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The transcription factor VAB-23 links vulval cell fate specification and morphogenesis

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During organogenesis, individual cells must commit to and execute specific cell fates. However, the molecular mechanisms linking cell fate specification to fate execution and morphogenesis remain a largely unexplored area in developmental biology. The *Caenorhabditis elegans* vulva is an excellent model to dissect the molecular pathways linking cell fate specification and execution during organogenesis. We have recently identified a conserved nuclear zinc finger transcription factor called VAB-23 that plays essential roles during vulval torid formation in the larva and ventral epidermal closure in the embryo. VAB-23 regulates the transcription of specific target genes including *smp-1* Semaphorin. EGFR/RAS/MAPK signaling upregulates via the HOX protein LIN-39 the expression of VAB-23 in the 1° vulval cell lineage, indicating that cell fate specification and execution are temporally overlapping and tightly linked processes. Here, we discuss the roles of VAB-23 in morphogenesis and the implications of its regulation on the spatio-temporal control of organogenesis.

Keywords: *C. elegans*, HOX gene, vulva, RAS, signal transduction, zinc finger

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Vulval Development as a Model for Cell Fate Specification and Organ Morphogenesis

The *C. elegans* vulva, the egg-laying organ in the hermaphrodite, has been extensively studied to understand how signaling networks regulate cell fate decisions.[1] However, the molecular mechanisms controlling the execution of these cell fates during the subsequent phase of morphogenesis are relatively poorly understood. This minireview focuses on the events occurring after the vulval cells have adopted their fates and organ morphogenesis is initiated. Recent findings indicate that the same signaling pathways and transcription factors used during vulval fate specification continue to act during morphogenesis in order to control the specific behavior of the differentiating cells.

Overview of *C. elegans* vulva development. In the adult hermaphrodite, the vulva forms a tubular organ connecting the uterus to the outside, permitting the animal to lay its eggs. Vulval development can be divided into three conceptual phases.[2] First, an equivalence group formed by six vulval precursor cells (VPCs) is established. During the second phase, called vulval induction, the cell fates of the VPCs are specified. Finally, during the execution phase three VPCs start dividing and differentiating into 22 cells that change their shape and size to form a functional vulva.

During the first phase in the L1 larva, six out of 12 epidermal blast cells (Pn.p cells) that are aligned along the ventral midline form the vulval equivalence group in the central body region (the VPCs P3.p through P8.p) (**Fig. 1**). All six VPCs have the potential to differentiate into vulval cells, while the more anterior (P1.p and P2.p) and posterior (P9.p-P12.p) Pn.p cells do not differentiate but instead fuse with the surrounding hypodermis (hyp7). During the third larval stage, three

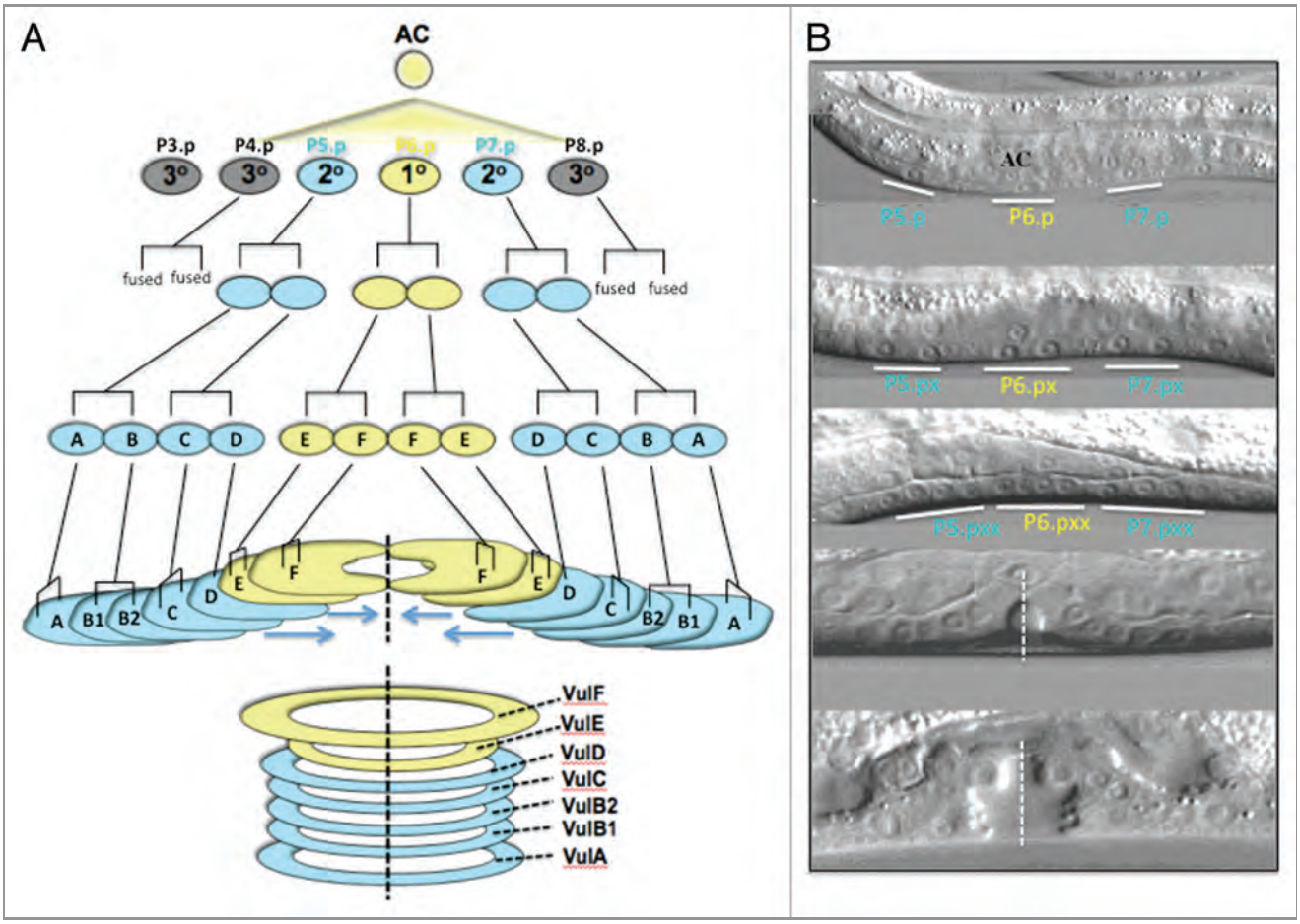

Figure 1. Overview of vulval development. (A) Schematic overview of the different stages of vulval development beginning with induction until the toroids are formed. The seven sub-fates VulA through VulF are indicated in the schematic drawings of the Pn.pxx and Pn.pxxx stages. The mature vulva (bottom panel) is formed by a stack of seven toroids drawn as rings, each comprised of 2 to 4 vulval cells of the same sub-fate. The panels in (B) show the corresponding stages as observed in live *C. elegans* larvae using Normarski optics.

different cell fates (1°, 2° and 3°) are assigned to the six VPCs (**Fig. 1**).[1,3,4] Only the three proximal VPCs (P5.p, P6.p and P7.p) that are induced by a signal from the gonadal anchor cell (AC) differentiate into vulval cells. P6.p adopts the 1° vulval fate, while P5.p and P7.p acquire the alternate 2° fate. The three distal VPCs, P3.p, P4.p and P8.p, are not induced by the AC and hence adopt the undifferentiated 3° fate, which is to divide once and then fuse with the surrounding hypodermis (known as the hyp7). However, enhanced inductive signaling in distal VPCs (e.g., through hyper-activation the RAS/MAPK pathway) results in a 3° to 1° or 2° fate transformation and the development of additional "pseudovulvae" characteristic of a multivulva (Muv) phenotype. On the other hand, reduced inductive signaling in P5.p through P7.p results in a 1° to 3° and 2° to 3° fate transformation and a vulvaless (Vul) phenotype. Since Vul animals cannot lay their eggs, they develop into "bags of worms" containing eggs that will hatch inside the mother.

At the beginning of the L3 larval stage, all VPCs begin to divide (**Fig. 1**). However, only the induced 1° and 2° cells (the P5.px to P7.px descendants) continue to divide for two more rounds until the beginning of the L4 stage. After these three rounds of cell division, P6.p has generated eight 1° cells, and P5.p and P7.p have produced seven 2° descendants each. These 22 vulval cells can be further subdivided into seven distinct sub-types, the five 2° sub-fates called VulA, VulB1, VulB2, VulC and VulD and the two 1° sub-fates called VulE and VulF (**Fig. 1**).[5] At the beginning of the L4 stage, the vulval cells begin to migrate dorsally and form an invagination, which marks the onset of the vulval morphogenesis phase. The cells form circumferential extensions (along the left/right axis) until each cell meets its contralateral partner cell of the same sub-fate at the vulval midline (indicated with the dashed line in **Fig. 1A**). This stage is commonly referred to as the "Christmas tree" stage of vulva development. After forming homotypic contacts, cells of the same sub-fates fuse (with the exception of VulB1 and VulB2) to form seven ring-like syncytial cells called the vulval toroids.[5] This process results in the formation of the vulval tube made out of seven distinct toroids stacked on top of each other, beginning with the ventral-most 2° Vul A toroid facing the outside and ending with the dorsal-most 1° VulF toroid that forms the connection to the uterus (bottom panel in **Fig. 1A**).

During the final phase of morphogenesis, the apical lumen of the toroids collapses and the organ everts, giving rise to the mature vulva. Defects in vulva morphogenesis can be easily recognized in the adults as they manifest as a "protruding vulva" (Pvl) phenotype, which is caused by the eversion of the vulval tissue.

Cell fate specification during early and late vulva development. The specification of the 1° and 2° cell fates is accomplished by a combination of EGFR/RAS/MAPK and NOTCH signaling. P6.p, the cell closest to the gonadal AC, receives most of the epidermal growth factor (EGF) from the AC, which activates the EGFR/RAS/MAPK pathway and induces the 1° cell fate.[6] As a consequence of adopting the 1° fate, P6.p sends a lateral signal by expressing several DSL NOTCH ligands that activate LIN-12 NOTCH signaling in the adjacent VPCs P5.p and P7.p. NOTCH then represses MAPK signaling and induces the 2° cell fate in these two VPCs.[7,8] The EGFR/RAS/MAPK pathway induces in combination with the Wnt pathway expression of the Antp class HOX transcription factor LIN-39 in the VPCs.[9,10] LIN-39 plays crucial roles both during early and late vulval development. Early on before induction, LIN-39 HOX prevents the VPCs from fusing with the hypodermis (hyp7) by inhibiting expression of the fusogen eff-1.[9,11] After vulval induction, LIN-39 HOX is required for vulval cell proliferation and for proper toroid formation.[11] The two HOX cofactors CEH-20 PBX and UNC-62 MEIS are similarly required to prevent ectopic cell fusions and regulate the cell movements during toroid formation, suggesting that they act together with LIN-39 at different stages of vulval development.[12] Despite its crucial roles during vulval morphogenesis, only a few transcriptional targets of LIN-39 HOX have been identified in order to explain the various functions of LIN-39.

Pioneering work from the Podbilewicz group described the stereotypic movements the vulval cells undergo during toroid formation and demonstrated for the first time a requirement for continuous RAS/MAPK signaling during vulval morphogenesis.[5] Using a *let-60* ras gain-of-function allele, the authors observed that a subset of cells of the developing vulva inappropriately fused with the surrounding hypodermis. Additionally, abnormal cell migrations were observed in the most distal vulva cells (VulA and VulB subfates).[5] This work provided the first evidence that RAS/MAPK signaling may perform additional functions during vulval morphogenesis that are distinct from the earlier functions of the RAS/MAPK pathway during 1° cell fate specification.

VAB-23 is a novel and essential regulator of morphogenesis regulated by the RAS/MAPK pathway. In order to identify novel regulators of vulval morphogenesis acting downstream of the RAS/MAPK pathway, we examined genes that were transcriptionally upregulated in the *let-60(gf)* background.[13] Among these candidates, we selected the genes that exhibit a Pvl phenotype when inactivated by RNAi,[14] which is indicative of a defect in vulval morphogenesis. Using this method we identified VAB-23, a conserved nuclear protein belonging to the C4H2 zinc finger family of transcription factors.[15] RNAi knockdown of VAB-23 or a conditionally rescued loss-of-function mutant of *vab-23* [referred to as *vab-23(0)*] resulted in embryonic lethality and severe abnormalities in vulval morphogenesis.[15,16] The three most notable phenotypes observed in the vulva were inter-toroidal fusion of the 3–4 dorsalmost toroids, irregular cell migrations toward the vulval midline and non-homologous cell contacts formed between ipsilateral vulval cells of different sub-fates (**Fig. 2**, adapted from ref. 16).

VAB-23 promotes cell migration and represses ectopic contact formation via transcriptional regulation of Semaphorin *SMP-1*. Semaphorins are a family of secreted and transmembrane proteins that activate transmembrane receptors of the Plexin family to control diverse processes during organ morphogenesis such as cell migration and guidance.[17] The *C. elegans* genome encodes three semaphorin family members; the Sema-1a homolog SMP-1, the Sema-1b homolog SMP-2, and the Sema-2A homolog MAB-20. All three Semaphorins play similar and partially redundant roles in multiple tissues of the worm. For example, loss-of-function mutants in *smp-1* and *smp-2* display defects in head and tail morphogenesis in the embryo, whereby epidermal cells are abnormally positioned.[18] Similarly, *mab-20(lf)* embryos arrest during ventral enclosure due to inappropriate contacts formed between non-homologous epidermal cells at the ventral midline.[19] During vulval morphogenesis, SMP-1 but not SMP-2 is required to guide the vulval cells toward the midline and prevent the formation of ectopic cell contacts between cells of different subfates.[20] This is accomplished by activation of the PLX-1 Plexin receptor, which induces a downstream signaling cascade involving the RAC GTPase CED-10.[20]

Two lines of evidence suggested a possible role of VAB-23 in the transcriptional regulation of *smp-1*. First, the defects in vulval cell migration and ectopic cell contacts in *vab-23(0)* animals were remarkably similar to those seen in *smp-1(lf)* mutants.[16,20] Second, VAB-23 and SMP-1 expression overlap spatially and temporally during vulval morphogenesis, with highest expression observed initially in the 1° cell lineage and lower expression in some of the 2° cells. As predicted, loss of *vab-23* function strongly reduced the expression of a $P_{smp\text{-}1}$*::gfp* transcriptional reporter (**Fig. 3**).[16] A further dissection of the *smp-1* promoter revealed a minimal regulatory region containing a VAB-23 binding site necessary for vulva expression, indicating a direct transcriptional regulation of *smp-1* by VAB-23. To our knowledge, VAB-23 is the first transcriptional regulator of Semaphorins discovered to date.

Similar functions of VAB-23 during vulval morphogenesis and ventral enclosure of the embryo. The defects in cell migration and the formation of ectopic contacts between non-homologous cells during vulval morphogenesis in *vab-23(0)* mutants are remarkably similar to the morphogenesis defects of *vab-23(0)* embryos during ventral epidermal enclosure.[15,16] In both tissues, homologous pairs of cells of identical fates must migrate toward a midline and form new contacts with their contralateral partner cells. Thus, VAB-23 may regulate a common set of target genes required for the interaction between homologous cells during embryogenesis and vulval morphogenesis.

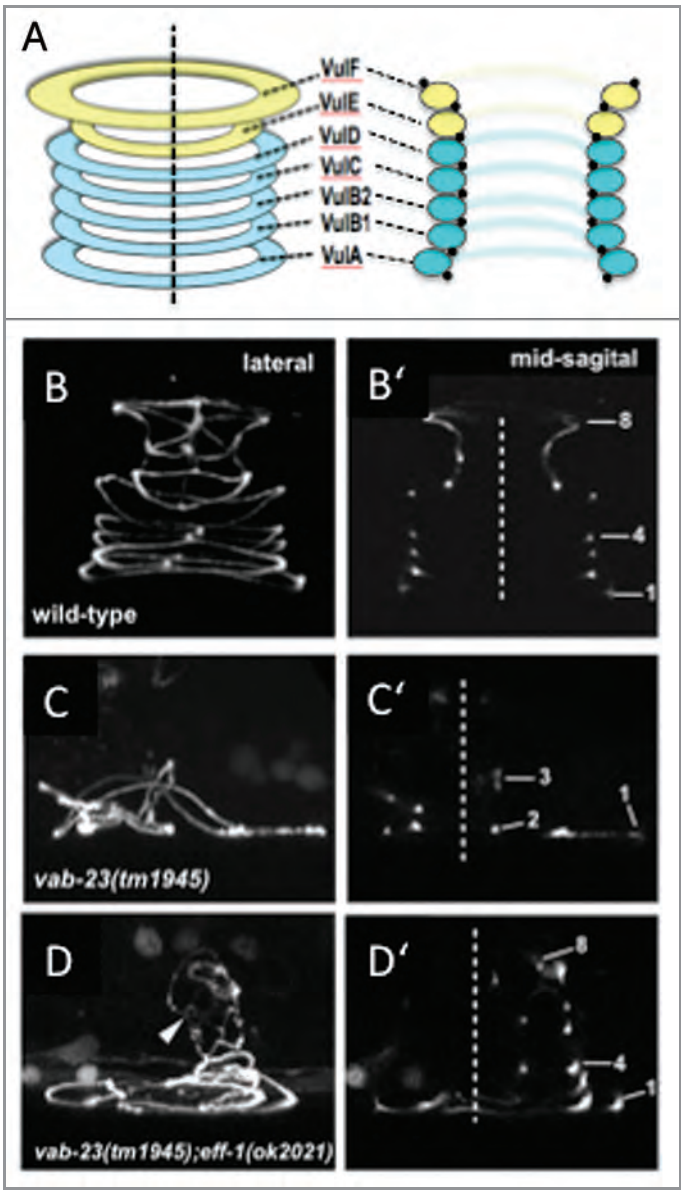

Figure 2. Vulva morphogenesis defects in vab-23(0) animals. (A) Schematic drawing of the seven toroid rings (left panel) and a mid-sagital cross-section through the toroids (right panel). (B) 3D reconstructions and (B') a mid-sagital optical section of the toroids in an L4 larva labeled with the apical junction marker AJM-1::GFP showing the eight rings (or dots in the section) that correspond to the junctions between the seven toroids. (C), (C') Abnormal toroids in a vab-23 (0) single and (D), (D') a vab-23(0); eff-1(0) double mutant, in which no cell fusions occur. Note the formation of ipsilateral cell contacts between non-homologous cells in (D) indicated with an arrowhead. This figure was adapted using pictures from reference 16.

Both, in the embryo and the vulva VAB-23 regulates cell migration and contact formation in a cell-non-autonomous manner.[15,16] During embryogenesis, VAB-23 functions in the underlying neuroblasts to guide ventral epidermal cells toward the midline, and in the vulva VAB-23 acts in the 1° cell lineage to control the migration of adjacent 2° cells toward the vulval midline. Since VAB-23 positively regulates *smp-1* semaphorin expression in the 1° vulval cells, it seems likely that other *C. elegans* semaphorins such as *mab-20* may be among the VAB-23 targets in the embryo.[16,19] However, both *smp-1(lf)* and *mab-20(lf)* mutants display incompletely penetrant defects in epidermal morphogenesis, whereas the *vab-23(lf)* phenotypes are completely penetrant, suggesting that VAB-23 regulates several other target genes in addition to the semaphorins.[15,16,18-20] Consistent with this notion, VAB-23 controls the expression of *egl-26*,[21] *lin-3*[6] and the fusogen *eff-1*[22] in the 1° VulF cells during late vulval morphogenesis (**Fig. 3**).[16]

LIN-39 HOX and VAB-23 link cell fate specification and morphogenesis. Expression of VAB-23 is highest in the 1° vulva cell lineage that receives most of the inductive LIN-3 EGF signal and expression depends on the activity of the RAS/MAPK pathway.[16] Further analysis of the *vab-23* promoter revealed direct binding of the LIN-39 HOX protein together with its co-factor CEH-20 PBX at two conserved PBX consensus sites in the *vab-23* enhancer region. During vulval induction, the MAPK negatively regulates the activity of the LIN-1 ETS transcription factor via phosphorylation of LIN-1,[23] and LIN-1 in turn represses LIN-39 HOX transcription in the VPCs (**Fig. 3**).[24] Thus, activation of the EGFR/RAS/MAPK pathway in the 1° vulval lineage induces *vab-23* expression through an increase in LIN-39 levels,[16] while lateral NOTCH signaling maintains LIN-1 in the unphosphorylated, active state. Hence, LIN-39 and VAB-23 expression remain low in the 2° cell lineage (**Fig. 3**). This regulatory network controlling *vab-23* expression also explains the previously identified functions of the RAS/MAPK pathway and LIN-39 HOX during toroid morphogenesis.[5,11] Continuous RAS/MAPK signaling is required after the vulval cell fates have been specified to maintain *vab-23* expression in the 1° lineage and allow proper morphogenesis of the toroids.[16] Thus, the same signaling pathways used during cell fate specification continue to act during vulval morphogenesis.

Several transcription factors involved in vulval morphogenesis are expressed only after cell fate specification has occurred.[25,26] In contrast, upregulation of VAB-23 in the 1° VPC is observed immediately after vulval induction, well before morphogenesis is initiated.[16] Thus, the previous notion that vulval cell fate specification and execution are temporally distinct events characterized by different

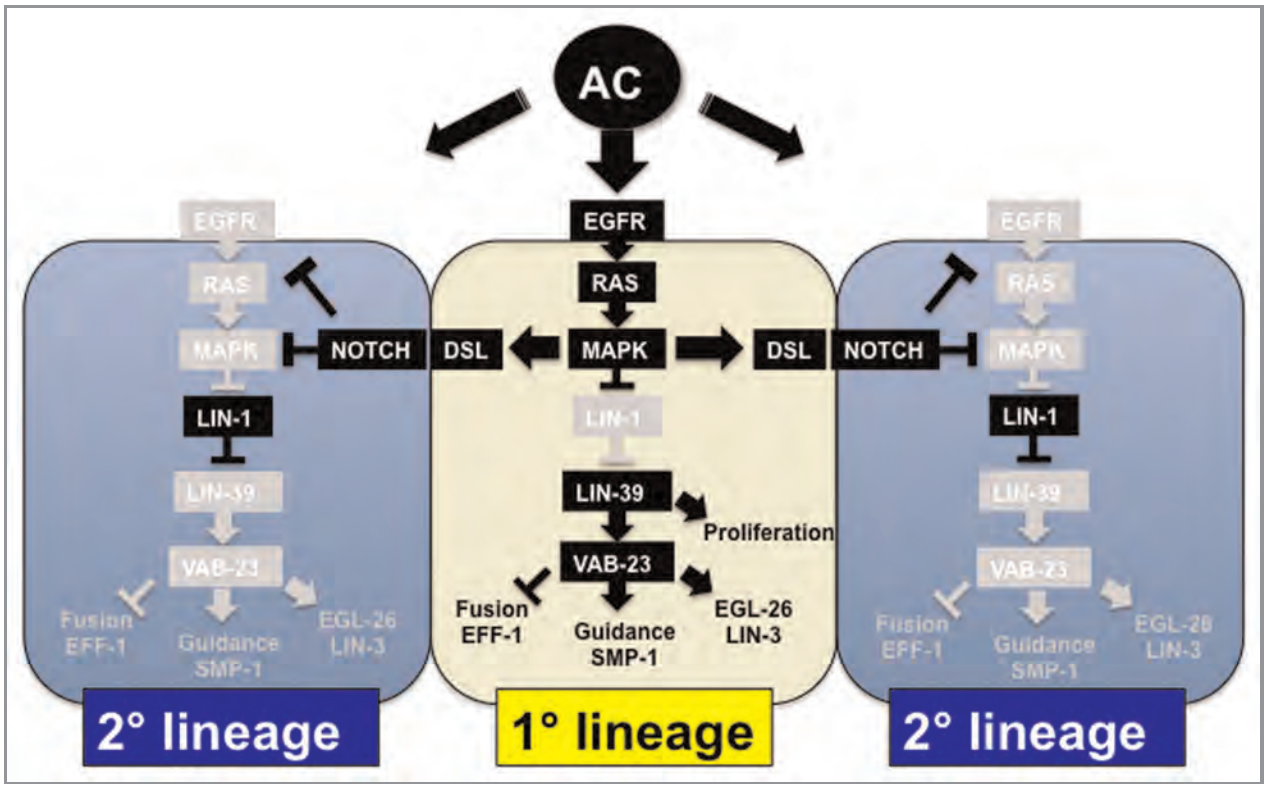

Figure 3. Diagram of the signaling network regulating vab-23 and its target genes in the 1° and 2° vulval cell lineages.

gene expression profiles is probably too simplistic. The link between RAS/MAPK signaling, LIN-39, VAB-23 and SMP-1 rather suggests that crucial regulators of tissue morphogenesis are already activated while the cell fate are specified. Thus, cell fate specification and execution overlap temporally and are tightly linked processes.

To better understand the question how cell fate specification and execution are linked during organ morphogenesis, it will be instrumental to better characterize more of the downstream targets of the RTK/RAS/MAPK and NOTCH pathways and the HOX genes and to identify the second and third layers of regulation (i.e., the targets of the targets and so forth). These indirect targets are probably the effectors that directly mediate the specific cellular behaviors as an output of the cell fates during morphogenesis. To date, we have seen only a small portion of a large regulatory network that is activated after the VPC fates have been specified by conserved signaling pathways such as the RTK/RAS/MAPK and the NOTCH pathways. Notably, chromatin immuno-precipitation experiments with VAB-23 have identified more than 400 potential binding sites in the *C. elegans* genome,[16] suggesting that VAB-23 controls a large number of target genes depending on the cellular context and developmental stage. VAB-23 may be one of the first examples for the second layer of regulators.

Finally, the key question in our research on model organisms such as *C. elegans* concerns the functional conservation. What about the VAB-23 homologs and their targets in higher animals? It is interesting to note that most metazoan genomes encode only a single copy of this unique C4H2 zinc finger protein, and all VAB-23 homologs show the highest degree of conservation in their zinc finger domains. The zebrafish *vab-23* homolog zC4H2 was recently isolated in a screen for genes that are essential for embryogenesis.[27] Interestingly, the zebrafish *vab-23* gene is predominantly expressed in the nervous system of the embryo, similar to the expression of *C. elegans vab-23* in the ventral neuroblasts during embryogenesis.[28] This similar expression pattern in the embryo might indeed hint at a conserved function of this class of C4H2 zinc finger proteins during metazoan development.

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