Anchor cell signaling and vulval precursor cell positioning establish a reproducible spatial context during *C. elegans* vulval induction

Stéphanie Grimbert a,1, Kyria Tietze b,1, Michalis Barkoulas c,2, Paul W. Sternberg b, Marie-Anne Félix c, Christian Braendle a,*

a Centre National de la Recherche Scientifique (CNRS) UMR7277 – Institut National de la Santé et de la Recherche Médicale (INSERM) U1091, Université Nice Sophia Antipolis, 06108 Nice cedex 02, France

b Howard Hughes Medical Institute and Division of Biology and Biological Engineering, California Institute of Technology, 1200 E. California Blvd., Pasadena, CA 91125, USA

c Institute of Biology of the Ecole Normale Supérieure, CNRS UMR 8197 and INSERM U1024, 46 rue d’Ulm, 75230 Paris cedex 05, France

**Abstract**

How cells coordinate their spatial positioning through intercellular signaling events is poorly understood. Here we address this topic using *Caenorhabditis elegans* vulval patterning during which hypodermal vulval precursor cells (VPCs) adopt distinct cell fates determined by their relative positions to the gonadal anchor cell (AC). LIN-3/EGF signaling by the AC induces the central VPC, P6.p, to adopt a 1° vulval fate. Exact alignment of AC and VPCs is thus critical for correct fate patterning, yet, as we show here, the initial AC-VPC positioning is both highly variable and asymmetric among individuals, with AC and P6.p only becoming aligned at the early L3 stage. Cell ablations and mutant analysis indicate that VPCs, most prominently 1° cells, move towards the AC. We identify AC-released LIN-3/EGF as a major attractive signal, which therefore plays a dual role in vulval patterning (cell alignment and fate induction). Additionally, compromising Wnt pathway components also induces AC-VPC alignment errors, with loss of posterior Wnt signaling increasing stochastic vulval centering on P5.p. Our results illustrate how intercellular signaling reduces initial spatial variability in cell positioning to generate reproducible interactions across tissues.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

Development of the egg-laying system requires the *de novo* formation of an opening from the mesodermal gonad to the outside through the ectodermal vulval epithelium. In *Caenorhabditis elegans*, a single cell of the somatic gonad, the anchor cell (AC), plays distinct key roles in the establishment of this opening. First, at the early-mid L3 stage, the AC acts as the main organizer of vulval cell fate induction. Second, a few hours later, at the late L3 stage, the AC invades the vulval epithelium and initiates the connection between the epidermis and the uterus. Therefore, a precise positional alignment between the AC and the vulva is of paramount importance for the development of the egg-laying system (Delattre and Félix, 1999). Although much is known on how the AC drives the induction of vulval cell fates (Sternberg, 2005) and later invades the vulval epithelium to initiate the connection between the epidermis and the uterus (Sherwood and Sternberg, 2003), the mechanisms mediating the initial alignment between cells of the two tissues remain unknown.

*C. elegans* vulval formation has served as a key developmental model system to study cell-cell interactions and cell fate patterning through cross-talk of EGF/Ras and Delta/Notch pathways. The vulva develops from a subset of vulval precursor cells (VPCs), called P3.p to P8.p, which form a row along the anteroposterior axis in the ventral epidermis (Sternberg, 2005) (Fig. 1A). During the L1 and L2 stages, these cells acquire the competence to adopt a vulval cell fate through expression of the Hox gene *lin-39* (Salser et al., 1993) and Wnt signaling from the posterior (Penigault and Félix, 2011). At the early-mid L3 stage, the AC induces vulva cell fate patterns by releasing the LIN-3/EGF-like ligand (Hill and Sternberg, 1992). LIN-3/EGF may act as a morphogen instructing the Pn.p cells to adopt the 1°, 2°, or 3° fates according to its local concentration and thus the relative distance to the AC (Hoyos et al., 2011; Katz et al., 1995). P6.p, the VPC generally closest to the...
Fig. 1. Quantifying the alignment process between anchor cell (AC) and vulval precursor cells (VPCs) (A) Schematic view of the alignment process of VPCs and AC during vulval induction. (B) DIC images of AC positioning (encircled in white) relative to P5.p, P6.p and P7.p at successive stages in C. elegans N2. P6.p is underlined with a white bar. (C) DIC images of progressive positioning of the AC relative to P5.p, P6.p and P7.p in C. briggsae AF16. (D) Quantifying the alignment process between VPCs and AC in the L2 and L3 stages. On the left is a schematic outline of measurements taken. First, a horizontal axis is defined that crosses the nuclei of P5.p and P7.p. The position of the AC nucleus is then projected onto this axis to allow measurement of the distance (red) between P6.p and AC nuclei. Right: Examples of measurements using DIC images of C. elegans N2 in mid L2 and early L3 stages. The AC nucleus is outlined in green, those of VPCs in yellow.
AC at this stage, receives the highest level of LIN-3/EGF and thereby acquires the 1° fate. Subsequently, P6.p expresses Delta ligands activating the LIN-12/Notch pathway in its neighbors, P5.p and P7.p, which then adopt the 2° cell fate and repress the EGF/Ras pathway (Berset et al., 2001; Shaye and Greenwald, 2005; Yoo et al., 2004). P3.p, P4.p and P8.p do not receive sufficient doses of either signal and adopt the 3°, non-vulval, fate. In addition to inducing vulval fates through expression of LIN-3/EGF, the AC is also involved in patterning uterine tissues through expression of the Delta ligand LAG-2 (Newman and Sternberg, 1996). The physical connection between the uterine and vulval lumen is established through a cell invasion event, following vulval cell fate induction, in the mid-to-late L3 stage: the AC becomes closely associated with the gonadal basement membrane, contacts the descendants of the 1° fate VPC and invades the epidermis, following a diffusible signal released by the primary vulval cells (Chang et al., 1999; Sharma-Kishore et al., 1999; Sherwood and Sternberg, 2003).

The vulval developmental system is often schematically represented with the AC being located immediately dorsal to P6.p, and with P5.p and P7.p at equidistance to P6.p, to the anterior and posterior, respectively. Although this is a simplification, it may actually be a fair representation of the average positioning of cells at the early L3 stage when vulval induction takes place. However, earlier on, the relative positioning of the vulval epithelium and the gonad primordium, each overlaid by a basal lamina (Kramer, 1994; White et al., 1976), is variable. This variation is due to the fact that the AC is not born directly dorsally to P6.p. In addition, the initial average position (L2 stage) of the AC relative to the VPCs shows variation among individuals (Braendle and Félix, 2008). Moreover, the vulval epithelium and the gonad slide past each other as the animal moves, thus constantly altering the position of the AC relative to the VPCs. Several observations indicate that the positioning of the VPCs relative to the anchor cell is crucial for correct vulval cell fate patterning. LIN-3/EGF is a secreted protein that can induce vulval cell fates across large distances, e.g., as observed in mutant animals with strongly displaced gonads (Thomas et al., 1990), where fate induction is often aberrant due to the abnormal position of the AC relative to the VPCs. LIN-3 acts in a concentration-dependent manner (Katz et al., 1995) and a three-fold excess of lin-3 genetic dose is sufficient to cause vulval hyperinduction: both P6.p and P5.p are then generally induced to a 1° fate, overriding the lateral inhibition mechanism mediated by the Notch pathway (Barkoulas et al., 2013). Therefore, regulation of the LIN-3 dose received by VPCs and of their position relative to the AC is essential for correct cell fate patterning.

In situations where the geometry is perturbed by mutations or cell ablations, the 1° fate may be adopted by a cell other than P6.p. For example, in lon-1 animals with an elongated body shape, the vulval fate pattern is centered on P5.p in some animals, with P4−6.p adopting a 2°−1°−2° fate pattern, and the 1° lineage is never shared between two daughters of P5.p and P6.p (Sternberg and Horvitz, 1986). This fact has been so far explained by the phenomenon of lateral inhibition, whereby the VPCs compare their level of induction from the AC through Delta-Notch lateral signaling (Sternberg, 1988).

Although the AC is almost invariably centered on P6.p in wild-type animals, stochastic, environmental or mutational perturbations may cause AC alignment with other VPCs, which leads to canonical, yet shifted 2°−1°−2° patterns or incomplete vulval induction (Braendle et al., 2010; Braendle and Félix, 2008; Grimbert and Braendle, 2014). In the C. elegans lab reference strain N2, such miscentering usually occurs on P5.p, which thus adopts the 1° fate, with a concomitant anterior shift of the 2°−1°−2° fate pattern. In contrast, in Caenorhabditis briggsae AF16, AC miscentering generally occurs on P7.p, resulting in a posterior shift of the 2°−1°−2° fate pattern. Interestingly, these anterior and posterior alignment shifts correlate with the early (mid L2) average AC position, which is biased towards P5.p in N2 and P7.p in AF16 (Braendle and Félix, 2008) (Fig. 1B and C). These observations indicate that the relative positioning of AC and VPCs is a significant determinant of the vulval cell fate pattern.

Here we investigate the dynamics and mechanisms of AC-VPC positioning before and during VPCs induction by the AC. We show that VPCs migrate towards the AC during the late L2 and early L3 stage, prior to vulval induction. As a result of this migration, the 1° cell, usually P6.p, comes fully in register with the AC at the early L3 stage. In contrast, the AC does not show any movement towards the VPCs during these stages. Using laser cell ablations and mutant analysis, we show that VPC migration depends on acquired, specific vulval cell fates. Thus, migration of the 1°-fate cell towards the AC creates a positive feedback loop on its level of induction and reinforces the adoption of the 1° fate by a single cell. We also demonstrate that the AC attracts the VPCs, at least in part, through the inductive signal LIN-3/EGF. Finally, we show that impairment of posterior Wnt signaling results in alignment defects and centering of the vulva pattern on P5.p in a fraction of the animals.

2. Results

2.1. Dynamics of the alignment between AC and VPCs

To study the dynamics of the alignment process between the AC and VPCs, we first quantified the position of the AC relative to P5.p, P6.p and P7.p in the C. elegans N2 reference strain from early L2 to early L3 stage (Fig. 1). Starting with AC birth in the early L2 stage, the positioning of the AC relative to VPCs throughout the L2 stage varies substantially among individual animals. Although P6.p is most often the VPC closest to the AC, it is rare to observe an exact alignment between the AC and a single VPC, P6.p, in the early L3 stage. Additional C. elegans and C. briggsae isolates, as well as isolates of other Caenorhabditis species (Caenorhabditis remanei, Caenorhabditis sinica, C sp. 2), show initial variability in AC-P6.p alignment, which is greatly reduced by the early L3 stage (Fig. 2A). The exact alignment between the AC and a single VPC, P6.p, is thus progressively established up to the early L3 stage, and prior to the first division of VPCs.

As previously noted (Braendle and Félix, 2008), in C. briggsae AF16, AC position relative to VPCs shows a bias of AC positioning towards the posterior side of P6.p during the L2 stage (Fig. 2B). Similar to C. elegans, we observed variability in AC-P6.p distances in the L2 stage, but an exact alignment of AC and P6.p in the early L3 stage. Additional C. elegans and C. briggsae isolates, as well as isolates of other Caenorhabditis species (Caenorhabditis remanei, Caenorhabditis sinica, C sp. 2), show initial variability in AC-P6.p alignment, which was greatly reduced by the early L3 stage (Fig. 2C). Therefore, precision in cell positioning is achieved through a progressive alignment of the AC and a single VPC, starting from different average positions in different Caenorhabditis species.

2.2. VPCs migrate towards the AC

The temporal alignment process between the AC and P6.p during development suggests that either the AC, or the VPCs, or both, may be able to move. To distinguish between these possibilities, we inferred the positions of the AC and VPCs using body wall muscle nuclei as cellular reference landmarks, as previously described (Sternberg and Horvitz, 1986) (Fig. S1A). To this end, we made use of lon-1(e185) mutant animals, which have increased body length and are thus easier to score for cell positions. In addition, because of the altered body shape of lon-1 mutant animals, either P5.p or P6.p aligns with the AC and adopts the 1° fate.
We selected animals with the AC initially positioned between these two VPCs during the L2 stage and then observed temporal changes in AC-VPC alignment. As previously observed (Sternberg and Horvitz, 1986), the AC always ended to be in register with either P5.p or P6.p and never ended in between two VPCs at the early L3 stage. Regardless of whether P6.p or P5.p became the 1° cell, 1°-fated VPCs always migrated towards the AC (N=6; P5.p adopted a 1° fate in three animals, P6.p in the three others) (Fig. S1B). In contrast, in none of these cases did the AC significantly change its position relative to body wall muscle nuclei, suggesting that the AC shows no or very minimal movement (Fig. S1B).

To confirm that VPCs migrate towards the AC and not vice versa, we performed laser ablation experiments in the wild-type strain (N2) to eliminate all VPCs except P8.p, the most posterior VPC (N=12) (Fig. S1B). In this experimental paradigm, the AC is found far from the single remaining VPC, yet we never observed any significant movement of the AC (relative to body wall muscle nuclei), whereas P8.p showed active movement towards the AC and adopted a 1° fate in 11 out of 12 animals (Fig. S1C). In some cases, P8.p became positioned exactly under the AC before division, while in other cases, only its progeny reached the AC, indicating that VPC progeny are also capable of migrating. In this situation where the AC and the isolated VPC are well apart, VPC migration occurs across a longer distance and may continue throughout the L3 stage.

To further validate that VPC movement is required for the AC alignment process, we performed P(3,4,-,6,7,-).p ablations at the L1 lethargus or early L2 stage and followed the capacity to migrate of the two remaining VPCs, P5.p and P8.p. The two isolated VPCs started migrating at the late L2 stage until the early L3 stage (Fig. 2C). In this experimental situation, P5.p typically reached the AC at the early to mid-L3 stage, generally before division of the dorsal uterine cells (Kimble and Hirsh, 1979), or before the first P5.p division at the latest. Taken together, these observations show that while the AC remains at a fixed position, with no or negligible movement, VPC movement is the driving force for establishing the alignment between the AC and the 1° fate VPC.
Fig. 3. Alignment of AC and VPCs after cell ablation. (A) and (B) show schematic outlines of the VPC ablation experiments that allowed us to distinguish non-directed movement due to spreading from directional migration of VPCs. Animals were ablated at the L1 lethargus or early L2 stage and the final position of VPC progeny was assessed at the L3 lethargus or early L4 stage. (A) Following P(3,4,6,7,-,-).p ablations, the movement due to spreading should be zero for P5.p, thus movement towards the AC reflects directional migration. (B) P(3,4,5).p ablations instead test the capacity of P6.p to resist spreading (moving non-directedly). The cell would tend to spread anteriorly into the space formerly occupied by the ablated cells, if an active migration force did not keep it in position (analogous to work done to resist falling due to gravity or vacuum). Thus, we infer an active directional migration if a cell does not move. (C-O) show the distribution of the progeny of the remaining VPCs (as judged by positions of their nuclei) with respect to the gonad in P(3,4,6,7,-,-).p ablations (C-I) and P(3,4,5).p ablation experiments (J-O). Horizontal axis: position; vertical axis lists positions of VPCs progeny in single animals. The genotype of the individuals is indicated in each panel. Cell fates were not scored in these animals, but for clarity the expected cell fate in the mutant context is indicated, for P5.p in (C-I) and P6.p in (J-O). Circle within somatic gonad: AC left intact; cross: “AC ablated”, i.e., the precursors of the AC/VU equivalence group (either Z1.pp and Z4.aa, or their daughters) were ablated, except in (I) where the AC is absent due to defects in LIN-12/Notch signaling caused by the lin-12 gain-of-function mutation.
2.3. Positioning of a VPC depends on its cell fate

While following VPC migration in the lon-1 mutant background, we observed that not only 1° VPCs, but also 2° VPCs (and occasionally 3° VPCs), move towards the AC, albeit to a lesser extent. We therefore hypothesized that cell positioning during AC-VPC alignment may depend on the specific fate adopted by each VPC. To address this hypothesis, we performed laser ablations of selected VPCs. We reasoned that after ablation of their neighbors, VPCs may not only move towards the AC (‘directed movement’), but might also diffuse into the space generated by ablation of cells (‘non-directed movement’). We thus designed two partially complementary ablation experiments to test for directed movement by eliminating the confounding effect of possible non-directed VPC movement and thereby characterized the migratory response of differently fated VPCs towards the AC (Fig. 3A and B).

First, we ablated the same number of VPCs on either side of P5.p, namely P(3,4).p and P(6,7).p, at the L1 lethargus or early L2 stage (Fig. 3A). We reasoned that in this experimental set-up, P5.p repositioning towards the space created by P(3,4).p ablation would represent non-directed movement, whereas its repositioning towards P(6,7).p would provide evidence for directed movement. In all cases examined, we found that P5.p moved directionally towards the AC and adopted a 1° cell fate (Fig. 3C). P8.p frequently moved toward the AC as well and usually adopted a 2° fate (Fig. 3C). In contrast, if the AC precursors were ablated at the same time as VPCs, P5.p and P8.p adopted a 3° cell fate and did not move directionally towards the center of the uterine primordium (Fig. 3D). These experiments show that VPCs undergo directed movement only when the AC is present, and they further show that both 1° and 2° VPCs move directionally towards the AC.

To further address whether 2° and 3° VPCs can also move towards an intact AC, we performed similar P(5,8).p isolation experiments in Vulvaless mutants with a defective induction and compared VPC positioning to that in intact and anchor-cell ablated wild-type animals (Fig. 3E and F). We observed reduced cell movement in animals harboring mutations in the EGF receptor let-23, in which VPCs acquired the 3° fate, such as in let-23(sy97) and let-23(n1045); lin-2(n768) mutants (Fig. 3E and F). We also observed a reduced level of P5.p movement in the lin-10(e1439); lin-12(n137)/lin-12(n137n220) background in which all VPCs adopt the 2° cell fate as a consequence of over-activation of Notch signaling. (Fig. 3I). Thus, 2° and 3° VPC movement was greatly reduced compared to that of 1° VPCs, and their alignment with the AC was very rarely complete.

In a second experimental approach, we ablated P(3,4,5).p and assayed repositioning of P(6,7,8).p (Fig. 3B). In this paradigm, P6.p will only become aligned with the AC if it resists moving towards the anterior space of ablated cells. After P(3,4,5).p ablation, P6.p always adopted a 1° fate and precisely aligned with the AC, while P7.p adopted a 2° fate and P8.p a 3° fate (Fig. 3I). In contrast, in AC-ablated animals, VPCs were not retained under the gonad center, and P6.p moved toward the anterior gonad primordium (Fig. 3K). Similarly, performing the same VPC ablations in Vulvaless mutants with an intact AC, VPCs showed variability in positioning relative to the AC, and P6.p moved anteriorly into the space formerly occupied by P(3–5).p (Fig. 3L and M). Therefore, 3° VPCs in Vulvaless mutants fail to align correctly with the AC. Together, these results indicate that induced VPCs undergo directed movement and that the extent of this movement is dependent on the presence of the AC and the vulval cell fate.

2.4. The AC is a key source of the VPC attraction signal

Since VPCs move towards the AC, a reasonable hypothesis is that the AC itself represents a source of the positioning signal. In such a scenario, AC ablation may have two combined, confounding effects: first, loss of inductive signal leads to 3° fate formation, leading to reduced movement of the VPCs; second, loss of the AC prevents a signal that attracts VPCs. To distinguish between these two possibilities, we used lin-15 mutations that result in vulval hyperinduction in the absence of the AC (Sternberg, 1988), due to transcriptional derepression and ectopic lin-3 expression in the epidermis (Cui et al., 2006; Saffer et al., 2011). In lin-15 animals with an intact AC, following P(3,4,5,6,7,8).p ablation, one of the two remaining VPCs assumed a 1° fate and aligned with the AC (Fig. 3G), i.e. the VPC closest to the AC at the beginning of the experiment (usually P5.p). In contrast, in AC-ablated lin-15 animals, the alignment of the 1° VPC with the gonad center was more variable (Fig. 3H), suggesting a role for the AC in providing an (attractive) positioning signal for the VPCs. A similar result was observed after P(3,4,5,6,7,8).p ablation experiments in lin-15 mutants in the presence versus absence of the AC (Fig. 3N and O). These results suggest that the AC is the source of an attractive short-range signal to VPCs. Based on the fact that we observed directed movement for the most distant VPCs, the effective range of the migration signal could be as great as 50 μm. However, it is of note that some residual VPC movement was observed in both AC- and gonad-ablated animals (Fig. 3). This attests to the possibility that other than the gonad tissues may also contribute to AC-VPC alignment; in addition, interactions among VPCs themselves, such as mutual attraction, cannot be excluded.

2.5. The molecular nature of the VPC migration signal

Our results indicate that a signal originating from the AC plays a key role in VPC positioning during the late L2 and early L3 stages. We thus tested the possibility that the AC-released attractive signal for VPCs corresponds to the inductive LIN-3 signal itself. For all tested lin-3 alleles, EGF receptor mutant let-23(sy11) and let-23(sy1); lin-3(e1417) double mutants, we found strongly reduced precision in the positioning of P6.p relative to the AC at the early L3 stage, with variances in AC-P6.p distances that were significantly higher than in the wild-type (Figs. 4A–C and 5A).

One caveat of the above experiments is that reduced LIN-3 levels lead to vulval hypoinduction, so that reduced precision in AC-VPC alignment in the examined genotypes may reflect indirect effects, i.e. due to a lack of induction of vulval fates (for examples of such patterning errors in lin-3(7f) mutants, see Fig. S2A and B). To test whether a 1°-fated cell was attracted to the AC in the absence of LIN-3 signal from the AC, we used lin-3(e1417); lin-15(e1763) animals, where mutation of lin-15 causes all VPCs to be induced due to increased, delocalized production of LIN-3 in the surrounding epidermis and other tissues (Cui et al., 2006; Saffer et al., 2011), with P6.p usually adopting a 1° fate (our observations). We reasoned that if LIN-3 is indeed the spatial cue for P6.p migration towards the AC, the lin-15(e1763) mutation should not be able to rescue the observed AC-VPC positioning defects in the lin-3(e1417) mutant. In contrast, if positioning defects occur indirectly due to loss of VPC induction or through another type of permissive action of LIN-3, lin-3(e1417); lin-15(e1763) animals should show correct AC-VPC positioning. Consistent with a direct and spatially instructive action of LIN-3, we found that the lin-15(e1763) mutation did not restore AC-VPC positioning defects in the lin-3(e1417) mutant. In contrast, if positioning defects occur indirectly due to loss of VPC induction or through another type of permissive action of LIN-3, lin-3(e1417); lin-15(e1763) animals showed a similar variance in P6.p-AC alignment as lin-3(e1417) animals (Brown-Forsythe test, F1,117 = 1.87, P = 0.17). Note that in the lin-15(e1763) single mutant (where lin-3 is expressed at normal levels by the AC and in addition ectopically expressed in various tissues), alignment is already mildly impaired, with an increased variance in AC-P6.p distances compared to the wild-type (Fig. 5A). This increased variance in the lin-15(e1763) mutant is
Fig. 4. Temporal progression of AC-P6.p alignment in lin-3(rf) and bar-1(0) mutant animals. Histograms of AC positions relative to the VPCs in early-mid L2, L2 lethargus and early L3 stages in (A) wild-type N2 (same data as in Fig. 2), for comparison with mutants (B) lin-3(e1417), (C) lin-3(n378), (D) bar-1(ga80) animals. In lin-3(e1417) mutant animals, P6.p adopts either a 3° fate or a 1° fate in about half of the cases (Barkoulas et al., 2013), while in bar-1(ga80) animals, P6.p adopts a 1° fate in 90% of the animals and a 2° fate in 10% (Milloz et al., 2008). In contrast to the wild type, in lin-3(e1417) mutants, the variance in AC-P6.p alignment is not reduced from early L2 to early L3 stage (Brown-Forsythe test, $F_{2,113} = 1.09$, $P = 0.34$), and only 25% of individuals show complete alignment at the early L3 stage. lin-3(e1417) animals also showed a significantly reduced proportion of individuals with complete AC-P6.p alignment (25% compared to 76% in wild type) (Fisher’s Exact Test, $P < 0.0001$). In lin-3(n378) mutants, the variance in AC-P6.p alignment is only slightly reduced from early L2 to early L3 stage (Brown-Forsythe test, $F_{2,89} = 4.16$, $P = 0.02$). In bar-1(ga80) individuals, variances in AC-P6.p alignment do not significantly differ between developmental stages (Brown-Forsythe test, $F_{2,73} = 0.65$, $P = 0.52$), and the variance remains high at the early L3 stage. V: Variance, n: Number of individuals examined. See also Fig. 5A.
Fig. 5. AC-VPC alignment in signaling pathway mutants. Box-Plots indicate variability of AC-P6.p distances at the early L3 stage in mutant backgrounds. For each mutant, variance (V) differences in AC-P6.p positioning relative to the wild type (N2) were tested using the Brown-Forsythe test; differences in median positioning of the AC relative to P6.p (M) were tested using Mood’s Median test. For both tests, significant differences are indicated (*P < 0.05, **P < 0.01, ***P < 0.001, ns: non-significant). See also Figs. S3–S5 for alternative presentation of the same data. (A) Mutants of the EGF/Ras/MAPK signaling pathway (blue) (B) Mutants of the Wnt signaling pathway (orange). bar-1 (ga80) is a putative null allele, while bar-1(mu63) is a weak allele (Natarajan et al., 2004). (C) Combination of mutants of the EGF/Ras/MAPK and Wnt signaling pathways (purple). Variances in AC-P6.p alignment of double mutants did not differ from the variance in single lin-3(n378) mutants (Brown-Forsythe Test, all P > 0.10).
likely caused by the ectopic LIN-3 expression in the epidermis that perturbs the focal positional information generated by AC-released LIN-3. Variance in AC-P6.p distance was significantly increased in lin-3(e1417); lin-15(e1763) relative to lin-15(e1763) (Brown-Forsythe test, \(F_{1,115} = 9.03, P = 0.0033\)), suggesting that reduction of LIN-3 levels in the AC is sufficient to deregulate AC-P6.p alignment, even when P6.p is always induced, usually adopting a 1° fate in this mutant context. Therefore, these results support the notion that AC-secreted LIN-3 provides a direct positional signal in the alignment process of VPCs and AC.

To further test the direct role of LIN-3 in AC-VPC alignment, we examined a lin-3(e1417) let-60(n1046) double mutant (Fig. 5A). Similar to lin-15(e1763) mutants, the Ras gain-of-function allele let-60(n1046) (Beitel et al., 1990; Ferguson and Horvitz, 1985) results in vulval induction in the absence of LIN-3 signal. lin-3(e1417) let-60(n1046) animals show very similar fate induction as let-60(n1046), with most animals adopting a correct 2°-1°-2° pattern for P5.p to P7.p, and variable hyperinduction due to P4.p and P8.p sometimes adopting vulval fates (data not shown). Again, we found no significant rescue of AC-VPC alignment in lin-3(e1417) let-60(n1046), which showed a similarly increased variance in P6.p-AC alignment as lin-3(e1417) (Brown-Forsythe test, \(F_{1,115} = 3.02, P = 0.08\) (Fig. 5A). (Note that the let-60(n1046) single mutant did not show a different variance in P6.p-AC alignment relative to the wild type (Fig. 5A). This result provides additional evidence that AC-released LIN-3 plays a direct role as a signal attracting VPCs towards the AC.

2.6. Stochastic centering of the vulva fate pattern on P5.p in posterior Wnt mutants

We next tested whether another key vulval cell fate specification pathway, the Wnt pathway, may contribute to AC-VPC alignment. Wnt signaling plays many roles in vulva development, including the formation and maintenance of the competence group (Eisenmann et al., 1998; Shemer and Podbilewicz, 2002; Wang et al., 1993), vulval induction (Gleason et al., 2002; Seetharaman et al., 2010) and P7.p polarity (Gleason et al., 2006; Inoue et al., 2004). In particular, two Wnt ligands, lin-44 and mom-2, were previously shown to be expressed in the AC (Inoue et al., 2004), thus providing potential candidates for a positioning signal from the AC to the VPCs. However, mutation of lin-44, RNAi knock-down of mom-2 in either wild type and lin-44 mutant did not cause any significant deviations in alignment of P6.p with the AC at the early L3 stage (Fig. 5B), suggesting that these Wnt ligands do not reflect AC signals involved in VPC positioning.

In contrast, we observed elevated variability in AC-VPC alignment in other Wnt ligand (e.g. egl-20) and receptor (lin-17, lin-18) mutants, and combinations of such mutations caused aggravated phenotypes (Fig. 5B). In double and triple mutant combinations for the Wnt ligands cwn-1, egl-20 and cwn-2 – expressed in partly overlapping domains along the anteroposterior axis (Harterink et al., 2011) - the AC was frequently positioned towards the anterior relative to P6.p, and at the early L3 stage the VPC that aligned with the AC was P5.p (and not P6.p) in a fraction of the animals (Fig. 5B). This P5.p alignment in some individuals is consistent with the observations that these animals show a partially penetrant displacement of the 1° fate from P6.p to P5.p (e.g. Fig. 5D). Thus, the posterior Wnts appear to act in preventing stochastic AC alignment on P5.p.

These mutant combinations of Wnt ligands cause partially penetrant epidermal fusion of VPCs during the L1 and L2 stages (Gleason et al., 2006; Myers and Greenwald, 2007). Therefore, observed AC-VPC mispositioning in these mutants could be partly due to the loss of migratory capacity of fused VPCs. Indeed, in lin-39(n1760) mutants, all VPCs fuse precociously to the epidermis (Maloof and Kenyon, 1998) and at the early L3 stage, the AC was strongly mispositioned towards the posterior (Fig. 5B). However, we also observed AC-P6.p misalignment in bar-1(1ga80) mutants (Figs. 4D and 5B) affecting β-catenin-mediated Wnt signaling, which show a relatively low fusion frequency of P(5–7).p (Braendle and Félix, 2008; Eisenmann et al., 1998): in this experiment, by scoring animals at the L4 stage for vulval induction, we found only approximately 5% of individuals with a fusion event in P(5–7).p, primarily in P5.p (N = 50). The variance in AC-P6.p alignment in bar-1(1ga80) mutants remained very high at the early L3 stage and only very few (25%) individuals showed complete alignment (Figs. 4D and 5B), suggestive of a role of BAR-1-mediated signaling in the AC-VPC alignment process. Consistent with these alignment defects, bar-1(1ga80) animals display a partially penetrant 1° fate adoption by P5.p or P7.p (Fig. 5C). Because Wnt pathway and BAR-1 signaling are involved in anteroposterior patterning of the larva (Harterink et al., 2011; Maloof et al., 1999), e.g. through regulation of Hox gene expression, such as mab-5 (Clandinin et al., 1997), we also tested whether AC-VPC alignment defects in Wnt pathway and bar-1 mutants could stem from an early aberrant position of the gonad primordium relative to newly born Pn.p cells. By measuring P6.p position relative to the center of the gonad at the L1 lethargus, no apparent differences were detected between bar-1(1ga80) mutants and wild-type animals (Fig. 5B). Therefore, early P6.p positioning relative to the gonad in the L1 stage does not explain errors in AC-VPC alignment in bar-1(1ga80) animals (Figs. 4D and 5C). However, in certain Wnt mutant contexts with increased AC-VPC alignment errors, such as cwn-1 (ok546); egl-20(n585) (Figs. 5B and 5D), variance in P6.p positioning relative to the gonad center was significantly increased compared to the wild type in the early L1 stage (Fig. 5B), suggesting that gonad positioning defects together with VPC fusion may contribute to the observed AC-VPC misalignment.

We further tested for possible synergistic effects between LIN-3 and Wnt/BAR-1 signaling pathways in AC-VPC alignment (Fig. 5C). We did not detect any significantly aggravated misalignment phenotypes in lin-3(n378); bar-1(1ga80) or let-23(sy1); bar-1(1ga80) backgrounds; similarly, overactivation of the Wnt pathway in a cwn-1(mu38) context (Korswagen, 2002) did not modify AC-VPC alignment in a lin-3(n378) background (Fig. 5C).

Finally, we addressed whether the Notch pathway acting in the VPCs may contribute to the directed movement of VPCs towards the AC, or may impact attraction and movement among VPCs themselves. Lateral signaling among Pn.p cells involves the Delta/Notch pathway (Sternberg, 2005). Delta/Notch signaling is also involved in AC specification (Seydoux and Greenwald, 1989). We previously showed that knocking down lin-12/Notch specifically in the VPCs resulted in a loss of 2° fates and only rarely to adjacent 1° fates, in contrast to a mutation or a whole-organism RNAi (Barkoulas et al., 2013). We thus performed VPC-specific RNAi knock-down of lin-12/Notch and one of the Delta ligands, apx-1 and assayed VPC positions (Chen and Greenwald, 2004; Barkoulas et al., 2013). However, these RNAi treatments did not deteriorate alignment between AC and P6.p at the early L3 stage (Fig. 5F), so Delta/Notch signaling seems unlikely to play a major role in the AC-VPC alignment process.

3. Discussion

Here we characterized the dynamical spatial context in which C. elegans vulval cell fates are specified, and determined cellular and molecular mechanisms involved in alignment between AC and VPCs, prior and during the early phase of vulval induction (Fig. 6). This alignment process is required for spatial coordination of cells to allow for correct cell fate patterned mediated by cell-cell
activity in the cell and its induction level compared to the neighboring VPCs. We further show that the key inductive signal of the AC, LIN-3/EGF, also acts as an attractive signal for VPCs, thereby coupling the alignment and inductive processes during the early steps of *C. elegans* vulval cell fate patterning in the late L2 stage.

This positive feedback results in centering the vulval cell fate pattern on a single VPC because two VPCs cannot pass left or right of each other. The VPCs indeed normally stay in a row, either due to simple steric hindrance or in part due to an inhibitory signaling system between them, such as semaphorin-plexin signaling that inhibits further cell movement upon VPC contact (Liu et al., 2005). Defects in this signaling system result in overlapping VPCs (when observed from a lateral view) and gaps along the anteroposterior axis, resulting in a low level of defects in Ras and Notch reporter inductions (Liu et al., 2005). Furthermore, defects in VPC elongation and movement upon *cdc-42* downregulation results in adjacent 1° fates (even in animals with a single AC) (Welchman et al., 2007).

A lack of precise positioning of P6.p potentially allows for shortened distances between the AC and P5.p (or P7.p). Thus, in addition to the classical Delta-Notch-mediated lateral inhibition, the regulation of cell positioning may further ensure that only a single Pn.p cell is in close proximity to the AC and adopts the 1° fate. In other terms, a secondary consequence of the positive feedback loop on the 1° fate is the prevention of 1° fate adoption by the other VPCs.

The alignment of the 1° VPC with the AC is followed by many other events that participate in the opening of the uterus lumen to the outside through the vulva. At the end of the L3 stage, the AC sends processes towards P6.p progeny, and the basement membrane is breached (Sherwood and Sternberg, 2003). In experimental situations, the connection can still be established if the two cells are at some distance from each other, for example after late P8.p isolation (Sherwood and Sternberg, 2003). Further reciprocal induction between the two tissues then proceeds, with formation of two tubes (uterine and vulval) in register (Schindler and Sherwood, 2013; Sternberg, 2005).

The Wnt pathway appears to play two roles in VPC positioning. First, as observed in *bar-1*/β-catenin mutants, the alignment between AC and P6.p is impaired at the early L3 stage when BAR-1-dependent signaling is disrupted (Fig. 4D), similar to the situation in which LIN-3 signaling is reduced or absent. In addition, lowering of Wnts secreted from the posterior of the animal results in a more posterior positioning of P6.p compared to the AC, leading to stochastic centering of the vulva pattern on P5.p (Fig. 5B). Posterior displacement of P6.p compared to the AC may result from a slightly more anterior gonad positioning during embryogenesis (Fig. 5B); alternatively, the posterior Wnts may act in repulsing the VPCs anteriorly. Reduced precision in AC-VPC alignment implies that the impairment of posterior Wnts also affects or counteracts the AC-mediated signal governing VPC migration. This in turn may impact cell fate patterning events. Consistent with this view, Wnt (e.g. *cwn-1(ok546); egf-20(n585)) or bar-1/β-catenin mutant animals frequently display P5.p with a 1° fate and P6.p with a 2° fate (Fig. S2C and D). Overall, while the inductive AC signal LIN-3 is a direct VPC alignment signal, the Wnt/BAR-1 pathway may affect VPC positioning indirectly through VPC competence. Wnt/BAR-1 signaling maintains VPC competence by preventing VPC fusion to the hyp7 epidermal syncytium (Eisenmann et al., 1998), and it increases the capacity to respond to the inductive signal LIN-3 for vulval fate specification (Myers and Greenwald, 2007). These same competence properties may similarly affect the capacity of VPCs to respond to LIN-3 with respect to movement towards the AC.

The alignment between VPCs and AC is not evolutionary conserved in all nematodes. The alignment of P6.p on the AC is conserved in the family Rhabditidae to which *C. elegans* belongs (Kiontke
et al., 2007). However, in the families Panagrolaimidae and Cephalobidae, the vulva cell fate pattern is centered in-between P6.p and P7.p, with the 1° fate being shared by the P6.p posterior daughter and the P7.p anterior daughter (Félix et al., 2000; Sternberg and Horvitz, 1981). In these species, the AC ends up being located between P6.p and P7.p and their daughters. In some species, the situation is further complicated by the posterior position of the vulval invagination, requiring net posterior migration of the VPCs (Félix et al., 2000). Which of these alignment mechanisms may represent the ancestral condition remains currently unclear.

The alignment process between VPC and AC illustrates how subtle interactions between a dynamical cellular spatial context and signaling pathways reinforce precision in cell fate patterning. From a broader perspective, the observed adjustment in cell position concomitant to an inductive fate patterning event is likely to be a recurrent feature of developmental systems, especially for those with long-range signaling and the specification of multiple cell fates. Such a positive feedback between induction and migration has been observed, for example, during neural tube patterning in zebrafish (Xiong et al., 2013). Taking into account the dynamics of cell positioning and spatial context of intercellular signaling events therefore represents a critical complement to molecular genetic analysis of developmental systems.

4. Materials and methods

4.1. Strains and general procedures

Strains were maintained on NGM agar plates (1.7% agar) carrying a lawn of Escherichia coli OP50 (Brenner, 1974; Wood, 1988). The list of mutants used in this study is presented in Table S1. The lin-3(mf75) allele is a new tissue-specific transcriptionally null allele, where cis-regulatory elements required for AC expression have been deleted using CRISPR-Cas9 (Dickinson et al., 2013); this allele will be described elsewhere.

4.2. Laser cell ablation

Gonads, AC precursors (i.e. either Z1.pp and Z4.aa, or their daughters) and VPCs were ablated at the L1 lethargus or early L2 stage, following previously described methods (Sternberg and Horvitz, 1986).

4.3. Cell lineage and anatomy

Live animals were observed using Nomarski optics (Sulston and Horvitz, 1977). In experiments where cell movements were not directly followed, individuals were anesthetized using 10 mM sodium azide. Criteria for identifying AC, as well as Pn.p cells and assigning 1°, 2° and 3° fates follow previous studies (Sternberg and Horvitz, 1986, 1989; Sulston and Horvitz, 1977).

4.4. Measurement of positions and distances

Cell positions were inferred by the localization of the nuclei of AC and P(5–7).p, as monitored morphologically using Nomarski optics (Fig. 1D–F). Images were acquired using the Imagel software on a Zeiss Axiolmage microscope at 100× magnification. To measure distances between the AC and P6.p, we first defined a horizontal axis crossing the center of P5.p and P7.p nuclei. The position of the AC nucleus was projected onto this axis to allow measurement of the horizontal distance between the nuclei of P6.p and of the AC. The alignment between the AC and P6.p was considered to be complete when the distance between the two nuclei was ≤ 2 μm. In addition, we measured the direct distances between nuclei of P5.p to P6.p, and P7.p to P6.p.

4.5. Position of nuclei and cells

Nuclei are easier to visualize by Nomarski optics than are cell contours and we thus score here the position of the nucleus as a proxy for cell position. The VPCs grow from the L1 to the L3 stage. When a VPC is still relatively small and distant from the others, as in the early and mid-L2 stage, its nucleus is a good proxy for cell position. The VPCs have grown sufficiently by the end of the L2 stage to contact each other and form a continuous epithelium. In the early L3 stage, the P6.p nucleus is equidistant to those of P5.p and P7.p (paired t-test, = 1.17, df= 44, p = 0.25), which reflects its central position in the cell. The P6.p nucleus then also resides just below the nucleus of the much smaller AC. The precise positioning of the nucleus and center of mass of P6.p is of relevance, because LIN-3 signaling will likely be integrated over the whole basal surface of the cell.

We observed that the spatial distribution of P5.p, P6.p and P7.p is asymmetric during the L2 stage. P5.p and P6.p are born as the daughters of P(5/6) cells, which are left-right counterparts, whereas P7 is the counterpart of P8 (Sulston and Horvitz, 1977). In the mid-L2 and lethargus L2 stages, the P6.p nucleus is significantly closer to that of P5.p than to that of P7.p (paired t-tests, mid L2: t = 3.91, df= 51, p = 0.0003; lethargus L2: t = 3.82, df= 46, p = 0.0004): the distance between P5.p and P6.p nuclei is on average approximately 10% shorter than the distance between P7.p and P6.p nuclei.

To study temporal changes in positioning of AC and VPCs during the L2 and L3 stages, we inferred their positions with respect to cellular landmarks, i.e. body wall muscle nuclei (BWM) in the vicinity of AC and studied VPCs (Sulston and Horvitz, 1977) using DIC microscopy observations as previously described (Sternberg and Horvitz, 1986) (Fig. S1A). Relative distances between AC, VPC and BWM were drawn to infer movements of AC and VPCs.

4.6. Statistical analyses and graphical representations

To test for differences in variance in AC-P6.p alignment between different developmental stages (Figs. 2 and 4) or between mutants and wild type (Fig. 5), we used the Brown-Forsythe Test. To test for differences in average AC-P6.p positioning (Fig. 5), we used the non-parametric Mood’s Median Test, which is robust to data lacking normal distributions (Mood, 1950).

Histograms of AC positions relative to the VPCs (Figs. 2 and 4) are based on absolute horizontal distances between the AC and P6.p, towards either P5.p or P7.p. Box-Plots (Figs. 2 and 5) show distributions of AC positioning relative to VPCs, expressed in relative distance (%) towards either P5.p or P7.p, and individuals with an AC-P6.p horizontal distance ≤ 2 μm, were considered to have complete AC centering on P6.p (i.e. 0% deviation). Therefore, an individual with full AC alignment on either P5.p or P7.p has a value of 100% deviation.

4.7. RNAi experiments

RNAi by bacterial feeding was performed using standard methods (Timmons et al., 2001). The HT115 bacterial strain carrying the empty RNAi expression vector L4440 was used as a control. RNAi plates were made of NGM containing 50 μg/ml of ampicillin and 1 mM of IPTG. Early L3 animals were placed on RNAi plates, and AC-VPC alignment was measured in the F1 generation at the early L3 stage. Construction of RNAi clones for lin-12 and apx-1 have been described previously (Barkoulas et al., 2013; van Zon et al., 2015). RNAi knock-down was confirmed as treated animals of the same experiments showed vulval hypoinduction at the L4 stage, consistent with reduced Delta/Notch signaling...
activity. Additional RNAi clones used were from the Ahringer RNAi library (Kamath et al. 2003). For Pnp-specific RNAi experiments, we used the strain JU2058 with the genotype ref-2(pk1426); mfs70 [lin-3::rde-1, nyo-2::GFP]; rde-1(ne219) (Barkoulas et al., 2013).

Competing interests

None.

Author contributions

Conceived and designed the experiments: CB, MAF, PWS. Performed the experiments: SG, KT, MB. Analyzed the data: CB, SG. Wrote the paper: CB, MAF, SG.

Funding

CB acknowledges financial support by the Centre National de la Recherche Scientifique (CNRS), the Fondation ARC pour la Recherche sur le Cancer, and the Fondation Schulermer for the Education and the Recherche. SG was supported by fellowships from the Ministère de l’Enseignement Supérieur et de la Recherche n° DOC20120605208. MAF acknowledges funding provided by the Agence Nationale de la Recherche (ANR12-BSV2-0004-01 and ANR10-LABX-54 MEMOLIFE) and the Bettencourt Schueller Foundation (Coup d’Elan 2011). MB was supported by a postdoctoral fellowship from the Fondation pour la Recherche Médicale.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.05.036.

References


Sternberg, P.W., Horvitz, H.R., 1981. Gonadal cell lineages of the nematode Pana-
grellus redivivus and implications for evolution by the modification of cell
Sternberg, P.W., Horvitz, H.R., 1989. The combined action of two intercellular sig-
naling pathways specifies three cell fates during vulval induction in C. elegans.
Cell 58, 679–693.
Thomas, J.H., Stern, M.J., Horvitz, H.R., 1990. Cell interactions coordinate the de-
can produce specific and potent genetic interference in C. elegans. Gene 263,
103–112.
Cells change their sensitivity to an EGF morphogen gradient to control EGF-
induced gene expression. Nat. Commun. 6, 7053.
Wang, B.B., Muller-Immergluck, M.M., Austin, J., Robinson, N.T., Chisholm, A.,
Kenyon, C., 1993. A homeotic gene cluster patterns the anteroposterior body
and the PAR-3/PAR-6/PKC-3 complex in diverse cell types. Dev. Biol. 305,
347–357.
ventral nerve cord of C. elegans. Philos. Trans. R. Soc. Lond. B Biol. Sci. 275,
327–348.
Xiong, F., Testmer, A.R., Huang, P., Gelas, A., Mosaliganti, K.R., Souhait, L., Rannou, N.,
progenitors sort to form sharp domains after noisy Shh signaling. Cell 153,
550–561.
Yoo, A.S., Bais, C., Greenwald, I., 2004. Crosstalk between the EGFR and LIN-12/
SUPPORTING INFORMATION

Supplementary Tables

Table S1. List of strains.

Table S2. Measurements of distances between AC and VPCs. Raw data, in microns.

Supplementary Figures

Figure S1. (A) Tracking AC positioning and VPC movement relative to cellular reference landmarks. To study temporal changes in positioning of AC and VPCs during the L2 and L3 stages, we inferred their positions with respect to cellular landmarks, i.e. body wall muscle nuclei in the vicinity of AC and studied VPCs (Sulston and Horvitz, 1977), as previously described (Sternberg and Horvitz 1986). The example DIC image depicts an early L2 larva (wild type, N2 strain) shortly after the birth of the AC. Body wall muscles (BWM, red) on right and left side, AC (blue) and VPCs (green) can be visualized by going through different focal planes. Relative distances between AC, VPC and BWM were drawn to infer movements of AC and VPCs. (B) VPC and AC movements in individuals with intact VPCs. Movement was assessed with respect to body wall muscle nuclei in lon-1(e185) individuals (Fig. S1A); cell positions were observed at multiple timepoints to establish movement patterns. We selected animals (N=6) with the AC initially positioned between P5.p and P6.p at the mid-late L2 stage and then observed temporal changes in AC-VPC alignment until the mid-L3 stage. In all individuals examined, one or more VPCs showed movement towards the AC; in contrast, the AC did not show any significant movement. Dash: no movement. Black arrow: VPC moved slightly towards the AC. Red arrow: VPC moved under the AC. nd: not determined. (C) Movement of isolated P8.p towards the AC. To observe the movement of an isolated VPC, we ablated all VPCs except P8.p at the early L2 stage (wild-type, N2 strain) and
assessed movement with respect to body wall muscle nuclei. Individuals (N=12) were followed from mid-L2 until the 4-cell stage (late L3 stage) to infer cell lineages/fates. In all cases, P8.p moved towards the AC while the AC did not show any significant movement.* P8.p movement was initially inhibited by cell debris of P7.p but reached the AC at 4-cell stage. ** P8.p initiated movement towards the AC but stopped at the debris of P7.p. (D) Migration of isolated P5.p and P8.p towards the AC. Example of temporal progression of P5.p (black) and P8.p (grey) nuclear positions after P(3,4,-,6,7,-) ablations at the L1 lethargus. P5.p and P8.p started migrating at the L2 lethargus stage, and P5.p aligned fully with the AC during the L3 stage.

Fig S2. Cell fate patterns of VPCs in mutant contexts. (A) lin-3(n378); (B) lin-3(e1417); (C) bar-1(ga80); (D) cwn-1(ok546); egl-20(n585). Each line represents the vulval pattern of a single individual, and individuals are ordered from highest to lowest index (I) of vulval induction. Vulval cell fate patterns of P3.p to P8.p were, whenever feasible, separately inferred for their anterior and posterior daughters (Pn.pa and Pn.pp). Black lines separate individuals with complete vulval induction (I=3), partial induction (0 < I < 3) and no induction (I=0). Colour coding of vulval cell fates (1°: blue, 2°: red) and non-vulval cell fates (3°: yellow, 4°: grey). Induced vulval cells that could not be clearly assigned to either a 1° or 2° fate are coded in purple; missing cells are coded in white. Vulval fate patterns were determined in early to mid L4 individuals using Nomarski microscopy, anaesthetized with sodium azide. We counted the Pn.p progeny and determined their fates as previously described (Sternberg and Horvitz 1986; Braendle and Félix 2008). The presented mutant cell fate patterns were obtained from a previously published data set, which reported levels of induction but not cell fate patterns as shown here (Braendle and Félix 2008).

Figure S3. AC-VPC positioning at the early L3 stage in EGF/Ras/MAPK mutants. The data shown in Figure 5A are plotted here as histograms of AC positioning relative to P(5-7).p. (A) lin-3(mf75), n=34; (B) let-23(sy1), n=27;
(C) let-23(sy1); lin-3(n378), n=24; (D) lin-3(mfls55), n=20; (E) lin-15(e1763), n=57; (F) lin-3(e1417); lin-15(e1763), n=60; (G) let-60(n1046), n=58; (H) lin-3(e1417) let-60(n1046), n=62.

Figure S4. AC-VPC positioning at the early L3 stage in Wnt pathway mutants. The data shown in Figure 5B are plotted here as histograms of AC positioning relative to P(5-7).p. (A) cwn-1(ok546), n=27; (B) egl-20(n585), n=26; (C) lin-44(n1792), n=28; (D) mom-2 RNAi, n=24; (E) mom-2 RNAi at 15°C, n=13; (F) lin-44(n1792) treated with mom-2 RNAi, n=15; (G) cwn-1(ok546); egl-20(n585), n=12; (H) cwn-1(ok546); egl-20(n585) cwn-2(ok895), n=14; (I) lin-44(n1792); cwn-1(ok546); cwn-2(ok895), n=12; (J) lin-17(n671), n=20; (K) lin-18(e620), n=26; (L) mom- 5(zu193), n=18; (M) mig-1(nu225), n=22; (N) cfz-2(ok1201), n=18; (O) bar- 1(mu63), n=30; (P) bar-1(ga80), n=30; (G) pry-1(mu38), n=15; (R) mab- 5(e1239), n=25 and (S) lin-39(n1760), n=17.

Figure S5. AC-VPC positioning at the early L3 stage in double mutants of the EGF and Wnt pathways. The data shown in Figure 5C are plotted here as histograms of AC position relative to P(5-7).p. (A) lin-3(n378); bar-1(mu63), n= 25; (B) lin-3(n378); bar-1(ga80), n= 29; (C) let-23(sy1); bar-1(ga80), n= 17; and (D) pry-1(mu38); lin-3(n378), n= 28.

Figure S6. Early VPC-gonad positioning in Wnt pathway mutants. (A) To test for early mispositioning of P6.p in the late L1 stage, we assessed its position relative to the centre of the gonad primordium as illustrated in the cartoon. (B) Box-Plots of gonad-P6.p alignment at the L1 lethargus: wild type N2, n=14; bar-1(ga80), n=15, (Brown-Forsythe test, F₁,27 = 0.49, P=0.49; Mood’s Median Test, Z=-0.055, P=0.58); lin-39(n1760), n=13, (Brown-Forsythe test, F₁,29 = 1.02, P=0.32; Mood’s Median Test, Z=5.10, P<0.0001); cwn-1(ok546); egl-20(n585), n=11, (Brown-Forsythe test, F₁,29 = 20.78, P<0.0001; Mood’s Median Test, Z=1.01, P=0.31); lin-44(n1792); cwn-
1(ok546); cwn-2(ok895), n=11, (Brown-Forsythe test, $F_{1,23} = 7.16$, $P=0.01$; Mood’s Median Test, $Z=-0.022$, $P=0.82$).

**Figure S7. AC-VPC positioning after Pn.p-specific RNAi.** (A) Box-Plots of AC-P6.p alignment in control animals and upon *lin-12* (n=18) or *apx-1* (n=18) RNAi knock-down in Pn.p cells. (B) Histogram of AC position relative to P(5-7).p after *lin-12* RNAi knock-down in Pn.p cells. *lin-12* RNAi treatment did not affect variance of AC-P6.p alignment (Brown-Forsythe test, $F_{1,30} = 0.04$, $P=0.85$) or median positioning of the AC relative to P6.p (Mood’s Median Test, $Z=0.72$, $P=0.47$). (C). Histogram of AC position relative to P(5-7).p after *apx-1* RNAi knock-down in Pn.p cells. *apx-1* RNAi treatment did not affect variance of AC-P6.p alignment (Brown-Forsythe test, $F_{1,37} = 0.84$, $P=0.37$) or median positioning of the AC relative to P6.p (Mood’s Median Test, $Z=0.90$, $P=0.37$).

**References**


Figure S1

A

posterior

right

BWM

left

anterior

dorsal

ventral

Figure S1

B

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td></td>
<td>→</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>←</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td></td>
<td>→</td>
<td>-</td>
<td>-</td>
<td>←</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>nd</td>
<td>nd</td>
<td>→</td>
<td>-</td>
<td>nd</td>
<td>nd</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>→</td>
<td>-</td>
<td>-</td>
<td>←</td>
<td>←</td>
<td>←</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>←</td>
<td>←</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>N</th>
<th>AC</th>
<th>P8.p</th>
<th>P8.p lineage</th>
<th>Fate</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>no movement</td>
<td>moves (under AC)</td>
<td>TTTT</td>
<td>1°</td>
</tr>
<tr>
<td>2</td>
<td>no movement</td>
<td>moves (partially under AC)</td>
<td>TTTL</td>
<td>1°</td>
</tr>
<tr>
<td>1</td>
<td>no movement</td>
<td>moves (close to AC)</td>
<td>NTTT</td>
<td>1°</td>
</tr>
<tr>
<td>1</td>
<td>no movement</td>
<td>moves*</td>
<td>TTTL</td>
<td>1°</td>
</tr>
<tr>
<td>1</td>
<td>no movement</td>
<td>moves**</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

D

AC position

L2  L2 leth.  L3

time (hrs)
Figure S2

A. lin-3(n378)

B. lin-3(e1417)

C. bar-1(ga80)

D. cwn-1(ok546); egl-20(n585)

Legend:
- 1° fate
- 2° fate
- Vulval fates
- Mixed fate
- 3° fate
- 4° fate
- Missing cell
Figure S3

A. *lin-3(mf75)*, N=25

B. *lin-3(mfts55[lin-3(+)])*, N=20

C. *let-23(sy1)*, N=27

D. *let-23(sy1); lin-3(n378)*, N=24

E. *lin-15(e1763)*, N=57

F. *lin-3(e1417); lin-15(e1763)*, N=60

G. *let-60(n1046)*, N=58

H. *lin-3(e1417) let-60(n1046)*, N=62
Figure S4

A cwn-1(ok546), N=27
B egl-20(n585), N=26
C lin-44(n1792), N=28
D mom-2 RNAi_20°C, N=24
E mom-2 RNAi_15°C, N=13
F lin-44(n1792) + mom-2 RNAi, N=15
G cwn-1(ok546); egl-20(n585), N=12
H cwn-1(ok546); egl-20(n585) cwn-2(Ok895), N=14
I lin-44(n1792); cwn-1(ok546); cwn-2(Ok895), N=12
J lin-17(n671), N=20
K lin-18(e620), N=26
L mom-5(zu193), N=18
M mig-1(nu225), N=22
N cfz-2(ok1201), N=18
O bar-1(mu63), N=30
P bar-1(ga80), N=30
Q pry-1(mu38), N=15
R mab-5(e1239), N=25
S lin-39(n1760), N=17
Figure S5

A  lin-3(n378); bar-1(mu63), N=25

B  lin-3(n378); bar-1(ga80), N=29

C  let-23(sy1); bar-1(ga80), N=15

D  pry-1(mu38); lin-3(n378), N=28
Figure S6

A

B

Figure: Graphs showing the distribution and frequency of wild type N2, bar-1(ga80), lin-39(n1760), cwn-1(ok546); egl-20(n585), lin-44(n1792); cwn-1(ok546); egl-20(n585) in different regions (P5.p, P6.p, P7.p).
Figure S7

A

Control RNAi

lin-12 RNAi

Control RNAi

apx-1 RNAi

B lin-12 RNAi N=18

C apx-1 RNAi, N=18