*, *mep-1* and *cct* complex.

Anchor cell invasion requires function of *fos-1a* (*fos* proto-oncogene family) (Sherwood et al., 2005), the long isoform of *egl-43* (*Evi1* proto-oncogene family) (Hwang et al., 2007; Rimann and Hajnal, 2007) and *nhr-67* (NHR family) (Verghese et al., 2011). Histone deacetylase *HDA-1*, zinc finger protein *MEP-1* and components of the cct chaperonin complex function to control expression of *fos-1a* as well as efficient assembly of the invasive membrane (Matus et al., 2010).

FOS-1a is necessary for *egl-43* expression but not vice versa (Hwang et al., 2007), and a *FOS-1* responsive element in the *egl-43* promoter contains a non-canonical binding site for *FOS-1*, suggesting direct regulation of *egl-43* by *FOS-1a* (Rimann and Hajnal, 2007). Both *FOS-1a* and *EGL-43* are required for expression of target genes *zmp-1*, *cdh-3* and *him-4* (Hwang et al., 2007; Rimann and Hajnal, 2007; Sherwood et al., 2005). The current model for the transcriptional network posits that *EGL-43* regulation of these targets is direct and that *FOS-1* may regulate them via *EGL-43* and perhaps directly as well (Figure 8).

5. Concluding remarks

Considerable progress has been made in the last decade towards identifying cell type-specific-transcriptional networks, cell polarization mechanisms, and morphogenetic changes that form the vulva and the vulval-uterine

connection. As a result we have learned that the polarity of the 2^o lineage vulval cells is controlled by a β -Catenin-TCF/LEF-mediated Wnt signaling pathway. The vulval toroids are formed by coordinated morphogenetic events and selective cell fusion. We also have expanded our understanding of the role of the AC in the formation of vulval-uterine connection. In spite of this our mechanistic insights of these events is far from complete. For example, we know very little about the downstream targets of transcriptional networks and how they confer unique cell identities, how cell divisions and cell fates are coupled and, how the timing of different cellular events in the vulva and uterus are coordinated. Likewise, we have rudimentary knowledge of the mechanisms regulating morphogenetic processes such as cell adhesion and cell migration. We expect that in the near future the availability of various genetics and genomics tools will help find answers to some of these questions thereby providing a more coherent picture of cellular and molecular processes in the formation of the egg-laying system.

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