# Patterning of the *C. elegans* 1° vulval lineage by RAS and Wnt pathways

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## SUMMARY

In *C. elegans*, the descendants of the 1° vulval precursor cell (VPC) establish a fixed spatial pattern of two different cell fates: E-F-F-E. The two inner granddaughters attach to the somatic gonadal anchor cell (AC) and generate four vulF cells, while the two outer granddaughters produce four vulE progeny. *zmp-1::GFP*, a molecular marker that distinguishes these two fates, is expressed in vulE cells, but not vulF cells. We demonstrate that a short-range AC signal is required to ensure that the pattern of vulE and vulF fates is properly established. In addition, signaling between the inner and outer 1° VPC descendants, as well as intrinsic polarity of the 1° VPC daughters, is involved in the asymmetric divisions of the 1° VPC daughters and the proper orientation of the outcome. Finally, we provide evidence that RAS signaling is used during this new AC signaling event, while the Wnt receptor LIN-17 appears to mediate signaling between the inner and outer 1° VPC descendants.

Key words: Pattern formation, 1° lineage, Execution, Asymmetric cell division, *C. elegans*

## INTRODUCTION

During animal development, two types of processes establish patterns of cell fates. First, unspecified precursor cells are induced through intercellular signaling to adopt different fates. Second, specified precursor cells execute their fates by dividing asymmetrically to form a defined pattern of different progeny cells. Although asymmetric cell division itself has been studied in a variety of developmental contexts (Horvitz and Herkowitz, 1992; Greenwald and Rubin, 1992; Schnabel and Priess, 1997; Jan and Jan, 1998), it remains unclear how precursors specified prior to their division generate correct patterns of descendants. *C. elegans* vulval development is an excellent system for studying both types of pattern formation due to the ease of manipulation at the single cell level and the invariance of cell lineage (Sulston and Horvitz, 1977; Sulston and White, 1980). Formation of a functional vulva can be divided into two major steps: the formation of the initial pattern of cell fates by the vulval precursor cells (VPCs), and the execution of different VPC fates through distinct cell divisions followed by morphogenesis of the descendants.

The first step comprises formation of the 3°-3°-2°-1°-2°-3° pattern of VPC fates. Six initially multipotential VPCs (P3.p-P8.p) are competent to adopt any of the three fates, 1°, 2° and 3° (Sulston and White, 1980; Kimble, 1981; Sternberg and Horvitz, 1986). The anchor cell (AC) in the somatic gonad produces an apparently graded inductive signal LIN-3, an Epidermal Growth Factor, and induces the closest VPC, P6.p, to adopt the 1° fate (Sternberg and Horvitz, 1986; Hill and Sternberg, 1992; Katz et al., 1995). The LIN-3 signal activates signal transducers in the inductive signaling pathway, including LET-23, a receptor tyrosine kinase (RTK), and LET-60, a RAS homolog (Arioan et al., 1990; Beitel et al., 1990; Han and

Sternberg, 1990). An induced 1° VPC laterally signals its immediate neighbors, P5.p and P7.p, to adopt the 2° fate through the LIN-12 receptor, a Notch homolog (Greenwald et al., 1983; Sternberg and Horvitz, 1989; Koga and Ohshima, 1995; Simske and Kim, 1995). *lin-15* negatively regulates the RAS signaling pathway and represses vulval fates in absence of LIN-3 (Ferguson and Horvitz, 1989; Clark et al., 1994; Huang et al., 1994).

Using the 1° lineage as an example, we dissect the mechanisms by which a specified VPC executes its fate decision to give rise to a distinct cell lineage. Previous studies suggest that the VPCs are specified before their first division (Greenwald et al., 1983; Sternberg and Horvitz, 1986; Ferguson et al., 1987; Euling and Ambros, 1996; Ambros, 1999). After P6.p is induced by the AC to adopt the 1° fate at the late L2 and early L3 stage (Kimble, 1981; Sternberg and Horvitz, 1986), it divides twice during the L3 stage. During L3 lethargus (the interval between L3 and L4), the four granddaughters of P6.p (P6.pxx) form a line along the anteroposterior axis. The AC attaches to the two inner P6.pxx cells (P6.pap and P6.ppa) and away from the two outer P6.pxx cells (P6.paa and P6.ppp) (Fig. 1A; also see Results). All four P6.pxx cells then divide transversely, and the eight great granddaughters (P6.pxxx) detach from the ventral cuticle, migrate dorsally and form a symmetric invagination at the L4 stage (Figs 1B, 2A). In wild-type animals, the 1° VPC descendants produce a fixed spatial pattern of cell fates: the four inner P6.pxxx cells become vulF, and the four outer P6.pxxx cells become vulE. The four vulE cells fuse to form a toroidal multinucleate cell, stacked beneath a ring formed by the analogous fusion of the four vulF cells (Sharma-Kishore et al., 1999). At mid-L4, establishment of the uterine-vulval connection is made primarily by the vulval cells most proximal

to the AC (the vulF cells), and involves the separation of the anterior and posterior vulF cells as well as the formation of the uterine-seam (utse) syncytium through AC fusion with the uterine π cells in the gonad (Newman et al., 1996). The vulval invagination starts to evert at the late L4 stage, and the vulva lips are formed during L4 lethargus (Fig. 1C).

Here we analyze how a specified 1° VPC executes its fate, generating an E-F-F-E pattern of descendants. We test possible roles for the AC, the VU cells, the descendants of the 1° VPC, and the neighboring 2° VPCs. We also manipulate the numbers of the AC as well as the relative positions of the AC to the 1° VPC descendants. We have found that the 1° lineage patterning may involve at least three different mechanisms. A short-range AC signal patterns the inner and outer 1° VPC descendants. Signaling between the inner and outer 1° VPC descendants and intrinsic polarity of the 1° VPC daughters contribute to the asymmetric divisions of the 1° VPC daughters.

## MATERIALS AND METHODS

### General methods and strains

*C. elegans* strains were handled at 20°C according to standard protocols (Brenner, 1974; Wood, 1988). The following alleles were used: for LGI, *lin-17(n671)*; for LGII, *vab-1(dx31)*; for LGIII, *dig-1(n1321), dpy-19(e1259), lin-12(n137), lin-12(n676n909), pha-1(n2123ts), unc-32(e189)*; for LGIV, *dpy-20(e1282), syIs49[zmp-1::GFP; dpy-20(+)]*; for LGX, *unc-6(ev400)* (Brenner, 1974; Greenwald et al., 1983; Katz et al., 1995).

Transgenic lines were generated using the standard microinjection protocol that produces high copy number extrachromosomal arrays (Mello et al., 1991). The *zmp-1::GFP* transgene *syEx282* was obtained by microinjection of pJB100 (J. Butler and J. Kramer, personal communication) at 100 ng/μl and pMH86 (*dpy-20(+)*) at 15 ng/μl, into *dpy-20(e1282)* mutant animals. Heritable lines bearing the marker DNA [scored by rescue of *dpy-20(e1282)*] and GFP expression were treated with X-rays (3800 rad) to promote chromosomal integration (Way et al., 1991) and generate *syIs49*. The *hs-ras* transgene strain PS3220 (*pha-1; dpy-20; syIs49; him-5; syEx312*) was obtained by injection of a *hs-ras(dn)* construct (C. Sigrist and R. Sommer, personal communication) with *pha-1(+)* as a marker.

### Cell ablations

Laser ablations were performed according to Sulston and White (1980). For the AC ablation experiments in Tables 1D, 5 and 6, Figs 2, 4C,D, the AC was ablated when P5.px and P7.px were dividing or had just divided, and P6.px had not divided yet, were dividing, or had just divided. P5.p, P6.p, P7.p and their descendants divide at approximately the same time, but the P6.px cells usually divide about 5-10 minutes later than the P5.px and P7.px cells (M. W. and P. W. S., unpublished). Therefore, the AC was ablated at the late P6.px stage or the early P6.pxxx stage. For the AC ablation experiments in a *lin-12(lf)* background in Table 3A,C and Fig. 3G,H, ACs were ablated at the early L3 stage when the DU cells had not divided. The success of the ablation was confirmed by observation under Nomarski optics 3-8 hours after ablation during L3 lethargus when the position of the AC was scored. Intact animals and ablated animals were mounted on agar pads containing sodium azide for about 15 minutes before being put back on individual plates.

### Heat shock of transgenic animals

Animals were mounted on agar pads, examined using Nomarski optics to confirm their stages (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979) and then recovered on seeded plates for 10 minutes before heat shock treatment. All heat shock pulses were performed at 33°C for 30 minutes. Animals were placed on prewarmed plates of desired temperature, sealed with Parafilm, and then floated in a covered water bath of the same temperature. After heat shock, animals were transferred immediately to plates kept at 20°C until the mid-L4 stage and L4 lethargus when they were scored for uterine-vulval connection and *zmp-1::GFP* expression in P6.pxxx cells.

### Scoring the 1° pattern

Expression patterns of *zmp-1::GFP* were observed using Nomarski optics and a Zeiss Axioplan microscope with a 200-watt HBO UV source, using a Chroma High Q GFP LP filter set (450 nm excitation/505 nm emission). Photographs were taken with a Hamamatsu digital camera and the Improvision Openlab 2.0.6 software.

The pattern of *zmp-1::GFP* expression in great granddaughters of the 1° VPC was scored during L4 lethargus. For experiments in Table 3C and Fig. 3, eight of the 16 P5.pxxx and P6.pxxx cells at either the left or right side (the upper focal plane) of the animals were scored. In many cases, the other side was difficult to score, and therefore not included in the data. For all other experiments, all P6.pxxx cells at both left and right sides of the animals were scored.

## RESULTS

### 1° vulval patterning in wild-type animals

In wild-type animals, all four granddaughters (P6.pxx) of the 1° VPC, P6.p, divide transversely, and the outer and inner great granddaughters of P6.p (P6.pxxx) produce two different cell types, vulE and vulF, respectively (Sulston and Horvitz, 1977; Sternberg and Horvitz, 1986; Sharma-Kishore et al., 1999; Fig. 1). Besides their different fusion behavior (Sharma-Kishore et al., 1999), the inner and outer P6.p descendants differ during vulval morphogenesis. For example, during L3 lethargus, the AC invariably attaches to the two inner P6.pxx cells (Fig. 1A; D. R. Sherwood and P. W. S., unpublished). Also, the inner P6.pxx cells divide slightly later and are more dorsally positioned compared with their outer counterparts (Sharma-Kishore et al., 1999). After P6.pxxx cells are generated, the inner P6.pxxx cells (vulF) invaginate to a greater extent than the outer P6.pxxx cells (vulE).

To look for markers that have differential expression patterns in vulE and vulF cells, we examined the distribution of *zmp-1::GFP*, a zinc metalloprotease fusion protein expressed in a subset of 1° vulval cells (J. Butler and J. Kramer, personal communication). We determined the expression pattern of an integrated *zmp-1::GFP* reporter gene in wild-type animals. We found that *zmp-1::GFP* was expressed in the AC throughout the L3 and the early L4 stage, but not in P6.p, P6.px, or P6.pxx cells. The expression in the P6.pxxx cells began in late-L4. During L4 lethargus, *zmp-1::GFP* was expressed in all four vulE cells, but not in any vulF cells, in all animals observed (*n*=138, Figs 1C, 2B,C). The expression in vulE cells persists in the adult stage. The transverse divisions of P6.pxxx result in two identical patterns of vulE and vulF cells at the left and right sides of the animal (Fig. 1C). We consider the pattern at each side as a half 1° lineage patterning unit to organize our data.

Based on several lines of evidence, *zmp-1::GFP* can be used reliably to distinguish the vulE and vulF fates. First, as described above, this marker has a spatially and temporally invariant expression pattern in wild-type 1° VPC great granddaughters. Second, the proper expression of *zmp-1::GFP* correlates with the morphological criteria of a properly

patterned 1° lineage, i.e. a correct vulval-uterine connection required for egg-laying. Mutant or experimentally manipulated animals with abnormal *zmp-1::GFP* expression in the 1° lineage lacked a proper connection (0%, *n*=174 animals; e.g. Fig. 2G-I). Occasionally the 1° VPC descendants with a wild-type *zmp-1::GFP* expression pattern also failed to form a connection between the uterus and the vulva (11%, *n*=207 animals; also see below). Therefore, correct expression pattern of *zmp-1::GFP* might reflect one aspect of the differentiation of the inner and outer great granddaughters of the 1° VPC.

## The neighboring 2° lineages are not required for 1° patterning

To examine whether any signaling from other cells in the vicinity of the 1° VPC or between neighboring cells of the 1°

VPC descendants is required for 1° patterning, we ablated both 2° VPC neighbors, part of the 1° VPC descendants, or some gonadal cells, and examined the 1° pattern formation by using *zmp-1::GFP* as a marker.

We ablated P5.p and P7.p, the two 2° VPCs flanking the presumptive 1° VPC P6.p, prior to the birth of any P6.p descendants. In these animals, the 1° lineage was correctly patterned as assessed by *zmp-1::GFP* expression (*n*=20 half 1° lineage patterning units; Table 1A), as well as by the morphological criteria of a proper vulval-uterine connection (data not shown). In addition, in *lin-12(lf)* mutants where the 2° fate is not specified, isolated 1° lineages invariably had the wild-type expression pattern of *zmp-1::GFP* (Greenwald et al., 1983; Table 3A; also see below). Therefore, the 2° VPC neighbors may not be required for 1° patterning.
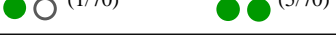
## Each anterior and posterior half of the 1° lineage patterns autonomously

The developing vulva is mirror-symmetric and previous studies have shown that the anterior and posterior halves of the vulva develop autonomously during vulval morphogenesis and cell fusion (Sharma-Kishore et al., 1999; Shemer et al., 2000). When we ablated P6.pa or P6.pp, the other P6.px underwent its normal division to produce two outer P6.pxxx cells that expressed *zmp-1::GFP* and two inner P6.pxxx cells that did not express the marker (*n*=18 half 1° lineage patterning units, Table 1B). The morphology of the half 1° vulva left was also relatively normal in these animals (data not shown). Each anterior and the posterior half of the 1° lineage can therefore pattern autonomously.

## The AC (but not VU) is required for 1° patterning

Besides the 2° VPCs and their descendants, the other set of

**Table 1. The AC is required for proper patterning of the 1° lineage, while the 2° lineages and the VU cells are not required**

| Experimental conditions | *zmp-1::GFP* expression in P6.pxxx during L4 lethargus (*n*) |
|---|---|
| Intact | (276/276) |
| (A) ablate P5.p and P7.p | (20/20) |
| (B) ablate one P6.p daughter | (18/18) |
| (C) ablate VU precursors before VPCs divide | (34/34) |
| (D) ablate AC at the late P6.px stage to early P6.pxx stage | (19/70) (5/70) (11/70) (1/70) (13/70) (12/70) (1/70) (2/70) (1/70) (5/70) |
| (E) ablate AC at the late P6.pxx stage | (20/20) |

L3 animals with integrated *zmp-1::GFP* were staged using Nomarski optics. In all panels, ventral is down. The panels indicate the expression patterns of *zmp-1::GFP* in P6.pxxx cells at the left or right side of the animal, observed by Nomarski optics during L4 lethargus. Filled circles indicate the P6.pxxx cells that expressed GFP; unfilled circles indicate the P6.pxxx cells that did not.

Intact, control animals that were not ablated.

*n*, the numerators indicate the number of half 1° lineage patterning units with the *zmp-1::GFP* expression pattern displayed; the denominators indicate the number of half 1° lineage patterning units scored.

(A) The 2° VPCs, P5.p and P7.p, were ablated.

(B) P6.pa or P6.pp was ablated.

(C) All the ventral uterine precursors (VU) were ablated at the early L3 stage (the three VU cells had not divided when ablated).

(D) The AC was ablated at the late L3 stage, just before, during, or right after the divisions of P6.px cells.

(E) The AC was ablated during L3 lethargus.

**Table 2. Failure of AC contact with 1° descendants disrupts patterning of the 1° lineage**

| Genotype | AC contact with 1° descendants* | 1° VPC descendants scored‡ | Wild-type *zmp-1::GFP* expression in Pn.pxxx |
|---|---|---|---|
| *dig-1* | Contact (ventral gonad, anteriorly shifted) | P4.p or P5.p | 44/45 |
| | No contact (dorsal gonad) | P4.p, P5.p or P6.p | 4/38 |
| *unc-6* | Contact | P6.p | 37/37 |
| | No contact | P6.p | 4/26 |

Animals were scored using Nomarski optics during L4 lethargus. The numerators indicate the number of VPCs whose descendants display the wild-type *zmp-1::GFP* expression pattern. The denominators indicate the number of VPCs scored. The full genotypes were: *dig-1(n1321); zmp-1::GFP* and *unc-6(ev400); zmp-1::GFP*.

*The position of AC at the Pn.pxx stage. In *dig-1* animals, when the gonad was positioned ventrally, but shifted anteriorly, it attached to the anterior P4.p or P5.p. When the gonad was mispositioned dorsally, it had no contact with any of the VPCs. In *unc-6* animals, the gonad was at the ventral side in all animals. However, the AC was sometimes misplaced at the dorsal part of the gonad, and therefore had no contact with VPCs and their descendants.

‡In *dig-1* animals, we scored *zmp-1::GFP* expression in descendants of P4.p, P5.p, or P6.p, whichever was closest to the AC. In *unc-6* mutants, P6.p was always scored.
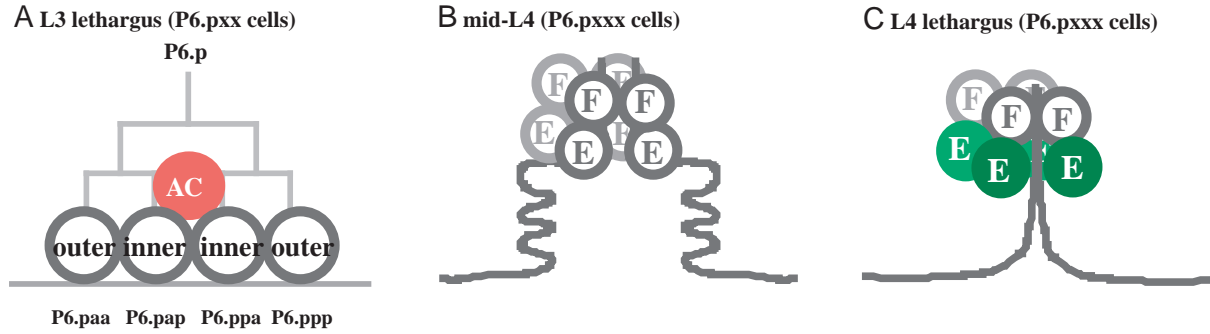
**Fig. 1.** Schematic outline of 1° lineage patterning. In all panels, ventral is down, anterior is to the left. In (C), filled circles indicate the P6.pxxx cells that expressed *zmp-1::GFP*; unfilled circles indicate the P6.pxxx cells that did not express *zmp-1::GFP*. In (B,C), the darker color indicates the P6.pxxx cells at the left side of the animal, and the lighter color indicates the P6.pxxx cells at the right side. AC, anchor cell; E, vulE cells; F, vulF cells.

cells in the vicinity of the 1° VPC and its descendants includes the AC and its surrounding VU precursors in the gonad (Kimble and Hirsh, 1979). Before the first division of the VPCs, the AC is surrounded by three VU precursors (Kimble and Hirsh, 1979). The VU precursors then divide twice, and their granddaughters become specified as π and ρ precursors, which generate uterine cells, such as utse and uv1 (Kimble and Hirsh, 1979; Newman et al., 1996).

When we ablated all three VU precursor cells before their division at the early L3 stage (VPCs were also undivided at

that time), the P6.pxxx cells had the wild-type *zmp-1::GFP* expression pattern (*n*=34 half 1° lineage patterning units, Table 1C, Fig. 2E,F). At mid-L4, the vulval-uterine connection was otherwise normal except that it was blocked by the unfused AC, which failed to form utse in the absence of π cells (Fig. 2D). Also, in *lin-12(lf)* mutants, where the π fate is not specified, isolated 1° lineages can invariably form a correct pattern (Newman et al., 1995; Table 3A; also see below). Therefore, the VU precursor lineages are not required to pattern the 1° lineage.
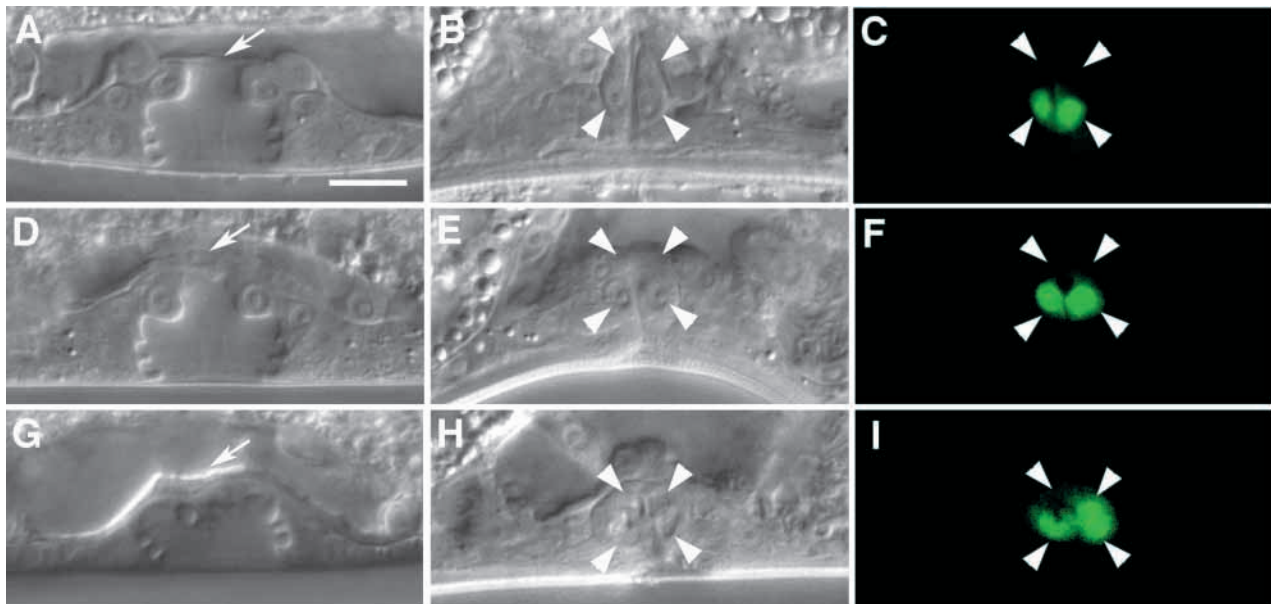


**Fig. 2.** The AC is required for proper patterning of the 1° VPC descendants. *zmp-1::GFP* animals from Table 1 are displayed. In all panels, ventral is down, anterior is to the left. Arrows point to the connection between the uterus and the vulva at mid-L4; arrowheads indicate the position of the great granddaughter nuclei of P6.p during L4 lethargus (some of the inner nuclei were out of focus). (A-C), intact animal; (D-F), animal with all VU precursor cells ablated before VPCs divide; (G-I), animal with the AC ablated at the early Pn.pxx stage. (A) Nomarski image of the vulval invagination at the mid-L4 stage of an intact animal. The vulva and the uterus were connected with a thin line of utse. (B,C) Nomarski and fluorescence images of the four P6.pxxx cells at the left side of the same animal during L4 lethargus. Neither of the inner P6.pxxx cells expressed *zmp-1::GFP*, while both outer cells expressed *zmp-1::GFP*. (D) Nomarski image of the vulval invagination at the mid-L4 stage of an animal with all VU precursors ablated. The unfused AC blocked the otherwise normal uterine-vulval connection. (E,F) Nomarski and fluorescence images of the four P6.pxxx cells at the left side of the same animal during L4 lethargus. The expression pattern of *zmp-1::GFP* was the same as that in intact animals. (G) Nomarski image of the vulval invagination at the mid-L4 stage of an animal with the AC ablated at the late P6.px or the early P6.pxx stage. The connection between the vulva and the uterus was abnormal. (H,I) Nomarski and fluorescence images of the four P6.pxxx cells at the left side of the same animal during L4 lethargus. Both outer P6.pxxx cells expressed *zmp-1::GFP* as in intact animals, but one of the two inner cells also expressed *zmp-1::GFP*. Scale bar, 10 μm.

**Fig. 3.** The expression pattern of *zmp-1::GFP* in the 1° VPC great granddaughters is determined by the proximity between the AC and the 1° VPC granddaughters. Animals from Table 3 are displayed. The strain used was *lin-12(lf); zmp-1::GFP*. In all panels, ventral is down, anterior is to the left. All animals were during L4 lethargus when photographed. Arrowheads indicate the positions of the great granddaughter nuclei of P5.p and P6.p. AC, the AC or multiple ACs remained unfused and expressed *zmp-1::GFP* during L4 lethargus in a *lin-12(lf)* background. (A-D) Two different animals with P5.p and P7.p ablated; (E,F) animal with P7.p ablated; (G,H) animal with P7.p and all ACs but one ablated. (A,B) Nomarski and fluorescence images of the four P6.pxxx cells at the left side of an animal. P5.p and P7.p were ablated at the early L3 stage. The four ACs attached to P6.pap and P6.ppa during L3 lethargus. During L4 lethargus, neither of the inner P6.pxxx cells expressed *zmp-1::GFP*, while both outer cells expressed *zmp-1::GFP*. (C,D) Nomarski and fluorescence images of the four P6.pxxx cells at the left side of an animal. P5.p and P7.p were ablated at the early L3 stage. The four ACs attached to P6.pap, P6.ppa and P6.ppp, but not P6.paa during L3 lethargus. During L4 lethargus, none of P6.papl, P6.ppal and P6.pppl expressed *zmp-1::GFP*, while P6.paal expressed *zmp-1::GFP*. (E,F) Nomarski and fluorescence images of the eight great granddaughters of P5.p



and P6.p at the left side of an animal. P7.p was ablated at the early L3 stage. The four ACs attached to the five innermost granddaughters of P5.p and P6.p (P5.ppa, P5.ppp, P6.paa, P6.pap and P6.ppa) during L3 lethargus. During L4 lethargus, P5.paal, P5.papl and P6.pppl expressed *zmp-1::GFP*, while P5.ppal, P5.pppl, P5.paal, P6.papl and P6.ppal did not express *zmp-1::GFP*. (G,H) Nomarski and fluorescence images of the eight P5.pxxx and P6.pxxx cells at the left side of an animal. P7.p and all ACs except one were ablated at the early L3 stage. The unablated AC attached to P5.ppp and P6.paa during L3 lethargus. During L4 lethargus, P5.ppal and P6.papl expressed *zmp-1::GFP*, while the other six cells did not express *zmp-1::GFP*. Scale bar, 10 µm.
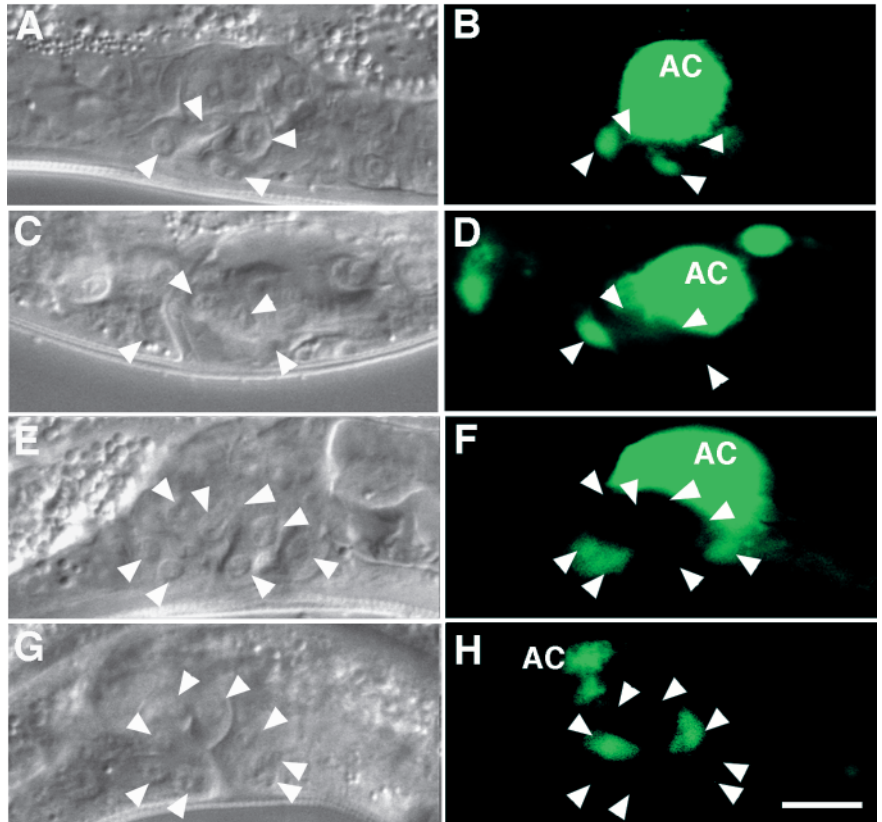
We then examined whether the AC is required for 1° lineage patterning. Previous AC ablation studies by Kimble (1981) suggest a possible role for the AC after the induction of VPCs in making a functional vulval-uterine connection. To separate a possible late role for the AC in 1° patterning from its earlier function in VPC fate specification, we ablated the AC at the late P6.px stage or the early P6.pxx stage, after VPC fates are specified (Greenwald et al., 1983; Wang and Sternberg, 1999). Although all P5.p, P6.p and P7.p were induced to divide three times after AC ablation as in intact animals, the inner P6.pxxx cells did not properly separate to form the uterine-vulval connection, and the vulva invariably had an abnormal morphology (Fig. 2G). When we scored *zmp-1::GFP* expression in P6.pxxx cells, the expression of *zmp-1::GFP* in P6.pxxx cells was disturbed in 51 of 70 resulting patterns (Table 1D, Fig. 2H,I). The patterns of P6.pxxx cells lacking the AC appear variable: we observed all ten possible patterns, but some patterns are obviously preferred (Tables 1D, 7; also see Discussion). We conclude that the AC is required after the initial induction of the 1° VPC fate to ensure the wild-type patterning of the 1° lineage.

When we ablated the AC at the late P6.pxx stage, all resulting patterns had the wild-type *zmp-1::GFP* expression in P6.pxxx cells (*n*=20 half 1° lineage patterning units, Table 1E), as well as normal morphology of the 1° vulva (data not shown).

Therefore, the AC may signal the P6.pxx cells, rather than P6.pxxx cells, to pattern the 1° lineage.

## Wild-type 1° patterning requires the AC at short range

To determine whether a proximity of the AC and the 1° VPC descendants is required for 1° lineage patterning, we examined *dig-1* or *unc-6* mutants, with or without AC attachment.

87% of *dig-1* mutant animals have an anteriorly shifted gonad (Thomas et al., 1990). In these animals, the AC is closest to P4.p or P5.p, rather than P6.p, and the VPC closest to the AC invariably adopts the 1° fate (Thomas et al., 1990). We found that the AC attached to the two inner P4.pxx or P5.pxx cells at L3 (*n*=16 animals). We scored either P4.p or P5.p, whichever was closest to the AC, and found that, in 44 of 45 these 1° VPCs, a wild-type *zmp-1::GFP* expression pattern was formed (Table 2). Therefore, P4.p and P5.p descendants are capable of forming a wild-type 1° pattern if the AC is positioned nearby.

In 13% of *dig-1* mutant animals, the gonad primordium is displaced to the dorsal side of the animal (Thomas et al., 1990). In these animals, the AC is mispositioned dorsally along with the gonad, and the AC is at least 20 µm (measured as a direct distance) from the VPCs (Thomas et al., 1990). Any of P4.p, P5.p or P6.p, can be the closest VPC to the AC and can then

**Fig. 4.** *ras* and *lin-17* might be involved in AC signaling and signaling between the 1° VPC descendants, respectively, in 1° vulval patterning. Animals from Tables 4 and 6 are displayed. The strains used were *hs-ras(dn); zmp-1::GFP*, and *lin-17(n671); zmp-1::GFP*. In all panels, ventral is down, anterior is to the left. Arrowheads indicate the position of P6.pxxx nuclei at the left side of the animal during L4 lethargus (some were out of focus). (A,B) An animal with *hs-ras(dn)* was heat shocked at the early Pn.pxx stage. Nomarski and fluorescence images of the four P6.pxxx cells at the left side of the animal during L4 lethargus. All four P6.pxxx cells expressed *zmp-1::GFP*. (C,D) An *lin-17(n671); zmp-1::GFP* animal with the AC ablated at the early P6.pxx stage. Nomarski and fluorescence images of the four P6.pxxx cells at the left side of the animal during L4 lethargus. The anterior inner and outer P6.pxxx cells expressed *zmp-1::GFP*, while the posterior inner and outer P6.pxxx cells did not express *zmp-1::GFP*. Scale bar, 10 µm.
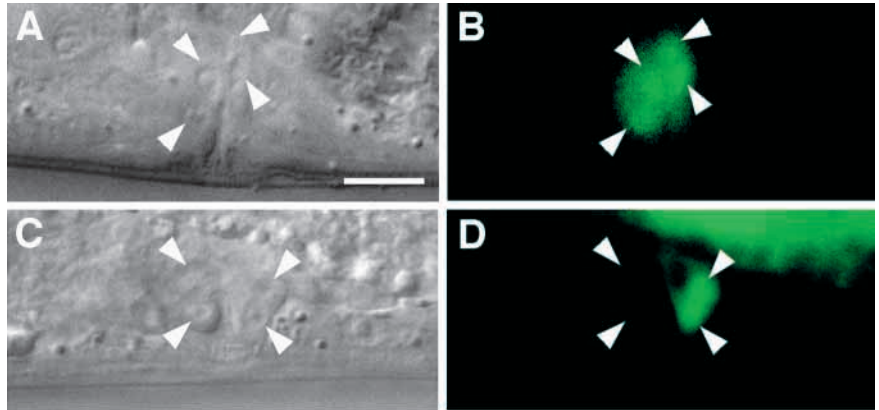
adopt the 1° fate. We found that only four of 38 1° VPCs (scored according to detachment of the descendants from ventral cuticle and symmetry of invagination; Katz et al., 1995) with a dorsal gonad displayed a wild-type *zmp-1::GFP* expression pattern in its descendants (*P*<0.0001, Fisher's exact test, Table 2), suggesting that the AC is able to pattern the descendants of a 1° VPC locally, but not at long range.

In *unc-6* mutants, the gonad is positioned ventrally as in wild type. However, in about 22% of the animals, the AC improperly migrates dorsally or laterally within the ventral gonad (the distance between the AC and P6.pxx cells is less than 20 µm), rather than attaching to the inner P6.pxx cells during L3 lethargus (Hedgecock et al., 1990; D. R. Sherwood and P. W. S., unpublished). In 26 animals with a displaced AC, only four of 26 P6.p cells had wild-type patterns of *zmp-1::GFP* expression in its progeny (Table 2). In contrast, when the AC was correctly positioned and attached to inner P6.pxx cells, all 37 P6.p cells formed the wild-type 1° pattern, indicating that the P6.p descendants are capable of forming a wild-type 1° pattern in an *unc-6* background, when the AC is correctly positioned (*P*<0.0001, Table 2).

We conclude that proper patterning of the 1° lineage utilizes a local signaling mechanism, possibly requiring direct contact between the AC and the 1° VPC granddaughters.

### Patterning of the 1° lineage with multiple ACs indicate that the AC signals the 1° VPC granddaughters it contacts to ensure the production of vulF progeny

To clarify how the AC patterns the 1° lineage, we examined the *zmp-1::GFP* expression pattern in VPC descendants in isolated 1° lineages with multiple ACs. In *C. elegans*, LIN-12 is used repeatedly as the receptor for lateral signaling. During early gonadal development, signaling between two initially equivalent cells, Z1.ppp and Z4.aaa, is mediated by *lin-12* and determines which cell becomes the AC and which becomes the ventral uterine (VU) precursor cell (Kimble, 1981; Greenwald et al., 1983; Seydoux and Greenwald, 1989). Afterwards, during vulval induction, LIN-12 functions again in lateral signaling between the VPCs to induce the 2° fate (Sternberg, 1988; Sternberg and Horvitz, 1989; Koga and Ohshima, 1995; Simske and Kim, 1995; J. Liu and P. W. S., unpublished).

In a *lin-12* loss-of-function mutant background, both Z1.ppp and Z4.aaa become ACs (Greenwald et al., 1983). In some cases, one or more of their sisters also undergoes transformation, resulting in more than two ACs (Sternberg and Horvitz, 1989). Out of 49 *lin-12(lf)* mutant animals examined, 32 had four ACs, 12 had three ACs, four had two ACs, and one had five ACs. Moreover, in the absence of LIN-12 activity, two or three central VPCs (P5.p and P6.p, or P5.p, P6.p and P7.p) adopt the 1° fate, while the distal VPCs (P3.p, P4.p and P8.p) adopt the 3° fate (Greenwald et al., 1983; Sternberg and Horvitz, 1989). We controlled the number of ACs and the 1° VPCs by laser ablation in a *lin-12(lf)* background.

We first ablated P5.p and P7.p, as well as all but one AC. In all six animals scored, the AC attached to the two inner P6.pxx cells during L3 lethargus. *zmp-1::GFP* was expressed in the outer P6.pxxx cells (as vulE), but not the inner cells (as vulF), during L4 lethargus (Table 3A). This indicates that a single 1° lineage with a single AC in a *lin-12(lf)* background patterns correctly, and that *lin-12* is not required for proper patterning of the 1° lineage.

We then examined patterning of isolated 1° lineages with multiple ACs: we ablated P5.p and P7.p, but left the multiple ACs intact. The ACs attached to the two inner P6.pxx cells in 12 of 14 animals, and the wild-type *zmp-1::GFP* expression pattern in P6.pxxx cells was observed (Table 3B, Fig. 3A,B). The ACs in the remaining two animals attached to one of the two outer P6.pxx cells, in addition to the two inner P6.pxx cells. Subsequently, the inner P6.pxxx cells, as well as the P6.pxxx progeny of the outer P6.pxx attached to the ACs, failed to express *zmp-1::GFP*, while the P6.pxxx progeny of the other outer P6.pxx expressed GFP (Table 3B, Fig. 3C,D). Therefore, the AC can signal the P6.pxx cells that it contacts to generate vulF progeny.

To examine the patterning of two adjacent 1° lineages, we ablated P7.p in a *lin-12(lf)* background. When we left multiple ACs intact, the ACs attached to various numbers of posterior P5.pxxx cells and anterior P6.pxxx cells (data not shown). Subsequently, 137 of 144 central Pn.pxxx cells (P5.ppax, P5.pppx, P6.paax and P6.papx) behaved as vulF and did not express the marker (e.g. Fig. 3E,F). When we ablated all ACs at early L3 before VPCs' first division, only 22 of 48 central Pn.pxxx cells did not express the marker (*P*<0.0001). We
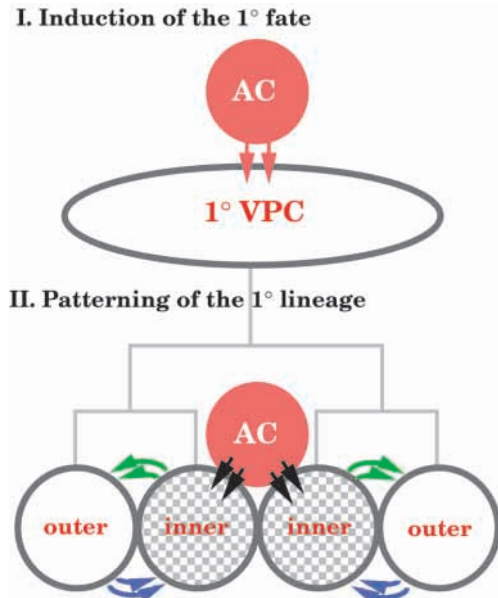
**Fig. 5.** Model for patterning of the 1° vulval lineage. After the early effect of the AC that induces the proliferation of the VPCs, the AC functions again later to pattern the 1° VPC descendants. The inner 1° VPC granddaughters attract the AC better than the outer cells and lead to the AC attachment. Through cell-cell contact, the AC signals the inner 1° VPC granddaughters to ensure that vulF progeny will be produced. The inner 1° VPC granddaughters are internally different from the outer cells, caused by intrinsic polarity of the 1° VPC daughters. In addition, signaling between the inner and outer 1° VPC descendants promotes the differentiation of the inner and outer cells. The 1° VPC descendants can variably adopt the vulE or vulF fate without forming a fixed spatial pattern when one or more of the three mechanisms is disrupted.

conclude that the AC may signal the P6.pxx cells that it contacts to ensure that vulF progeny will be generated.

### RAS signaling may be used by the AC to pattern the 1° lineage

We tried to elucidate the signals and pathways that are utilized in 1° patterning. As shown above, *lin-12* is not required to pattern the 1° lineage. Since LET-60 RAS is the signal transducer during the initial AC induction of VPC fates, we tested whether RAS also functions later in patterning the 1° lineage.

To test the effect of interrupted RAS signaling on 1° patterning, we used a heat shock construct, which encodes a putative dominant negative form of RAS from the closely related nematode *Pristionchus pacificus* (*hs-ras(dn)*; C. Sigrist and R. Sommer, personal communication). This RAS variant is about 90% identical to LET-60 and contains a G10R mutation (Sommer et al., 1996). In *C. elegans*, a G10R mutation of *let-60 ras* has a dominant negative effect on the inductive signaling activity and causes a vulvaless phenotype (Han and Sternberg, 1991).

We crossed *zmp-1::GFP* into the transgenic line carrying *hs-ras(dn)*, heat shocked the animals at different times, and then scored the *zmp-1::GFP* expression pattern in the 1° lineage. When we heat shocked animals before VPCs' first division, P5.p, P6.p or P7.p always adopted the 3° fate (*n*=16 animals),

### Table 3. The effect of adjacent 1° lineages and multiple ACs
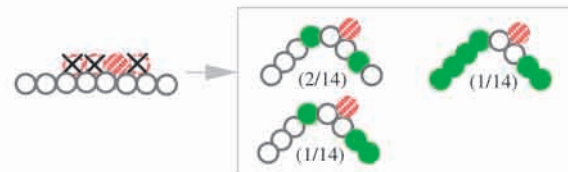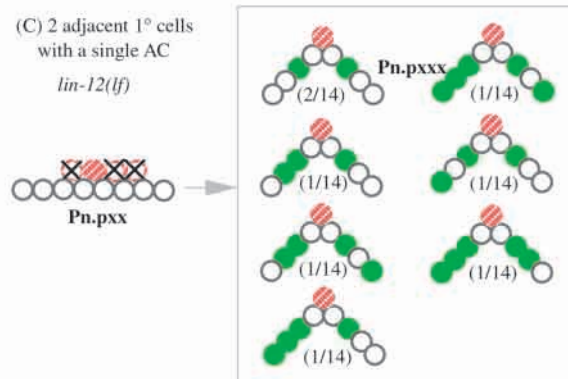


*lin-12(lf); zmp-1::GFP* animals were scored using Nomarski optics. In all panels, ventral is down. All ablations were performed at the early L3 stage before VPCs' first division. The expression patterns of *zmp-1::GFP* in Pn.pxxx cells were scored by Nomarski optics during L4 lethargus. Filled circles indicate Pn.pxxx cells that expressed GFP; unfilled circles indicate those that did not. In C, only the expression patterns of *zmp-1::GFP* in P5.pxxx and P6.pxxx at the upper focal plane (either the left or right side of the animal) were scored. In all panels, the relative position of the AC(s) to P5.pxx and P6.pxx cells during L3 lethargus was scored and indicated. In *lin-12(lf)* mutants, the ACs fail to fuse with uterine p cells and therefore persist throughout larval stages.

Full genotypes: *unc-32(e189) lin-12(n676n909); zmp-1::GFP* and *unc-6(ev400); zmp-1::GFP*.
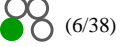
*n*, the numerators indicate the number of animals whose 1° VPC descendants display the *zmp-1::GFP* expression pattern shown; the denominators indicate the number of animals scored.

(A) P5.p and P7.p, along with all ACs except for one, were ablated in *lin-12(lf)* mutants. The P6.p lineage was scored.

(B) P5.p and P7.p were ablated in *lin-12(lf)* mutants, and the P6.p lineage was scored.

(C) All ACs but one were ablated in *lin-12(lf)* mutants. P7.p was ablated, and the P5.p and P6.p lineages were scored.

### Table 4. Expression of a dominant negative form of *ras* disrupts patterning of the 1° lineage

| Experimental conditions | zmp-1::GFP expression in P6.pxxx during L4 lethargus (*n**) | |
|---|---|---|
| No *hs-ras(dn)*‡ | ⬤⬤ (top), ⬤⬤ (bottom)  (68/68) | |
| Induce *hs-ras(dn)* at the late P6.px or the early P6.pxxx stage§ | ○○ / ⬤⬤  (11/38) | ⬤○ / ○○  (2/38) |
| | ○○ / ⬤○  (7/38) | ⬤⬤ / ○○  (1/38) |
| | ○○ / ○⬤  (6/38) | ⬤⬤ / ⬤○  (4/38) |
| | ⬤○ / ○⬤  (1/38) | ⬤⬤ / ⬤⬤  (2/38) |
| | | ⬤⬤ / ⬤⬤  (4/38) |
| Induce *hs-ras(dn)* at the late Pn.pxx stage¶ | ⬤⬤ / ⬤⬤  (14/14) | |

L3 animals were staged using Nomarski optics. In all panels, ventral is down. The panels indicate the expression patterns of *zmp-1::GFP* in P6.pxxx cells at the left or right side of the animal, observed by Nomarski optics during L4 lethargus. Filled circles indicate the P6.pxxx cells that expressed GFP, and unfilled circles indicate the P6.pxxx cells that did not. Full genotype: *hs-ras(dn); zmp-1::GFP*.

*The numerators indicate the number of half 1° lineage patterning units with the *zmp-1::GFP* expression pattern displayed. The denominators indicate the number of half 1° lineage patterning units scored.

‡*hs-ras(dn); zmp-1::GFP* animals that were not heat shocked.

§*hs-ras(dn); zmp-1::GFP* animals were heat shocked at late-L3, just before, during or right after the divisions of P6.px cells.

¶*hs-ras(dn); zmp-1::GFP* animals were heat shocked during L3 lethargus.

### Table 5. Patterning of the 1° lineage in mutants of the Ras signaling pathway components

| Background | AC ablation at the late P6.px or early P6.pxx stage | zmp-1::GFP expression in P6.p lineage* |
|---|---|---|
| Wild-type | Intact | 276/276 |
| Wild-type | AC ablated | 6/35 |
| *let-23 (gf)* | Intact | 49/49 |
| *let-23 (gf)* | AC ablated | 13/16 |
| *lin-15 (lf)* | Intact | 39/41 |
| *lin-15 (lf)* | AC ablated | 2/19 |
| *hs-ras(dn)* | Untreated | 34/34 |
| *hs-ras(dn)* | Heat shocked | 3/19 |
| *lin-1 (lf)* | Intact | 5/23 |
| *lin-31 (lf)* | Intact | 31/31 |

Animals were scored using Nomarski optics during L4 lethargus. AC ablation or heat shock were performed during the late P6.px or the early P6.pxx stage.

*The numerators indicate the number of P6.p cells whose descendants display the wild-type *zmp-1::GFP* expression pattern. The denominators indicate the number of P6.p cells scored.

The full genotypes were: *let-23(sa62); zmp-1::GFP, lin-15(e1763); zmp-1::GFP, hs-ras(dn); zmp-1::GFP, lin-1(sy254); zmp-1::GFP* and *lin-31(n301); zmp-1::GFP*.

indicating that wild-type RAS activity was effectively blocked by heat shock at this stage. When we disrupted RAS signaling after the 1° VPC was specified by heat shocking animals during the late Pn.px or the early Pn.pxx stage, three central VPCs (P5.p-P7.p) were induced to divide three times as in wild type, but the pattern of *zmp-1::GFP* expression in P6.pxxx cells was abnormal (Table 4, Fig. 4A,B). The distribution of these abnormal patterns was very similar to the patterns of 1° lineage resulting from AC ablation (*P*=0.83, Table 1), suggesting that RAS may be used to transduce the AC signal to program the 1° VPC granddaughters. However, it remains possible that RAS is involved in other aspects of the 1° patterning process, or affects the process indirectly, such as by facilitating the function of the AC.

If the AC utilizes RAS signaling to pattern the 1° lineage, constitutive activation of the receptor LET-23 might be able to bypass the requirement of the AC. We used a gain-of-function allele of *let-23*, *sa62*, to constitutively activate RAS signaling (Katz et al., 1996), and ablated the AC in this background (Table 5). When the AC was ablated at the late P6.pxx or the early P6.pxxx stage in *let-23(sa62)* mutants, 13 out of 16 animals had wild-type *zmp-1::GFP* expression, which is significantly different from the result obtained from AC ablation in a wild-type background (*P*<0.0001, Table 5). The observation that the 1° patterning defects in the absence of the AC can be rescued by the ligand-independent activated LET-23 is consistent with RAS activation being involved in transducing the AC signal to pattern the 1° lineage.

To examine whether all components connected with the RAS pathway during initial 1° VPC fate specification are also required in patterning of the 1° lineage, we examined *zmp-1::GFP* expression in various mutant backgrounds. Since reduction-of-function mutations of positive components of the RAS pathway disrupts the initial 1° VPC fate specification, we scored only mutants with at least wild-type levels of VPC induction (three or more VPCs induced).

In a *lin-15* loss-of-function background, all VPCs often adopt vulval fates, and P6.p is always the closest VPC to the AC and adopts the 1° fate. We observed wild-type *zmp-1::GFP* expression in 39 of 41 P6.p lineages (Table 5; the remaining two animals had an abnormal gonad). Therefore, *lin-15* is not required for normal 1° patterning. Furthermore, unlike *let-23(gf),* the *lin-15(lf)* mutation does not suppress the 1° patterning defect caused by the absence of the AC. When the AC was ablated at the late P6.px stage or the early P6.pxx stage, the expression patterns of *zmp-1::GFP* in P6.pxxx cells were disturbed in 17 of 19 animals examined (*P*<0.0001, Table 5). Thus, *lin-15* is likely not involved in negatively regulating RAS signaling during 1° lineage patterning.

*lin-1* encodes an ETS domain transcription factor and is a downstream inhibitor of vulval induction that is negatively regulated by *let-60 ras* (Beitel et al., 1995). Similar to *lin-15* mutants, *lin-1* loss-of-function mutants have all VPCs adopting vulval fates, and the AC is closest to P6.p, which always becomes 1°. We found that the *zmp-1::GFP* expression pattern in P6.pxxx was abnormal in 18 of 23 animals examined (Table 5), suggesting that *lin-1* is involved in 1° lineage patterning.

LIN-31, a winged helix transcription factor, functions downstream of RAS signaling (Miller et al., 1993). In a *lin-31* mutant background, VPCs adopt vulval or non-vulval fates randomly, although P6.p is always the closest VPC to the AC. We scored the patterning of P6.p descendants only when P6.p adopted the 1° fate (scored according to detachment of the

descendants from ventral cuticle and symmetry of invagination; Katz et al., 1995), and found that it displayed the wild-type *zmp-1::GFP* expression pattern in its progeny in all cases (*n*=31 animals, Table 5). Therefore, *lin-31* is likely not required during 1° lineage patterning.

## Patterning of adjacent 1° lineages with a single AC reveals signaling between the inner and outer 1° VPC descendants

To determine whether there are any other intercellular signaling mechanisms involved in 1° lineage patterning besides AC signaling, we ablated P7.p and all but one AC in a *lin-12(lf)* background to examine patterning of adjacent 1° lineages with a single AC. In all 14 animals examined, the Pn.pxx cells that attached to the AC gave rise to progeny that did not express GFP (as vulF), while their flanking Pn.pxx cells always produced progeny that expressed GFP (as vulE) (Table 3C, Fig. 3G,H). The rest of the Pn.pxxx cells showed a random pattern of GFP expression. Therefore, the AC signals the 1° VPC granddaughters it contacts to produce vulF progeny. Their immediate neighbors invariably generate vulE progeny. However, the progeny of the more distal neighbors do not form an invariant pattern. Notably, the Pn.pxxx cells surrounding the AC that form the E-F-F-E pattern do not always descend from the same 1° VPC daughter and therefore do not always possess internal differences through intrinsically polar mother cells. We infer that in addition to AC signaling, there is likely signaling between the inner and outer 1° VPC descendants (P6.pxx or P6.pxxx cells) in patterning the 1° lineage.

In this experiment, all 28 vulF cells that attached to the AC

had a vulE neighbor (100%, Table 3C). In contrast, only 64 of 105 inner vulF cells in wild-type animals without an AC had an outer vulE neighbor (61%, Table 1D). Therefore, signaling between the inner and outer 1° VPC descendants might be partly triggered by AC signaling the inner 1° VPC descendants. It is also conceivable that the AC signals the outer 1° VPC descendants from a distance to produce the vulE progeny (see Discussion).

## Bias of 1° lineage patterning in the absence of AC signaling reveals intrinsic polarity of the 1° VPC daughters

Is there an intrinsic mechanism that generates the polar 1° VPC daughters during 1° lineage patterning? Two lines of evidence suggest that the inner 1° VPC granddaughters are different from the outer cells prior to AC-dependent patterning of the 1° lineage.

First, positioning of the AC with respect to the 1° VPC progeny is biased in wild-type animals. Before P6.p's first or second division, the AC is not always in the middle of P6.p daughters. However, the AC invariably migrates and ends up attaching to the inner P6.pxx, but not the outer P6.pxx during L3 lethargus (D. R. Sherwood and P. W. S., unpublished). When both 2° neighbors and one of the 1° VPC daughters were ablated, the AC invariably attached only to the one presumptive inner granddaughter left, but not to the presumptive outer granddaughter (*n*=9 animals). Strikingly, when multiple ACs were present in a *lin-12(lf)* background, they attached to only the inner P6.pxx cells in 12 of 14 cases examined (Table 3B). Considering the relatively random position of the AC before the 1° VPC granddaughters were born, if the inner and outer granddaughters of the 1° VPC were equally attractive to the AC, the AC should have been positioned more randomly.

Second, the inner and outer 1° VPC descendants are predisposed in adopting the vulF and vulE fates, respectively.

### Table 6. *lin-17* is involved in proper expression of *zmp-1::GFP* in the 1° lineage

| Experimental conditions *lin-17(lf)* | *zmp-1::GFP* expression in P6.pxxx at L4 molt (*n*)* |
|---|---|
| Intact‡ | (144/144) |
| Ablate AC at the late P6.px to the early P6.pxx stage§ | |
| Ablate AC at the late P6.pxx stage¶ | (16/16) |

L3 animals with integrated *zmp-1::GFP* were staged using Nomarski optics. In all panels, ventral is down. The panels indicate the expression patterns of *zmp-1::GFP* in P6.pxxx cells at the left or right side of the animal, observed by Nomarski optics during L4 lethargus. Filled circles indicate the P6.pxxx cells that expressed GFP; unfilled circles indicate the P6.pxxx cells that did not.

Full genotype: *lin-17(n671); zmp-1::GFP*.

*The numerators indicate the number of half 1° lineage patterning units with the *zmp-1::GFP* expression pattern displayed. The denominators indicate the number of half 1° lineage patterning units scored.

‡Control animals that were not ablated.

§The AC was ablated at late-L3, right before, during, or right after the second divisions of P6.p.

¶The AC was ablated during L3 lethargus.

### Table 7. Distribution of patterns of *zmp-1::GFP* expression in P6.pxxx cells

| | | P6.pxxx pair with different inner and outer cells | | Proper orientation | |
|---|---|---|---|---|---|
| Background | Perturbation | % | *n* | % | *n* |
| Wildtype | Intact | 100 | 552 | 100 | 552 |
| Wildtype | AC ablated | 50 | 140 | 91 | 70 |
| *hs-ras (dn)* | Untreated | 100 | 136 | 100 | 136 |
| *hs-ras (dn)* | Heat shocked | 50 | 76 | 87 | 38 |
| *lin-1* | Intact | 57 | 92 | 92 | 52 |
| *lin-17* | Intact | 100 | 288 | 100 | 288 |
| *lin-17* | AC ablated | 12 | 60 | 86 | 7 |

Results from Table 1, 4, 5 and 6 were summerized to compare the results of AC ablation in wild type, a heat shock induced Ras(dn) background , a *lin-1(sy254)* background, and AC ablation in a *lin-17(n671)* background. AC ablation or heat shock were performed during the late P6.px or the early P6.pxx stage. The expression patterns of *zmp-1::GFP* in P6.pxxx cells were scored during L4 lethargus. Inner and outer P6.pxxx pairs that expressed *zmp-1::GFP* differently were scored as P6.pxxx pairs with different inner and outer cells. Among these P6.pxxx pairs with different inner and outer cells, those expressed *zmp-1::GFP* in the outer cells were scored as those with the proper orientation.

*n*, the number of inner and outer P6.pxxx pairs scored.

Simply activating RAS signaling by *let-23(gf)* can partially rescue the 1° patterning defect caused by the absence of the AC (Table 5), suggesting that the inner and outer 1° VPC descendants can differentiate from one another independent of the AC. Furthermore, when the AC was ablated, RAS signaling disrupted by heat shock induced RAS(dn) after the induction of VPC fates, or *lin-1* mutated, the 1° VPC descendants expressed *zmp-1::GFP* in a variable fashion (Tables 1D, 4, 5). However, a careful examination of the pattern of *zmp-1::GFP* expression shows that among the pairs of 1° inner and outer P6.pxxx cells that adopted different fates, 64/70, 33/38 and 48/52 had the proper orientation with vulF facing the normal position of the AC, respectively (Table 7). Since unbiased signaling between the inner and outer 1° VPC descendants can not produce invariably oriented asymmetric divisions of the 1° VPC daughters, such a bias towards specifically oriented 1° lineage pattern might involve an intrinsic mechanism that forms the polar 1° VPC daughters.

## LIN-17 may mediate signaling between the inner and outer 1° VPC descendants

*lin-17* encodes a seven-transmembrane protein similar to *Drosophila Frizzled*, a Wnt receptor (Vinson et al., 1989; Sawa et al., 1996). In *lin-17* mutants, asymmetric divisions of many different types of cells are disrupted (Ferguson et al., 1987; Sternberg and Horvitz, 1988; Way et al., 1992; Chamberlin and Sternberg, 1995; Jiang and Sternberg, 1998). In the vulva, the asymmetric division of P7.p is either abolished or reversed. We suspected that LIN-17 could be involved in the asymmetric divisions of the 1° VPC daughter P6.px, despite that the 1° lineage patterning is wild-type in *lin-17(lf)* mutants (Table 6). First, a *lin-17::lacZ* reporter gene was expressed in all P6.pxx cells (Sawa et al., 1996). Second, and more importantly, double mutants of *lin-17* and *lin-18*, another gene involved in asymmetric divisions in the 2° vulval lineage, show defects in *zmp-1::GFP* expression in P6.pxxx cells, although neither single mutant does (Table 6; Ferguson et al., 1987; M. W., W. Katz and P. W. S., unpublished). This suggests that *lin-17* may function redundantly with other genes during 1° patterning.

To create a sensitized background to examine the effect of *lin-17* on 1° patterning, we ablated the AC. We found that AC ablation in *lin-17* mutants during the late Pn.px or the early Pn.pxx stage resulted in abnormal expression patterns of *zmp-1::GFP* in P6.pxxx cells (Table 6, Fig. 4C,D), and that these patterns were distinct from those caused by AC ablation or disruption of RAS activity in a wild-type background (Table 7). Strikingly, in *lin-17* mutants lacking an AC, only 12% of the inner and outer P6.pxxx cells adopted different fates. In contrast, in wild-type animals lacking an AC, 50% of the inner and outer P6.pxx cells adopted different fates (*P*<0.0001, Table 7). Similarly, in animals with heat shock induced RAS(dn) and *lin-1* mutants, 50% and 57% of the inner and outer P6.pxx cells adopted different fates, respectively (Table 7). Therefore, *lin-17* appears to function in parallel to the AC signaling to promote the distinction of the inner and outer 1° VPC descendants.

Among the pairs of inner and outer P6.pxxx cells that adopted different fates in AC ablated wild-type animals, RAS(dn) induced animals, or *lin-1* mutants, 64 of 70, 33 of 38 and 48 of 52, respectively, had the proper orientation with vulF facing the normal position of the AC (Table 7). In *lin-17* mutants, six of seven also had the proper orientation (*P*>0.5,

Table 7). Therefore, the intrinsic bias of the inner and outer cells to adopt a proper orientation in the 1° pattern is relatively normal when *lin-17* is mutated. If *lin-17* were involved in establishing the intrinsic polarity of the 1° VPC daughters, we would expect such a bias to be disrupted. We infer that *lin-17* could facilitate the differentiation of the 1° VPC descendants by mediating signaling between the inner and outer 1° VPC descendants (P6.pxx or P6.pxxx).

## DISCUSSION

We have examined the mechanisms of 1° lineage patterning during *C. elegans* vulval development in order to understand how a precursor cell produces a pattern of different progeny cells. We show that the AC, which induces the VPC to adopt the 1° fate, functions again later to ensure an appropriate pattern of cell fates. During AC patterning of the 1° lineage, the AC appears to function within a short range and might require direct contact to signal the inner 1° VPC granddaughters. Our results suggest that additional mechanisms, including intrinsic polarity of the 1° VPC daughters and signaling between the inner and outer 1° VPC descendants, might also be involved in pattern formation of the 1° lineage. Finally, we find that the AC may use the RAS signaling pathway to pattern the 1° lineage, and the Wnt receptor LIN-17 may function during signaling between the inner and outer 1° VPC descendants.

## A model for 1° lineage patterning

In wild-type animals, a final pattern of eight 1° VPC descendants is established by specification of two distinct cell types, vulE and vulF. We used *zmp-1::GFP* as a molecular marker to distinguish the vulE and vulF fates adopted by the 1° VPC great granddaughters. Our results suggest a model for 1° lineage patterning that is dependent on at least three different mechanisms: AC signaling the inner 1° VPC granddaughters, signaling between the inner and outer 1° VPC descendants, and intrinsic polarity of the 1° VPC daughters (Fig. 5).

First, in wild-type animals, the AC signals the inner granddaughters of the 1° VPC through direct contact to ensure the production of vulF progeny (Fig. 5). Results of AC ablation after the induction of VPC fates demonstrate that the AC signal is required for 1° lineage patterning. Analysis of situations in which the AC properly attached to, or was at a distance from the 1° granddaughters, indicates that the AC functions locally. A dorsally or laterally displaced AC is incapable of programming a wild-type pattern of the 1° lineage. Moreover, when in contact, the AC can signal an outer 1° VPC granddaughter to generate the vulF descendants.

Second, signaling between the inner and outer 1° VPC descendants (granddaughters or great granddaughters) may ensure proper differentiation of the vulF and vulE fates (Fig. 5). This hypothesis is based on the observation that vulF cells patterned by the AC are always flanked by vulE cells, even when they do not descend from the same 1° VPC daughter. In addition, the *lin-17(lf)* mutant phenotype suggests that in the absence of the AC, the distinction between the inner and outer descendants can be significantly disrupted without affecting the proper orientation of the outcome in rare cases with differentiated inner and outer cells.

Third, the inner 1° VPC granddaughters are internally different from their outer sisters, probably a consequence of intrinsic polarity of the 1° VPC daughters (Fig. 5). Such an intrinsic program may lead to asymmetric segregation of cytoplasmic determinants during divisions of the 1° VPC daughters. By examining the 1° lineage patterning in the absence of the AC, we have found that the orientation of the pattern of the inner and outer cell pair is strongly biased, independent of AC signaling. This bias is striking even if the inner 1° VPC descendants might signal between them to differentiate from one another and therefore work antagonistically to the intrinsic polarity mechanism. Also, homoiogenetic signaling between the inner 1° VPC descendants could not explain the bias of the patterning orientation, should there be no intrinsic polarity of the 1° VPC daughters.

## Multiple mechanisms help ensure the precision of 1° patterning

Our results indicate that no single mechanism is sufficient and multiple mechanisms likely act together to increase the reproducibility of 1° lineage patterning. On the one hand, the combined mechanisms of signaling between the inner and outer 1° VPC descendants and intrinsic polarity of the 1° VPC daughters are apparently not enough to pattern the 1° lineage, since the AC is an indispensable component in patterning the 1° lineage precisely. On the other hand, AC signaling alone may not be sufficient to determine precisely the fates of the VPC descendants.

It is unlikely that AC signaling at a distance is the only mechanism involved in patterning the vulE fate. We have found that vulF cells formed in the absence of the AC have more variable neighbors than vulF cells with an AC. Since the neighboring vulE and vulF cells do not always descend from the same VPC daughter, we can exclude intrinsic asymmetric division as the only other mechanism. Either signaling between the inner and outer 1° VPC descendants partly relies on AC signaling, or the AC also signals from a distance to produce vulE cells, or both. If there were no signaling between the neighboring 1° VPC descendants, each cell would have to solely depend on interpretation of absolute levels of the AC signal received, or, the AC would have to send out different signals to the VPC descendants in contact and those at a distance. However, this does not seem to be the case. In *unc-6* mutants, when the dorsally or laterally mispositioned AC had a similar distance from the outer 1° VPC granddaughters as in wild-type, the outer descendants did not invariably become vulE (Table 2, data not shown).

It is probable that the mechanism of signaling between the inner and outer 1° VPC descendants is partially redundant with other mechanisms. LIN-17 activity is involved in signaling between the inner and outer 1° VPC descendants. However, the 1° lineage patterns correctly in *lin-17(lf)* mutants. Ablation of a subset of the 1° VPC granddaughters does not affect patterning of the remaining granddaughters. When both inner P6.pxx cells (P6.pap and P6.ppa) were ablated, the two remaining outer cells (P6.paa and P6.ppp) underwent their normal divisions, and the four resulting progeny all expressed the marker (*n*=14 animals). When P6.paa and P6.ppp were ablated, P6.pap and P6.ppa developed normally, and none of the four resulting progeny expressed the marker (*n*=12

animals). Since the ablation of P6.pxx cells could only be performed after the mitosis of P6.px cells had completed, the ablation may not disrupt signaling between the inner and outer P6.pxx cells if they signal each other right after they are born. This is true of many Wnt signaling events in *C. elegans*. Alternatively, their signaling is redundant with other mechanisms in 1° patterning.

Our results indicate that the 1° patterning process might employ a triple assurance strategy: the AC signals the inner 1° VPC granddaughters, the 1° VPC granddaughters are intrinsically different, and signaling between the inner and outer 1° VPC descendants reinforces their difference (Fig. 5). Utilizing multiple mechanisms to ensure the precision of cell fate pattern formation may be a general scenario during pattern formation in many developmental systems, e.g. differentiation of mating type between mother and daughter cells in *S. cerevisiae*, and the four different progeny cells produced by the sensory organ precursors in *Drosophila* (reviewed by Herskowitz, 1989; Jan and Jan, 1994).

## Proteins that are involved in each mechanism

Our results suggest that some components in the RAS pathway that act during initial VPC fate specification may also be involved in the later role of the AC in patterning the 1° lineage. Terminating RAS signaling after VPC fate specification has an equivalent effect to ablating the AC at this time. These results might explain the finding that RAS is involved in vulval cell migration and cell fusion (Shemer et al., 2000), since the distinct morphogenesis and cell fusion behavior of vulE and vulF cells are likely downstream events of their specification. Rescue of the 1° patterning defect after AC ablation by a ligand-independent activated form of the LET-23 RTK is also consistent with this scenario. Among other downstream effectors of RAS, LIN-1, a common effector in multiple tissues, is required in patterning of the 1° lineage, but the tissue-specific LIN-31 (reviewed in Tan and Kim, 1999) is not. LIN-15, a negative regulator of the pathway during VPC induction, does not seem to be involved in 1° lineage patterning. Finally, we speculate that the ligand of the AC signaling pathway might be a membrane-bound protein, due to requirement of the AC to function at short range. Consistent with this speculation, heat shock induced LIN-3EGF expression (presumably diffusible) does not have any effect on 1° lineage patterning (data not shown).

Among other signaling pathways that may function in the vulva, the LIN-12/Notch signaling pathway is unlikely to be involved in AC signaling to pattern the 1° lineage. In *lin-12(lf)* mutants, a 1° lineage with a single AC has a wild-type pattern of *zmp-1::GFP* expression. Also, an RTK of the Eph receptor family, VAB-1, is likely not required in 1° patterning, since *vab-1* mutants do not display any defects in 1° VPC specification and 1° lineage patterning (George et al., 1998; data not shown).

We show here for the first time that the Wnt receptor LIN-17 functions in asymmetric divisions of the 1° VPC daughters. Decreasing LIN-17 activity significantly inhibits the inner and outer 1° VPC descendants from becoming different from one another, but does not affect the orientation of the resulting pattern if they do become different. LIN-17 might thus be involved in signaling between the inner and outer 1° VPC descendants (which affects only distinction), rather than

intrinsic polarity of the 1° VPC daughters (which affects both distinction and polarity). Further characterization of molecules in each pathway involved may further clarify how multiple redundant mechanisms regulate 1° lineage patterning.

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