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# Signal transduction during *C. elegans* vulval development: a NeverEnding story

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The *Caenorhabditis elegans* hermaphrodite vulva is one of the best studied models for signal transduction and cell fate determination during organogenesis. Systematic forward genetic screens have identified a complex and highly interconnected signaling network formed by the conserved EGFR, NOTCH, and WNT signaling pathways that specifies an invariant pattern of cell fates among the six vulval precursor cells (VPCs). Multiple inhibitory interactions between the EGFR and NOTCH pathways ensure the selection of a single 1° VPC that is always flanked by two 2° VPCs thanks to lateral NOTCH signaling. Building on this 'central dogma' of cell fate specification, scientists have investigated a broad spectrum of novel questions that are summarized in this review. For example, vulval development is a unique model to study the intracellular trafficking of signaling molecules, such as NOTCH or EGFR, to investigate the interactions between the cell cycle and cell fate specification pathways, and to observe epithelial tube morphogenesis and cell invasion at single-cell resolution. Finally, computer scientists have integrated the experimental data into mathematical and state-based '*in silico*' models of vulval development, allowing them to test the completeness and limits of our current understanding.

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## A central dogma: the interplay of Wnt, EGFR, and NOTCH signaling determines the 1° and 2° vulval cell fates

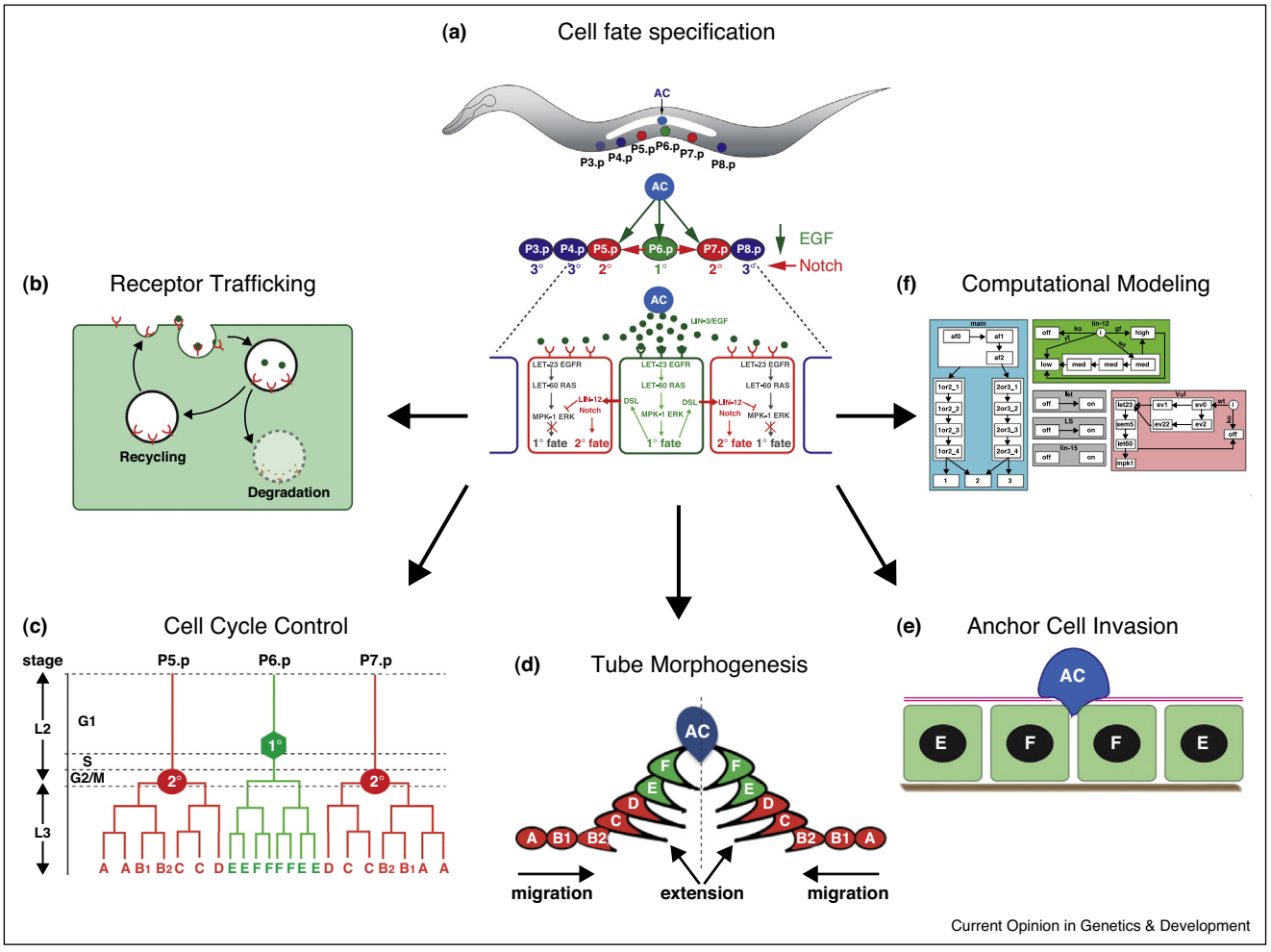
### From the P lineage to the vulval competence group: Wnt and EGFR signaling maintain VPC competence

The *C. elegans* vulva originates from the ventral epidermal P cells that divide during the first larval stage (L1) into Pn.a and Pn.p daughter cells [1,2••]. The anterior Pn.a cells will later differentiate into ventral cord neurons, whereas the posterior Pn.p cells form the epidermis. At the end of the L1 stage, a Wnt signal from the posterior body region selects six Pn.p cells (P3.p through P8.p) in the mid-body region to become the vulval precursor cells (VPCs) and form the vulval competence group (Figure 1a) [3–5]. Canonical Wnt signaling maintains the VPCs as polarized epithelial cells by inducing the *hox* gene *lin-39* [6–8]. Among other functions (see below), *lin-39* prevents the fusion of the VPCs with the surrounding syncytial epidermis (hyp7) by repressing the expression of the fusogen *eff-1* [9–11]. The anterior (P1.p and P2.p) and posterior (P9.p to P11.p) Pn.p cells fuse with hyp7 and loose their potential to differentiate. An interesting case is P3.p, the VPC at the anterior border of the competence group; in around 50% of the animals, P3.p looses its competence before the end of the L2 stage and fuses with hyp7 [2••,12]. However, P3.p fusion can be prevented by overexpression of the EGF growth factor LIN-3, indicating that EGFR signaling acts redundantly with the Wnt pathway to induce *lin-39* expression [13,14]. Thus, the vulval equivalence group is specified by cooperative Wnt and EGFR signaling.

### 1° cell fate specification by the anchor cell

Beginning in the L2 stage, the anchor cell (AC) in the somatic gonad secretes the LIN-3 protein, a member of the epidermal growth factor family (Figure 1a) [15,16]. Even though LIN-3 is produced as a transmembrane precursor similar to mammalian TGFα, LIN-3 is released from the AC in a graded manner, and activates the LET-23 EGFR in all VPCs [17–19]. However, when expressed at a normal dosage LIN-3 is efficiently sequestered by the VPC closest to the AC, P6.p, which presents the highest levels of LET-23 on its basolateral membrane [20•,21]. Since P6.p receives most of the LIN-3 signal, it is the only VPC that adopts the 1° vulval cell fate. Downstream of the EGFR tyrosine kinase, a canonical RAS/MAPK pathway transduces the signal into the nucleus. Conserved components of the core pathway include the adaptor protein SEM-5 (GRB2) [22], the guanine exchange factor SOS-1 [23] and the RAS protein LET-60 [24], which activates the LIN-45 RAF [25], MEK-2 MEK [26] and MPK-1 MAP kinase cascade [27]. MPK-1 activation is both necessary and sufficient to induce the 1° vulval cell fate [28]. To date, the ETS family LIN-1 and *forkhead* LIN-31 transcription factors are the only known MPK-1 substrates [29–31]. In their unphosphorylated state, LIN-1 and LIN-31 form a complex that inhibits vulval induction by repressing 1°-specific transcription [32]. After

**Figure 1**




Overview of vulval development. Different aspects of vulval development that have been investigated so far include **(a)** the mechanisms of cell fate specification and pattern formation, **(b)** the intracellular trafficking of NOTCH and EGFR, **(c)** the interactions between cell cycle and cell fate specification, **(d)** the morphogenesis of the vulval cells into an epithelial tube, **(e)** the invasion of the AC into the vulval epithelium, and **(f)** the computational modeling of the cell fate specification pathways.

phosphorylation by MPK-1, the LIN-1/LIN-31 complex dissociates, phospho-LIN-1 cannot repress anymore, while phospho-LIN-31 is turned into an activator of 1° gene expression [29]. Other transcription factors acting downstream of the RAS/MAPK pathway include SUR-2 and LIN-25, which are both components of the transcription mediator complex [33,34]. One key target of the LIN-1/LIN-31 repressor complex is the *lin-39 hox* gene [8,35]. *lin-39* is required for 1° cell fate execution by coordinating vulval cell proliferation and morphogenesis [9,36–38]. Again, Wnt and RAS/MAPK signaling act in parallel to induce *lin-39* expression during fate specification, as Wnt signaling can partially compensate for a reduction in EGFR signaling [39]. Besides the core components of the EGFR pathway, systematic genetic screens have identified a number of modifiers and negative regulators of the EGFR pathway. For example, the tyrosine kinase *ark-1* [40], the *cbl* homolog *sli-1* encoding an E3 ubiquitin ligase [41] or the receptor tyrosine phosphatase *dep-1*, which de-phosphorylates LET-23 [42], all function as negative regulators of the EGFR.

### 2° cell fate specification: sequential induction by NOTCH and graded LIN-3 EGF signaling

The 1° VPC P6.p up-regulates the expression of three partially redundant ligands of the Delta/Serrate family, *lag-2*, *dsl-1*, and *apx-1* [43]. These ligands activate the NOTCH receptor LIN-12 in the adjacent VPCs P5.p and P7.p (Figure 1a). While *lag-2* and *apx-1* encode membrane-bound ligands, *dsl-1* encodes a secreted protein that may activate NOTCH signaling without direct cell contact [44•]. One outcome of NOTCH signaling is the repression of the EGFR signaling pathway in P5.p and P7.p, a classical example of lateral inhibition [45]. LIN-12

NOTCH induces the transcription of several negative regulators, such as the MAPK phosphatase *lip-1*, *ark-1*, the adaptin homolog *dpy-23*, which may regulate EGFR endocytosis, or the *lst* genes [46,47]. However, LIN-12 NOTCH signaling also plays an instructive role during the subsequent 2° vulval fate specification, as a constitutively active NOTCH receptor causes all VPCs to adopt the 2° fate even in the absence of the inductive LIN-3 signal [16,48]. The distal VPCs P3.p, P4.p and P8.p that receive neither inductive the LIN-3 nor the lateral NOTCH signal adopt the 3°, non-vulval cell fate [1]. These VPCs divide once and fuse with hyp7.

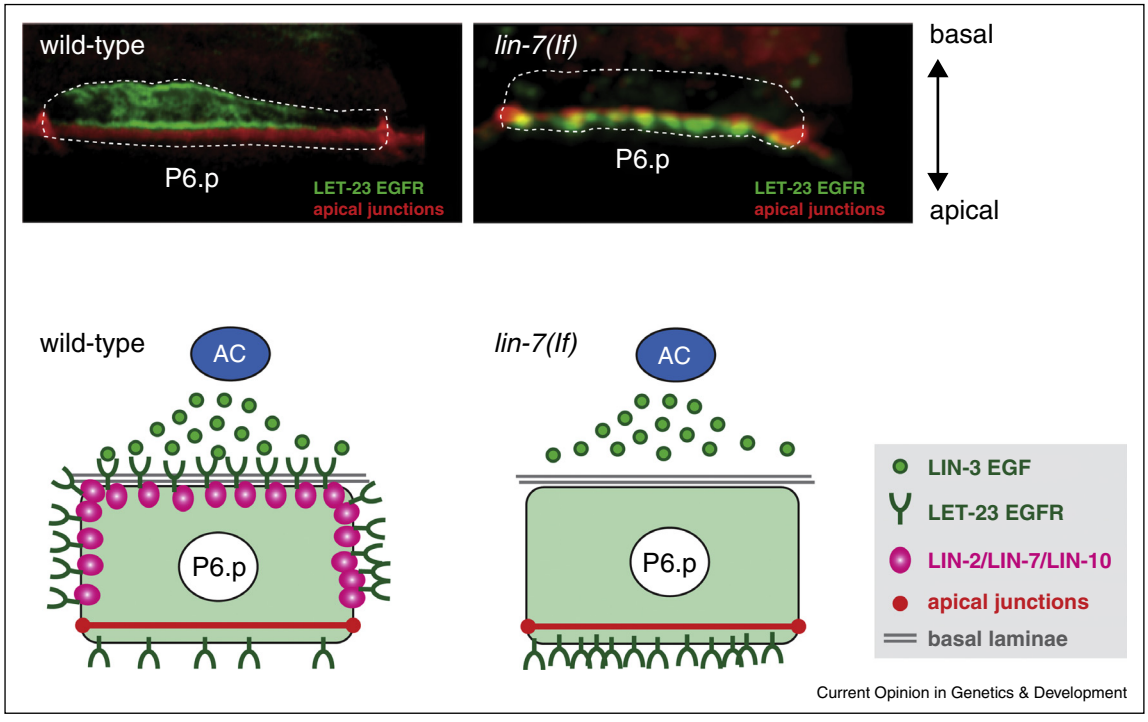
One hotly debated question has been the relative contribution of EGFR and NOTCH signaling towards 2° fate specification [18,49,50]. While *let-23* mosaic experiments demonstrated that VPCs lacking *let-23* or other components of the RAS/MAPK pathway can adopt the 2° fate, as long as they are adjacent to a 1° VPC [49], *lin-3* dosage experiments indicated that an isolated VPC can adopt a 2° fate if exposed to an intermediate LIN-3 concentration [18]. Furthermore, it has been proposed that VPCs receiving intermediate levels of LIN-3 may adopt the 2° fate through autocrine stimulation via the secreted NOTCH ligand DSL-1 [44•]. However, Zand *et al.* [51••] identified a RalGEF as an alternate RAS effector that antagonizes the canonical RAF/MAPK pathway in P5.p and P7.p and inhibits 1° fate specification. Thus, the lateral NOTCH signal together with a graded LIN-3 signal activating an alternate RAS/RAL pathway ensure that a 1° VPC is always flanked by two 2° VPCs.

## The story goes on: new topics in vulval development

### Receptor localization and trafficking control signaling

Thanks to the transparent body, it is possible to observe in live larvae the intracellular trafficking of signaling molecules that determine the VPC fates (Figure 1b). In particular, the two receptors LIN-12 NOTCH and LET-23 EGFR show a dynamic expression pattern and rapid protein turnover depending on the VPC fates [52–54]. *lin-2*, *lin-7*, and *lin-10*, which were among the first lineage defective (*lin*) mutants identified, encode components of a conserved protein localization complex that retains the EGFR on the basolateral plasma membrane of the 1° VPC (Figure 2) [53,55]. Basolateral EGFR localization is essential for receptor activation because only the basolateral membrane compartment of the VPCs is exposed to the LIN-3 EGF signal from the gonadal AC. On the other hand, the small GTPase ARF together with its exchange factor AGEF-1 and the AP1 component UNC-101 antagonize basolateral EGFR localization via the LIN-2/LIN-7/LIN-10 complex [56•]. Other factors regulating EGFR trafficking include the EPS-8 protein, which

**Figure 2**




LET-23 EGFR localization. A tripartite LIN-2/LIN-7/LIN-10 localization complex retains the LET-23 EGFR on the basolateral membrane, allowing efficient receptor activation in the 1° VPC P6.p. In *lin-7(lf), lin-2(lf)* or *lin-10(lf)* mutants, the EGFR is mislocalized to the apical compartment and cannot bind the LIN-3 growth factor ligand.

inhibits RAB-5 mediated receptor endocytosis [57], and the actin binding protein ERM-1, which regulates EGFR mobility [54]. Especially, in the 2° VPCs, RAB-5 and RAB-7 mediated receptor endocytosis removes the EGFR from the basolateral membranes, resulting in rapid receptor degradation [58].

The expression pattern and localization of the LIN-12 NOTCH receptor is a mirror image of LET-23 EGFR expression [59,60]. After vulval induction, LIN-12 is rapidly degraded in the 1° lineage and up-regulated in the 2° VPC, where it accumulates on the apical plasma membranes [61]. Binding of a DELTA ligand induces proteolytic cleavage of NOTCH via the presenilin intramembrane protease SEL-12, releasing the intracellular domain that translocates into the nucleus and activates 2°-specific gene expression [62]. However, activation of the RAS/MAPK pathway in the 1° VPC inhibits NOTCH signaling by inducing endocytosis from the apical membrane and lysosomal degradation [61]. This NOTCH destruction pathway requires a di-leucine containing endosomal sorting signal and serine/threonine phosphorylation of the intracellular domain. Trans-factors controlling LIN-12 trafficking include ALX-1, a homolog of mammalian Alix, the ubiquitin ligase WWP-1 and the neurobeachin homolog SEL-2 [52,63]. Thus, specific intracellular trafficking pathways are used to establish the differential activation of the NOTCH and EGF receptors in the 2° and 1° VPCs, respectively.

### Cell cycle control and signal transduction

After the vulval competence group has been specified, the VPCs spend most of the L2 stage arrested in the G1 phase of the cell cycle (Figure 1c) [64]. Even though MAPK signaling is sufficient to induce the 1° cell fate, premature MPK-1 activation does not induce precocious VPC proliferation [28]. VPC quiescence depends on the heterochronic genes *lin-14* and *lin-28*, which are part of a miRNA network that controls the transition from one larval stage to another [65]. In *lin-14(lf)* or *lin-28(lf)* mutants, the VPCs divide precociously in early L1 or L2 larvae, respectively, yet they adopt a normal 3°–2°–1°–2°–3° fate pattern [66]. Thus, the EGFR and NOTCH pathways can function at earlier time points. One key downstream target of the heterochronic pathway is *cki-1*, a cyclin-dependent kinase (*cdk*) inhibitor homologous to mammalian p21 that is required for G1 arrest [64,67]. *cki-1* expression in quiescent VPCs is promoted by LIN-1 and LIN-31 [68]. Hence, *lin-31* or *lin-1* mutants occasionally exhibit precocious VPC divisions [31]. A key factor promoting VPC proliferation after fate specification, once *cki-1* levels have declined, is the *lin-39 hox* gene. In *lin-39(lf)* mutants, in which cell fusion is blocked through an *eff-1(lf)* mutation, the VPCs maintain their competence and can respond to the inductive LIN-3 signaling (Roiz, Hajnal, unpublished data), but they do not proliferate [9].

Certain components of the cell cycle machinery control the activities of the cell fate specification pathways. For example, mutations in the cyclin E homolog *cye-1* not only lengthen the G1 phase and affect cell divisions, but they also perturb 1° and 2° fate specification [69]. Especially, the outcome of LIN-12 NOTCH signaling depends on the cell cycle phase; during G1, LIN-12 inhibits 1° fate specification by repressing RAS/MAPK signaling, while the instructive signal specifying the 2° fate only occurs during the G2 phase (Figure 1c) [70]. The heterochronic LIN-14 protein inhibits LIN-12 signaling before induction [71•], and LIN-12 NOTCH protein turnover is induced by cell cycle progression [72•]. The G1 cyclin CYD-1 stabilizes LIN-12 NOTCH at the apical membrane, while the cyclin B homolog *cyb-3* promotes the degradation of the intracellular NOTCH fragment in the 1° VPC during the G2 phase. After vulval fate specification, the VPCs undergo three rounds of cell divisions generating 22 vulval cells. The *cullin* gene *cul-1* and *lin-23*, which encode components of a SCF ubiquitin ligase complex, are required for cell cycle exit after the third division round, possibly by inducing CDK/Cyclin degradation [73,74].

### Vulval morphogenesis and epithelial tube formation

The 22 descendants of the induced VPCs further differentiate into seven subfates. The seven 2° cells on each side adopt the vulA, vulB1, vulB2, vulC, and vulD subfates and form the outer part of the vulva, the eight 1° cells adopt the vulE and vulF subfates and form the inner part of the organ (Figure 1d) [75,76]. The opposing orientation of the 2° subfates along the anterior-posterior axis (ABCD in P5.p versus DCBA in P7.p descendants) is established by two superimposed WNT signals [77••]. EGL-20 WNT secreted by tail cells establishes the 'ground' polarity ABCD, while the MOM-2 and LIN-44 WNTs secreted by the AC specifically reverse the orientation of the P7.p subfates by activating the LIN-18 RYK and LIN-17 Frizzled receptors. This results in a mirror symmetrical ABCDEFFEDCBA pattern with a central axis between the vulF cells defining the vulval midline.

The vulval cells migrate towards this midline and extend circumferential protrusions until they meet their contralateral partner cells and form homotypic cell contacts [75]. The two anterior vulE cells connect with the posterior vulE cells and so on. Finally, homotypic cell fusions mediated by the fusogens *eff-1* and *aff-1* yield the vulval toroids, ring-like shaped syncytia with a central hole formed by the apical surface (except for the vulB1 and vulB2 toroids, which remain unfused) [78•]. Thus, the vulva is formed by a stack of seven toroids, each consisting of cells with the same subfate. The vulA toroid forms the outer, ventral and the vulF toroid the inner, dorsal part of the organ.

A Semaphorin/Plexin signaling pathway guides the cells to the midline and mediates homotypic contact formation

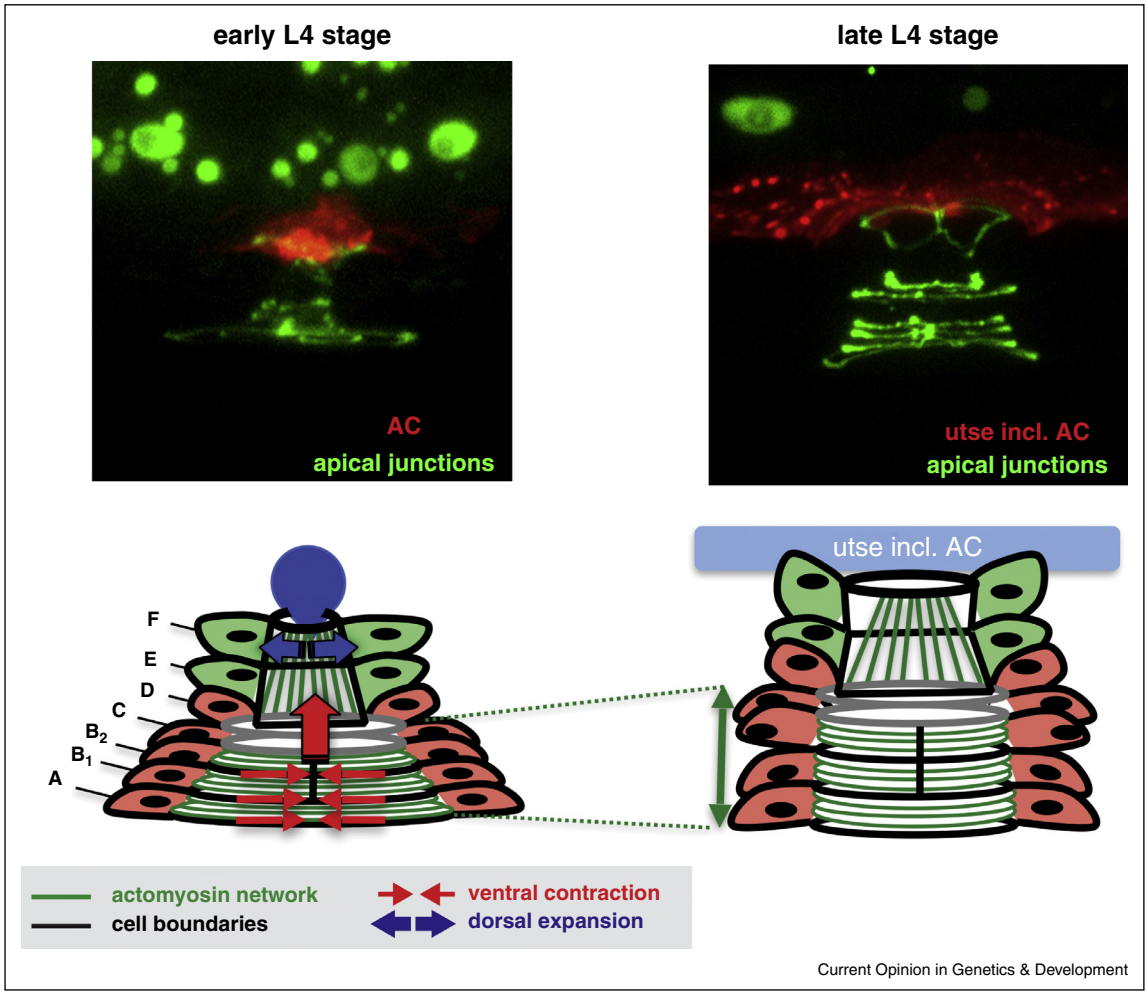
[79,80]. SMP-1 Semaphorin, which is initially produced by the AC, activates via the PLX-1 receptor a CED-10/MIG-2 RAC signaling pathway. The signal then propagates from the dorsal to the ventral vulval cells, as the signal receiving vulF and vulE cells become signal producing cells that activate PLX-1 signaling in the adjacent vulD cells. Unlike Semaphorin signaling in other systems, SMP-1 has an attractive rather than a repulsive effect on the vulval cells [81]. Besides controlling cell proliferation, the *hox* gene *lin-39* is also involved in toroid formation [9]. LIN-39 induces the expression of the VAB-23 zinc finger protein, which in turn activates *smp-1* expression [38].

The cylindrical shape of the apical toroid lumen is established through the sequential contraction of the ventral and expansion of the dorsal toroids (Figure 3) [82•]. Ventral contraction is mediated by the RHO kinase LET-502, which is up-regulated by NOTCH signaling in 2° cells and induces the constriction of the circular actomyosin network in the vulA and vulB1/2 toroids. Dorsal expansion, on the other hand, requires the EGL-26 palmitoyltransferase in vulE cells and the prior invasion of the AC into the dorsal lumen (Figure 1e) [83,84]. At the same time, the secretion of chondroitin and heparan sulfate carrying glycoproteins into the apical lumen creates a hydrostatic pressure that keeps the lumen expanded [85–88].

## Anchor cell invasion during morphogenesis

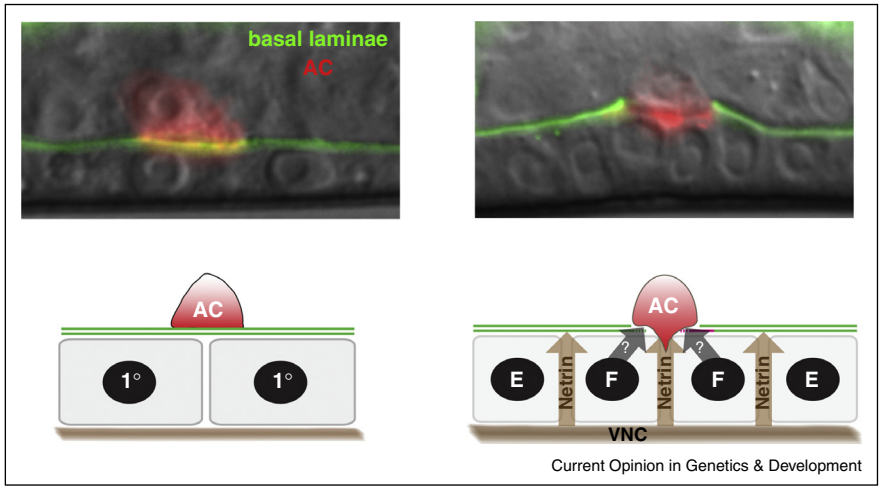
A key event during vulval morphogenesis is the invasion of the AC into the vulval tissue (Figure 1e) [89••]. Before the last round of cell divisions, the AC undergoes an epithelial to mesenchymal transition, breaches two basal laminae that separate the somatic gonad from the ventral epidermis and extends actin-rich filopodia into the 1° vulval tissue (Figure 4). *fos-1*, a homolog of the mammalian *fos* proto-oncogene, encodes a key regulator of AC

**Figure 3**




Lumen morphogenesis. The top panels show microscopic images of the apical toroid junctions stained in green and the AC stained in red before (left) and after (right) lumen contraction. Contraction of the ventral toroids (red arrows) via the actomyosin network (green lines) followed by expansion of the dorsal toroids (blue arrows) by the invading AC shape the vulval lumen during the L4 stage. Cell boundaries are indicated with black lines.

**Figure 4**




AC invasion. The top panels show two mid L3 larvae before (left) and after (right) basal laminae breaching. The AC is stained in red and the basal laminae are labelled with Laminin::GFP in green. A Netrin signal from the ventral nerve cord (VNC) (brown arrows) together with an unknown guidance cue from the 1° vulval cells (gray arrows) polarize the AC during invasion.

invasion [90]. The FOS-1 transcription factor induces via the EGL-43 zinc finger protein the expression of several pro-invasive factors, such as the *zmp-1* metalloprotease, the *him-4* hemicentin, or the *cdh-3* proto-cadherin [90,91]. *fos-1* thus allows the AC to cross the basal laminae and establish direct contact with the vulF cells. Furthermore, an UNC-6 Netrin signal from the ventral nerve cord together with an unknown guidance cue from the 1° cells polarize the AC along the dorso-ventral axis, which is necessary to guide the invasive protrusions ventrally [92•,93]. AC invasion permits the expansion of the dorsal toroids during the final phase of morphogenesis (Figure 3) [83]. After having completed these tasks, the AC fuses with surrounding uterine cells, forming a syncytial sheet called utse [78•].

Normal AC invasion resembles in many aspects the changes that occur in invasive tumor cells that migrate away from their tissue of origin and enter blood or lymph vessels. Hence, the developmental control of AC invasion may shed light on the molecular events occurring during tumor metastasis [94].

### Computational modeling of vulval development: are we done?

To integrate and formalize our knowledge about vulval fate specification, a number of computational *in silico* models have been constructed (Figure 1f). Two principal approaches were taken: first, mathematical models represent the components of signaling pathways as differential equations [44•,95,96]. Such models can make quantitative predictions about the activity changes of each component, thus permitting a detailed analysis. However, mathematical models require the input of quantitative parameters, such as reaction rates and affinity constants, that are difficult to measure in the VPCs and can only be estimated. Second, in state-based models, each component passes through a defined number of discrete activity states [72•,97,98•,99,100]. Although, state-based models are less detailed, they can include larger numbers of components and thus permit the analysis of entire signaling networks. Even though the field is still in its early stages, both types of models have yielded new insights into VPC fate specification, especially with respect to the kinetics of induction and the temporal order of the signaling events.

## Concluding remark

More than 25 years after the seminal papers by Sternberg and Horvitz on pattern formation during vulval development [2••,16], many questions about the molecular mechanisms underlying cell fate specification have been answered. Yet, these answers have left us with a wide spectrum of new questions to be investigated in this seemingly simple model of organogenesis.

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