C. elegans PlexinA PLX-1 mediates a cell contact-dependent stop signal in vulval precursor cells

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Abstract

PLX-1 is a PlexinA transmembrane protein in Caenorhabditis elegans, and the transmembrane-type semaphorin, SMP-1, is a ligand for PLX-1. The SMP-1/PLX-1 system has been shown to be necessary for proper epidermal morphogenesis in the male tail and seam cells. Here, we show that the SMP-1/PLX-1 system also regulates vulval morphogenesis. In plx-1 and smp-1 mutants, hermaphrodites sometimes exhibit a protruding vulva or multiple vulva-like protrusions. Throughout the vulval development of plx-1 and smp-1 mutants, the arrangement of vulval cells is often disrupted. In the initial step of vulval morphogenesis, vulval precursor cells (VPCs) are generated normally but are subsequently arranged abnormally in mutants. Continuous observation revealed that plx-1 VPC fails to terminate longitudinal extension after making contact with neighbor VPCs. The arrangement defects of VPCs in plx-1 and smp-1 mutants are rescued by expressing the respective cDNA in VPCs. plx-1::eGFP and smp-1::eGFP transgenes are both expressed in all vulval cells, including VPCs, throughout vulval development. We propose that the SMP-1/PLX-1 system is responsible for a cell contact-mediated stop signal for VPC extension. Analyses using cell fate-specific markers showed that the arrangement defects of VPCs also affect cell fate specification and cell lineages, but in a relatively small fraction of plx-1 mutants.

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Introduction

Morphogenetic movements of epithelial cells are major and prominent phenomena in animal morphogenesis, and include such a wide variety of processes as epiboly, invagination, convergent extension, and tubule formations (Gilbert, 2000). When analyzing morphogenesis, it is important to understand the mechanisms underlying how cells initiate, maintain, and terminate morphogenetic changes. Cells may terminate movement not due to increased adhesiveness but by specific signals suppressing cell motility. In neural development, it is well known that multiple factors negatively regulate the growth cone movement of growing axons through their repulsive effects (for a review, see Dickson, 2002; Tessier-Lavigne and Goodman, 1996). In contrast to neuronal cells, the roles played by repulsive interactions in the morphogenetic processes of non-neuronal cells, including epithelial cells, have had relatively little documentation. Nonetheless, recent observations suggest that repulsive factors, such as Eph/ephrin, are also involved in the regulation of the proper migration and arrangement of cells (Chin-Sang et al., 1999; George et al., 1998; Xu et al., 1995, 1999).

Semaphorins and their receptors, plexins, are known to have repulsive effects on the guidance of growing axons in vertebrates and Drosophila (Nakamura et al., 2000; Raper, 2000). Many members of the semaphorin family cause the growth cone collapse of cultured vertebrate neurons and deflect their trajectories. In the case of Sema3A, this collapse is known to accompany cytoskeleton reorganiza-
tion (Fan and Raper, 1995; Fan et al., 1993). In Caenorhabditis elegans (C. elegans), PLX-1 belonging to the plexinA subfamily, and its ligands, SMP-1 and SMP-2, transmembrane-type class I semaphorins, are involved in epidermal morphogenesis in the male tail and seam cells in the lateral body walls (Fujii et al., 2002; Ginzburg et al., 2002). However, the exact mechanism of how the SMP/PLX-1 system regulates the arrangement of epidermal cells remains to be elucidated. In this paper, we report the role of the SMP-1/PLX-1 system in the morphogenesis of the vulva.

The vulva is a passage for eggs and sperm, localized at the ventral midline of adult hermaphrodites, and consists of 22 identifiable epithelial cells. The vulva originates from epithelial cells known as vulval precursor cells (VPCs), which develop in the first larval (L1) stage (Kimble, 1981; Sulston and Horvitz, 1977). The fate of VPCs is determined in the early L3 stage through multiple cell–cell interactions. Major morphogenetic development of the vulva then occurs, including divisions, fusions, migrations, and rearrangements of vulval cells (Sharma-Kishore et al., 1999).

The cell fate specification of VPCs has been extensively studied (for a review, see Greenwald, 1997). Six VPCs, P3.p–P8.p, have the initial potential to adopt any of 1°, 2° or 3° fates. In normal development, an EGF-like LIN-3 signal emanating from a somatic gonadal cell, the anchor cell (AC), activates the RKT-RAS-MAPK signaling pathway in P6.p, the closest VPC, to adopt the 1° fate (Hill and Sternberg, 1992; Katz et al., 1995; Sternberg and Horvitz, 1986). The induced P6.p laterally signals its neighboring P5.p and P7.p to adopt the 2° fate through the Notch-like LIN-12 receptor (Chen and Greenwald, 2004; Greenwald et al., 1983; Sternberg, 1988; Yochem et al., 1988; Yoo et al., 2004). The products of synMuv genes, including LIN-15 and components of the Rb/E2F pathway, negatively regulate the RAS signaling pathway and repress vulval fates in the absence of LIN-3, inducing P3.p, P4.p, and P8.p to adopt the 3° fate and fuse to the surrounding giant hypodermal syncytium, hyp7 (Ceol and Horvitz, 2001, 2004; Herman and Hedgecock, 1990; Lu and Horvitz, 1998; Thomas and Horvitz, 1999). The Wnt pathways (Eisenmann and Kim, 2000; Eisenmann et al., 1998; Inoue et al., 2004) also play a role in the cell fate specification of VPCs.

Compared to cell fate specification, the cellular and molecular mechanisms underlying vulval morphogenesis are poorly understood. The details of vulval morphogenesis have been described only recently (Sharma-Kishore et al., 1999). In brief, each of the 6 VPCs first extends longitudinally to make contact with each other, divides longitudinally (first division), and 6 of the 12 daughter cells fuse to the surrounding hyp7 syncytium. The remaining 6 VPC daughters divide longitudinally to give rise to 12 VPC granddaughters (second division). Finally, 22 epithelial cells constituting the vulval primordium are generated through terminal division. During these processes, cells constituting the vulval primordium are aligned in rotational two-fold symmetry along the ventral midline. Cells from the anterior and posterior halves of the vulval primordium then start to migrate toward the center of the primordium, meet and fuse with their symmetrical partners from the other half to form the ring precursor at the center of the vulval primordium. The ring precursors invaginate successively, and a premature vulva, which is a depression consisting of a stack of 7 toroidal cells (vulA-vulF), eventually forms. Finally, eplosion transforms the depression into a functional mature vulva.

Genetic screening for mutations affecting the vulval function (egl) or causing such morphological defects as protruding (pvl), everted (evl) or squashed (sqv) vulvae has identified various genes that appear to be specifically involved in vulval morphogenesis rather than cell fate specification (Antoshechkin and Han, 2002; Eisenmann and Kim, 2000; Hanna-Rose and Han, 2002; Herman et al., 1999; Mohler et al., 2002; Seydoux et al., 1993; Trent et al., 1983). Mutations in genes cad-10, mig-2, and unc-73, encoding two members of Rac GTPases and Trio guanine nucleotide exchanging factor in C. elegans, respectively, have been shown to affect the proper orientation of the division and migration of vulval cells (Kishore and Sundaram, 2002). Ras GTPase encoded by let-60 is involved in cell migration, in addition to its well-known role in cell fate specification (Shemer et al., 2000). Depletion of PAR-1, a known regulator of cell polarity, causes failure of the symmetrical halves of the vulval cells to fuse to each other (Hurd and Kemphues, 2003). The list of molecules involved in this process, however, is currently incomplete.

The molecular mechanisms underlying many essential aspects of vulval morphogenesis remain to be elucidated. Even confining our studies to the earliest stage of vulval morphogenesis, we do not know what regulates the stereotypic morphological changes of VPCs. Here, we report vulval formation defects in pvl-1 and smp-1 mutants, and provide evidence that the SMP-1/PLX-1 system mediates a signal required for the cessation of the longitudinal extension of VPCs.

Materials and methods

Strains and general methods

C. elegans strains, N2, DR466 him-5(e1490), NL2099 rrf-3(pk1426), AH142 zhs4[pTB10(lip-1::gfp)], and NH2246 ayIs4[egl-17::gfp]; dpy-20(e1282) were obtained from the C. elegans Genetic Stock Center, care of Theresa Sternagile (The University of Minnesota). NW1358 smp-l(ev715) and NW1335 smp-2(ev709) were provided by Joe Culotti (Ginzburg et al., 2002); SU93 (jcs1) by Jeff Simske (Mohler et al., 1998); arIs92[egl-17::cfp-lacZ] by Iva Greenwald (Yoo et al., 2004) and MT10865 ced-10::gfp::ced-10 by Erik Lundquist (Lundquist et al., 2001).
phrodites to generate lines with extrachromosomal arrays. From intensely fluorescent lines, integrated strains were generated by γ-ray-irradiation as described (Shioi et al., 2001).

**Observation of vulvae**

To examine the GFP expression, worms were mounted on 4% agarose containing 1 mM levamisol and were examined with a Zeiss Axioplan microscope using Zeiss filter set #10. Images were recorded with a Nikon Coolpix-900CCD camera. To examine the CFP expression, Chroma Technology filter 31044V2 was used.

For continuous observation, worms were mounted with 1 mM levamisole and observed at 1-h intervals. To avoid desiccation, agar pads of twice the usual thickness (ca. 0.6 mm) were used and distilled water was added to the pad after each observation.

For laser confocal microscopic observation, Olympus Fluoview was used. 3-D images were constructed from stacks of image files along the z axis using the “voltex” function of amira 2.3 software (TGS). Worms were mounted between the pad and the coverslip with 10 µl of 10 mM NaN₃ for complete immobilization. After each observation, worms were recovered from the pad with a micro capillary filled with M9 buffer, and were transferred to seeded NGM plates to allow further development.

**Results**

The vulva is deformed in plx-1 mutants and smp-1 mutants

In adult plx-1(nc37); him-5 hermaphrodites, 11% of animals had a deformed vulva, including a protruding vulva (PvI), and 2% had extra vulva-like structures (Multivulvae: Mv) along the ventral midline (n = 115) (Fig. 1C) under dissection microscopic observations. Control him-5 hermaphrodites had no protruding or extra vulvae (n = 100). These phenotypes were rescued by a plx-1(+) transgene; in plx-1(nc37); him-5; Ex[plx-1LA-PCR, rol-6(su1006)] animals, none showed protruding or extra vulvae (n = 100). By visualizing their apical junctions with the genetic background of jcIs1 carrying a ajm-1::gfp transgene (Koppen et al., 2001), we found that the arrangement of the vulval cells was often disrupted in plx-1 mutants, deviating from symmetric arrangements in wild-type animals (data not shown).

We have previously shown that the transmembrane class 1 semaphorin, SMP-1, and probably also SMP-2, is a physiological ligand for PLX-1 during ray morphogenesis in the male tail (Fujii et al., 2002). To examine whether vulval defects in plx-1 mutants are dependent on SMP ligands, we examined the vulva in smp-1(ev715) and smp-2(ev709) mutants (Ginzburg et al., 2002). smp-1 hermaphrodites exhibited PvI and Mv phenotypes, vulval defects similar to those of plx-1 mutants (Fig. 1B). On the other hand, smp-2
vulvae appeared normal (Fig. 1D). The RNA interference (RNAi) of smp-1, but not smp-2, caused similar vulval defects (data not shown).

**Defective arrangement of VPCs**

The vulva originates from 6 VPC cells, P3.p–P8.p, which develop in L1 and extend anterior–posteriorly at the L2 stage. By visualizing the apical surface of epithelial cells with the ajm-1::gfp transgene, the vulval defects detected earliest in plx-1 mutants was abnormal morphology and aberrant arrangements of extended VPCs. At this stage, the VPC usually adopts an elongated oval-shape in wild-type animals (Figs. 2A, B), whereas in plx-1 mutants, the size and shape of VPCs were often irregular (Fig. 2C). In wild-type animals, VPCs made contact with each other at their anterior and posterior tips, usually at one point, resulting in the formation of a contiguous single cellular row along the ventral midline. In plx-1 mutants, extended VPCs often overlapped each other and made contact at their lateral sides instead of at their anterior/posterior tips. In addition, there were sometimes more than 7 areas demarcated with AJM-1::GFP in the mutants (Fig. 2C inset). This appeared to be caused by fragmentation of the apical surface of VPCs without cell division, since there were 6 nuclei corresponding to VPCs in these animals and some of the areas were much smaller than the usual vulval cells (data not shown), suggesting that the apical surface of a VPC was partly

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**Fig. 1.** The vulva of plx-1 and smp-1 mutants. (A–D) The lateral view of the mid-body of N2 wild-type (A), plx-1(C), smp-1 (B), and smp-2 (D) mutant animals. In plx-1 and smp-1 mutants, an extra protrusion (black arrows) was seen beside the vulva (white arrows). The smp-2 vulva appeared normal. Scale bar is 20 μm.

**Fig. 2.** Vulval Precursor Cells (VPCs) in plx-1 and smp-1 mutants. The ventro-lateral view of the mid-body of wild-type (N2) (A, B), plx-1 (C) and smp-1 (D) L3 larvae. The apical domain of VPCs before (A, C, D) and shortly after (B) the initiation of the first division (arrows) was visualized with the ajm-1::gfp transgene. (A, B) Wild-type VPCs contacted with each other at the anterior/posterior tips throughout until first division. plx-1 (C) and smp-1 (D) VPCs attached to each other on the lateral sides. The shape of VPCs in the mutants was irregular, distinct from the oval shape of wild-type VPCs. The inset in (C) shows the magnification of 2 overlapping cells whose apical surface demarcated with AJM-1::GFP appeared to be fragmented into smaller areas (asterisks) before the first division. Scale bar is 10 μm.
overlaid with hyp7, a surrounding giant epidermal syncytium, or with other VPCs, and apical junction formed ectopically. The formation of the apical junction between locally folded portions of the surface of a VPC also appeared to lead to fragmentation. In addition, VPCs sometimes did not appear to make contact and were separated by hyp7, forming a gap in the cellular row (Fig. 2D). To quantify this arrangement defect, we scored the number of animals having at least one VPC overlapping other VPCs along more than one third of its length (Table 1). Wild-type animals rarely had overlapping cells (2%), whereas almost all plx-1(nc37) animals had overlapping cells (98%). The defects were rescued by the plx-1(+) transgene: in ncls13[ajm-1::gfp]; plx-1(nc37); him-5; Ex[plx-1LA-PCR, rol-6(su1006)] animals, only 37% had overlapping cells. We also scored the presence of gaps along the row when VPCs initiated the first division, and found that 23% of plx-1(nc37) animals had gaps (n = 100), whereas no gap was found in wild-type animals.

smp-1 VPCs exhibited arrangement defects similar to those of plx-1 VPCs (Fig. 2D): smp-1 mutants always had overlapping VPCs, and gaps were found in 28% (n = 98). In smp-1; plx-1 double-mutants, VPCs were affected in a similar way and to a similar extent to either of the single mutants, suggesting that SMP-1 and PLX-1 function in the same genetic pathway in regulating the arrangement of VPCs. In contrast, the arrangement of smp-2 VPCs was normal.

To reveal how the arrangement defect was generated, we continuously observed VPCs in single worms visualized with the ajm-1::gfp transgene. VPCs developing at the L1 stage were initially round-shaped and separated from each other by hyp7. At L2, each VPC changed its shape and extended longitudinally (Fig. 3B). When a VPC eventually contacted with its neighboring VPC, the extension stopped. Since the positioning and morphology of 6 VPCs in plx-1 animals were normal at the L1 stage (Fig. 3A), the extensive overlapping between plx-1 VPCs at the L3 stage implies that the regulation of longitudinal extension is affected in mutants. Continuous observation confirmed that plx-1 VPCs continued to extend along the lateral side of the neighboring cells even after contact (Fig. 3C). We observed a case in which a VPC made contact with a non-neighboring VPC, resulting in the reversal of the anterior–posterior order of VPCs (Fig. 3C arrow). Occasionally, a VPC appeared to extend preferentially in one direction, either anteriorly or posteriorly, and failed to contact with one of its neighboring VPCs, which appeared to lead to the formation of a gap in a VPC row.

Since our expression analysis indicated that both plx-1 and smp-1 genes are expressed in VPCs, but not in hyp7 (see below), we examined whether the expression of plx-1 and smp-1 genes in VPCs is sufficient for the proper arrangement of VPCs. The lin-31 promoter drives gene expression in Pn.p cells (including VPCs), which decreases after vulval cell divisions are completed and before cell migrations occur (Kishore and Sundaram, 2002; Tan et al., 1998). The arrangements of VPCs in plx-1 mutants were rescued efficiently by the lin-31::plx-1 cDNA transgene: in ncls15[ajm-1::gfp]; plx-1(nc37); Ex[lin-31::plx-1 cDNA, rol-6(su1006)] animals, only 24% had overlapping cells. The lin-31::smp-1 cDNA transgene also rescued the VPC arrangement in smp-1 mutants: in ncls15[ajm-1::gfp];
smp-1 (ev715); Ex[lin-31p::smp-1 cDNA, rol-6(su1006)] animals, only 29% had overlapping cells. On the other hand, the lin-31p::smp-2 cDNA transgene did not rescue the arrangement defects of VPCs in smp-1 mutants in two lines we have examined.

Arrangement defects of vulval primordium in later stages

In all stages thereafter, arrangement defects of vulval cells were detected in plx-1 and smp-1 mutants. In wild-type animals, vulval cells were arranged in almost bilateral symmetry and were in contact with each other (Figs. 4A, C, E). In contrast, the configuration of the vulval primordium was disrupted and the shape of individual cells was affected in the mutants. We scored the morphological defects of the vulval primordium after completion of the second division of VPCs up to the eversion of immature vulvae in plx-1(nc37) mutants. Throughout this time window, symmetric configuration was severely disrupted in nearly half of the mutant animals, and some vulval cells were separated by gaps: At 31–33 h after hatching, 15% of larvae had asymmetric vulval primordia and 31% of larvae had gaps (n = 65) (Fig. 4B). At 33–37 h, 31% and 17% of larvae had asymmetric vulval primordia and gaps, respectively (n = 46) (Fig. 4D), and no defects were scored in wild-type larvae. In the premature vulvae of plx-1 mutants, not only the apical structure, but also the morphology of the toroids was sometimes abnormal (data not shown).

Continuous observation revealed that various aspects of vulval development in the later stages were abnormal in plx-1 mutants. We found that coordinated development of the vulval primordium was sometimes disrupted. In plx-1 mutants, we identified a case in which invagination, which normally occurs after the second division, started before the completion of selective fusion of the descendants of P3/4/8.p, which normally occurs before the second division (Fig. 6A). Unlike wild-types, the synchrony in the development between the anterior and posterior halves of the vulval primordium was also sometimes disrupted in plx-1 mutants. We also found cases where a gap was newly generated in originally contiguous vulval cells (Fig. 6B).

In order to thoroughly examine the morphology of vulval cells, we used a transgenic line expressing ced-10::gfp::ced-10 (Lundquist et al., 2001), in which GFP is localized close to the cell cortex, to visualize the entire cell contour. Reconstruction of the 3-D configuration revealed that vulval cells are simple columnar epithelial cells, with their apical surface adjacent to the ventral cuticle, while their basal surface extends up to the overlying gonad in wild-type animals (Figs. 7A, B). In plx-1 mutants, some vulval cells no longer adopted columnar morphology, but were squeezed against the overlying gonad, and their apical surfaces did not reach the ventral cuticle (Fig. 7C). Vulval cells sometimes failed to form a single cell cluster, being completely separated by a large epidermal cell, hyp7. These observations confirmed that some vulval cells do not make contact with each other and their shapes are aberrant not only at the apical surface but in terms of their three dimensional configurations in plx-1 mutants.

To examine whether the plx-1 gene is required in later stages of vulval morphogenesis, we followed the development of vulval primordia in plx-1 animals whose VPCs had

**Fig. 4.** Vulval cells in plx-1 mutants. (A–F) Cells constituting the vulval primordium visualized with the ajm-1::gfp transgene in wild-type (A, C, E) and plx-1 (B, D, F) hermaphrodites at the early L3 (32 h) (A, B), at the mid L3 (34 h) (C, D), and at the early L4 (E, F) stages. (A, C) In wild-type animals, cells of the vulval primordium were in contact with each other and were arranged in rotational, two-fold symmetry. (B, D) In plx-1 mutants, cells were sometimes separated by a gap (arrowheads) and the symmetric configuration was disrupted (arrows). (F) Some cells (arrowhead) were separated from the main vulval primordium undergoing invagination. (G) A schema indicating the developmental stages of vulval primordia is shown in (A)–(F). Scale bars are 20 μm.
been arranged normally by introducing the \textit{lin-31::plx-1} transgene. Out of 62 \textit{ncIs13[ajm-1::gfp]; plx-1(nc37); Ex[lin-31::plx-1 cDNA, rol-6(su1006)]} animals that exhibited a normal VPC arrangement, the vulval primordium developed normally in 57 animals (Fig. 5). In addition, out of 68 \textit{ncIs15[ajm-1::gfp]; smp-1(ev715); Ex[lin-31::smp-1 cDNA, rol-6(su1006)]} animals with normally arranged VPCs, the vulval primordium developed normally in 65 animals. The frequency of the occurrence of defective vulvae in those animals was comparable to those in wild-type animals. Since the \textit{lin-31} promoter is not active after vulval cells have completed their division, the results indicate that the SMP-1/PLX-1 system is dispensed with during later vulval morphogenesis.

![Fig. 5](image1.png)

\textbf{Fig. 5.} Morphological defects in late L4 vulval primordia. Vulval primordia whose overall configuration severely deviated from two-fold symmetry without gaps (hatched bars), and primordia with gaps (white bars) were scored in N2, \textit{plx-1(nc37), smp-1(ev715), and smp-1(ev715); plx-1(nc37)}. Defects in \textit{plx-1(nc37) and smp-1(ev715)} animals, in which the arrangement defects of VPCs had been rescued with the respective cDNAs driven under the \textit{lin-31} promoter or defects in \textit{plx-1(nc37)} animals, whose VPCs had adopted the 2' fate normally, were also scored: \textit{plx-1; Ex[lin-31::plx-1] (VPC rescued), smp-1; Ex[lin-31::smp-1] (VPC rescued) and plx-1 (normal lip-1::gfp)}. We did not examine the arrangement of individual cells extensively and some primordia not scored as asymmetric may have an aberrant cell arrangement. The numbers of animals examined were shown in parentheses at the top of bars.

![Fig. 6](image2.png)

\textbf{Fig. 6.} Sequential changes in \textit{plx-1} vulval primordia. (A) The most distally located cell (small arrowheads) in the left half remained unfused after the next proximal cell (large arrowheads) and two distal cells in the right half had completed cell fusion. Even after the central region of the primordium started to invaginate (an arrow), the cell had not fused with hyp7. (B) A gap was newly generated (an arrow) in the primordium. Scale bars are 5 \(\mu\text{m}\).
The vulva of wild-type and plx-1 animals carrying ced-10::gfp::ced-10, ced-10::gfp::ced-10 was examined with a confocal microscope. The lateral view of the vulval primordium was reconstructed by combining optical sections projected onto the sagittal plane using “amira” software. Dorsal side is up. Note that, as the vulva is located at the ventral side of the body, the apical surface of vulval cells (“ap.” in A, C) is at the bottom and the basal surface (“ba.” in A, C) is at the top in these figures. (A, B) In the wild-type, vulval cells formed a simple columnar epithelium, with each cell extending from the basal surface adjacent to the overlying gonad to the apical surface adjacent to the ventral cuticle at the L3 (A) and L4 (B) stage. (C, D, E) The vulva of a single plx-1 animal was examined at the L3, L4 and adult stages. (C) At L3, hyp7 epidermal cell (white arrowheads) separated the vulval primordium into three cell clusters (horizontal arrows). Some vulval cells did not display the columnar shapes typical of epidermal cells, and some did not reach the apical surface adjacent to the ventral cuticle (vertical arrows). (D) At L4, the gaps persisted and three cell clusters (horizontal arrows) each started to invaginate. (E) In adults, each cell cluster formed a vulva-like protrusion. Scale bars are 10 μm.

Fig. 7. The vulva of wild-type and plx-1 animals carrying ced-10::gfp::ced-10. ced-10::gfp::ced-10 was examined with a confocal microscope. The lateral view of the vulval primordium was reconstructed by combining optical sections projected onto the sagittal plane using “amira” software. Dorsal side is up. Note that, as the vulva is located at the ventral side of the body, the apical surface of vulval cells (“ap.” in A, C) is at the bottom and the basal surface (“ba.” in A, C) is at the top in these figures. (A, B) In the wild-type, vulval cells formed a simple columnar epithelium, with each cell extending from the basal surface adjacent to the overlying gonad to the apical surface adjacent to the ventral cuticle at the L3 (A) and L4 (B) stage. (C, D, E) The vulva of a single plx-1 animal was examined at the L3, L4 and adult stages. (C) At L3, hyp7 epidermal cell (white arrowheads) separated the vulval primordium into three cell clusters (horizontal arrows). Some vulval cells did not display the columnar shapes typical of epidermal cells, and some did not reach the apical surface adjacent to the ventral cuticle (vertical arrows). (D) At L4, the gaps persisted and three cell clusters (horizontal arrows) each started to invaginate. (E) In adults, each cell cluster formed a vulva-like protrusion. Scale bars are 10 μm.

plx-1::gfp and smp-1::gfp transgenes are expressed throughout vulval development

We examined the expression of plx-1 using a reporter transgene ncEx[plx-1::egfp, rol-6(su1006)], containing a translational fusion construct (Fujii et al., 2002). All the vulval precursor cells and their descendants, vulA-vulF, expressed GFP intensely throughout development (Figs. 8A–D). No intense expression was detected in hyp7.

We also examined the expression of smp-1 and smp-2 genes in the vulval primordia using translational fusion constructs with GFP. The smp-1::egfp expression was detected in all vulval precursor cells and their descendants (Figs. 8E–G). In addition to the vulva, smp-1::egfp was detected in larval seam cells, the epidermal cells of a larval male tail, and some neurons in the ventral cord and around the nerve rings. The ventral and dorsal cords also expressed GFP. hyp7 did not appear to express the transgene. Interestingly, the expression pattern resembled that of plx-1::egfp.

The expression pattern of the smp-1 mRNAs revealed by in situ hybridization is available on NEXTO_DB (http://nematode.lab.nig.ac.jp/cgi-bin/dbest/SrchByClone.sh?clone=yk422b11). The signal was detected in cells of the vulval primordia under invagination and motor neurons of the ventral nerve cords, which is consistent with the expression patterns of the smp-1::egfp transgene. These results suggest that the plx-1 and the smp-1 genes are both expressed in all cells of the vulval primordium including VPCs.

The expression of the smp-2::egfp transgene was not detected in VPCs. It was detected in vulA and vulE in the late L4 stage (Fig. 8H), when invagination was nearly complete.

Cell specification and cell lineage in plx-1 animals

Observation with ajm-1::gfp suggested that some plx-1 primordia appeared to contain more than 22 cells (Fig. 9A), whereas the wild-type L4 vulval primordium is composed of 22 cells. This prompted us to examine cell fate specifications and cell lineages in plx-1 mutant vulvae. First, we examined the number of cells expressing GFP in the genetic background of ayIs4[egl-17::gfp]. At the L4 stage, egl-17::gfp was expressed in vulC and vulD cells (Inoue et al., 2002), and the number of cells expressing GFP was almost always six in wild-type animals (134/135) (Fig. 9B). In plx-1 mutants, some worms expressed GFP in more or less than 6 cells (4.6%, n = 151) (Fig. 9C), indicating that the P5/7.p lineage is abnormal.

The fate determination of a VPC is critically dependent on its position relative to the anchor cell (AC) and its association with other VPCs. Since the arrangements of VPCs are disrupted in plx-1 mutants, this may result in the adoption of aberrant fates in some VPCs. First, we analyzed the 1° fate specification. The strong expression of egl-17::cfp-lacZ in the genetic background of arls92, a sensitive marker for the activation of the Ras-MAPK cascade by AC-mediated induction (Yoo et al., 2004), was mainly restricted to P6.p in plx-1 mutants, although the frequency at which the neighboring cells strongly expressed the reporter was higher in the mutant (10%, n = 176) than in wild-type animals (2%, n = 90). Out of 10 plx-1 animals that had a gap between P6.p and the neighbors at the time of observation, the separate neighbor expressed the marker in 3 animals. This may indicate that some neighbor cells were induced strongly due to the aberrant position, and some received diminished lateral signaling due to the gap in the mutants. However, they did not seem to eventually adopt the 1° fate, since egl-17::gfp, which is a less sensitive marker for the activation of the Ras-MAPK cascade, was expressed exclusively in a single VPC in plx-1 mutants (n = 60) with the genetic background of ayIs4.

Next, we analyzed the 2° fate specification by examining the lip-1::gfp expression (Berset et al., 2001) in jclsl1[ajm-1::gfp] plx-1(nc37); zhs4[pTB10(lip-1::gfp)] animals.
Both in wild-type and \textit{plx-1} mutant animals, the majority of worms expressed the reporter in either P5.p and/or P7.p. In \textit{plx-1} mutants, the number of worms that expressed the reporter in cells other than P5/7.p was slightly higher than in wild-type animals: 21% and 14% for \textit{plx-1} mutants and wild-types, respectively (Table 2). Since lateral signals play critical roles in the 2\textsuperscript{o} fate specification, we were interested in how gaps between P6.p and P5/7.p would affect it. We examined the \textit{lip-1::gfp} expression in 15 \textit{plx-1} animals whose P6.p was separated from either of its neighbors by a gap before as well as after the division of VPCs. Before the first division, the expression of the marker in a VPC next to the gap was negative in 10 animals, and positive in 5 animals. The expression of \textit{lip-1::gfp} in P5/7.p appeared strongly dependent on the contact with P6.p, but not completely. When a VPC and its descendants next to a gap consistently failed to express the marker, their development were often arrested: of 8 animals, we have observed that P7.p failed to divide in one animal (Figs. 9D, E, F), and the descendants were arrested after the first division in one
animal (data not shown). For the other 6 animals, the exact fate of the descendants was not clear, as we did not count the number of nuclei. Out of 6, 5 animals had 4 apical areas marked with AJM-1::GFP at the position expected for the descendants of the VPC and its distal neighbor, suggesting that the VPC was arrested after either the first or second division. One animal had 6 apical areas marked with AJM-1::GFP, suggesting that it continued to divide after the second division.

In addition, in order to examine the correlation between the arrangement defects of the vulval primordium and the fate specification defects of VPCs, we followed the vulval development of animals whose VPCs appeared to adopt the 2° fate normally. Out of 60 plex-1 animals, in which P5/7.p exhibited the wild-type lip-1::gfp expression pattern and VPCs had no gaps at L2 (Fig. 9G), 8 animals (13%) had the primordium separated by a gap at L4 (Fig. 9H). In another 8 animals (13%), the symmetric arrangement of vulval cells was severely disrupted. The frequency of defects is comparable with plex-1 animals as a whole (Fig. 5), indicating that the arrangement defects in the vulval primordium in later stages, including gaps, can be generated.

Fig. 9. Cell lineages and cell specifications in plex-1 mutants. (A) In a plex-1 mutant at the L4 stage, labeling with ajm-1::gfp suggested the presence of more than 22 cells in the vulval primordium. (B, C) egl-17::gfp marks vulCs and vulDs in the L4 vulval primordium. In the wild type (B), egl-17::gfp is expressed in six of the vulval cells at the L4 stage: 4 vulCs (asterisks) and 2 vulDs (out of the focal plane). In a plex-1 mutant at a similar stage, egl-17::gfp was expressed in 10 vulval cells (asterisks). (D–G) lip-1::gfp marked the nuclei of the 2° lineage cells (arrowheads) in VPCs, which were also visualized with ajm-1::gfp. (D) In a plex-1 mutant, lip-1::gfp was expressed intensely in P5.p (an arrow), but not in P7.p (an arrowhead) that was separated from P6.p by a gap. In this animal, P7.p had two apical surface areas marked with AJM-1::GFP due to a fragmentation. (E, F) The same animal as in (D) after the first division of P5.p (arrows) and P6.p (E), and during invagination (F). The development of P7.p (an arrowhead) was arrested prior to the first division while the anterior half of the vulval primordium appeared to develop normally. (G) From a plex-1 mutant VPCs whose P5/7.p expressed lip-1::gfp normally, a severely disrupted vulval primordium with a gap (H) developed. Scale bars are 5 μm for B and C, and 20 μm for D and G.
independently of initial defects in cell fate specifications of VPCs.

**Discussion**

**SMP-1/PLX-1 system mediates contact-dependent stop signals in VPCs**

Plexins, functioning as receptors for semaphorins, are known to mediate repulsive signals to growth cones of growing axons in vertebrates. In vertebrates, a secreted-type semaphorin, Sema3A, is profuse in epidermal tissues, with apparently minor defects in the nervous systems (Fujii et al., 2002). In this study, we examined the development of morphological defects of the vulva in plx-1 and smp-1 mutants. We found that vulval formation in plx-1 mutants is affected from the earliest stage of vulval morphogenesis: the arrangements of vulval precursor cells (VPCs) are aberrant. Continuous observations suggest that this defective arrangement is in part caused by the failure of VPCs to prevent longitudinal extension after making contact with neighboring VPCs. We also found that worm mutants for SMP-1, a putative PLX-1 ligand, exhibited similar VPC arrangement defects. The arrangement defects of VPCs and the vulval primordium did not deteriorate further by combining smp-1 and plx-1 mutations, supporting the notion that these genes function in the same pathway. The expression analysis of plx-1 and smp-1 strongly suggests that both genes are expressed in VPCs, albeit that the exact localizations of PLX-1 and SMP-1 remain to be determined by more direct means, such as immunostaining, in future studies. Finally, the VPC-specific expression of plx-1 and smp-1 cDNA in the respective mutants was sufficient for rescuing the arrangement defects. Based on these observations, we propose that PLX-1 and SMP-1 mediate a stop signal for an extending VPC when it makes contact with neighboring VPCs.

In vertebrates, a secreted-type semaphorin, Sema3A, causes rearrangement of the cytoskeleton, which eventually causes the growth cone collapse. A member of the class 6 transmembrane-type semaphorin family of vertebrates, which is structurally most closely related to the class I semaphorin family, to which SMP-1 belongs, possesses repulsive activity on growth cones (Xu et al., 2000). Our results suggest that a cellular mechanism similar to that in the vertebrate growth cones may operate downstream of PLX-1 and cause cessation of the longitudinal extension of VPCs. The simplest model is that PLX-1 expressed on a VPC receives a repulsive signal from the membrane-bound SMP-1 on neighboring VPCs and on the cell contact, and terminates the longitudinal extension. Since each VPC expresses both PLX-1 and SMP-1, contacts between VPCs generate bi-directional stop signals. An alternative possibility is the presence of a reverse signaling mechanism for the semaphorin/plexin system, as in the Eph/ephrin system. Recent analyses showed that transmembrane-type semaphorins of Drosophila as well as mice can cause reverse signaling (Godenschwege et al., 2002; Toyofuku et al., 2004), although we have not identified any intracellular signaling motif in SMP-1. Further mosaic analysis would be helpful to determine whether PLX-1 regulates the behavior of VPCs in a cell-autonomous fashion.

It was previously shown that seam cells, a group of epidermal cells arranged in a row along the lateral body, exhibit arrangement defects in plx-1 (Fujii et al., 2002), smp-1, and smp-2 mutants (Ginzburg et al., 2002). In the wild-type, seam cells make contact with each other at their anterior/posterior side. Aberrant contact along the dorso-ventral sides of plx-1 seam cells (Fujii et al., 2002) is reminiscent of overlapping plx-1 VPCs, suggesting that PLX-1 plays a similar role in both systems. Interestingly, gaps were sometimes formed in a row of plx-1 seam cells. Continuous observation suggested that the failure to terminate longitudinal VPC extension in one direction and concomitant failure to extend in the other direction might lead to gap formation. It remains to be examined whether this also applies to gap formation in a row of seam cells. Although there were cases in which gaps were observed without changes in the number of apical areas of VPCs demarcated with AJM-1::GFP (Z.L. and S.T., unpublished observation), some gaps might be generated when VPCs are overlaid with hyp7. The possibility that the fusion of VPCs with hyp7 might lead to the formation of gaps cannot be
excluded, although we currently have no data to support this. The proposed model does not appear to account for the finding that plx-1 and smp-1 VPCs often adopt irregular morphologies, including fragmentation, suggesting that the SMP-1/PLX-1 system may be involved in other morphogenetic aspects of VPCs. One may speculate that this system helps to keep a proper morphology of VPCs by preventing formation of unnecessary contacts not only between neighboring cells, but also between folded areas on the surface of a single cell.

In ray precursor cells and seam cells, smp-2 mutants exhibit arrangement defects similar to those of smp-1 mutants. In contrast, our phenotypic as well as expression analyses indicated that SMP-2 is not involved in regulating the arrangement of VPCs. We also found that the lin-31::smp-2 cDNA transgene did not rescue the arrangement defect of smp-1 VPCs. This raises an interesting possibility that the mode of function of SMP-2 may not be identical to that of SMP-1, albeit that the lin-31::smp-2 cDNA transgene is expressing properly remains to be examined in future study.

**VPC fate specification in plx-1 mutants**

Although the arrangements of VPCs were usually disrupted in plx-1 mutants, the cell-fate specification of VPCs appeared to be affected only in a relatively small fraction of the mutants.

Previous studies showed that specification, particularly of the 2° fate, depends on complicated cellular interactions: the lateral signal through LIN-12 is critical, and the LIN-3 signal is not usually necessary for the 2° fate specification, since VPC can become a 2° fate cell even if it does not express a LIN-3 receptor (Koga and Ohshima, 1995; Simske and Kim, 1995). Recent studies showed that the LIN-12 signal inhibits the Ras-MAPK cascade downstream to LIN-3 (Yoo et al., 2004). On the other hand, however, LIN-3 overexpression can induce adjacent VPCs to assume the 1° fate, indicating that high levels of LIN-3 can override the lateral inhibition signal (Katz et al., 1995). It was also shown that low levels of LIN-3 can induce an isolated VPC to adopt the 2° fate, suggesting that the lateral signal can be dispensed with under certain circumstances (Katz et al., 1995; Sternberg and Horvitz, 1986). Our analysis on the effect of a gap between P6.p and its neighbors suggested that direct contact between P6.p and its neighbors is important for the 2° fate specification, but is not always necessary. In the absence of direct cell–cell contact, the 2° fate might be induced either by low levels of the LIN-3 signal generated by AC, or by a lateral signal mediated through secreted ligands for LIN-12, such as DSL-1 (Chen and Greenwald, 2004). It should be noted that, although the contours of VPCs on the apical surface marked with AJM-1::GFP would appear separate, we cannot exclude the possibility that VPCs made contact with each other beneath the surface.

**Roles of SMP-1/PLX-1 in later stages of vulval morphogenesis**

We have found that the development of the vulval primordium is affected in plx-1 and smp-1 mutants. Continuous observation revealed a variety of defects, including gap formations and asynchronous development in the primordium. On the other hand, our rescue experiments using the lin-31 promoter showed that the SMP-1/PLX-1 system does not play a major role in the later development of the vulva. Defects in the plx-1 and smp-1 vulval primordia are therefore likely to be secondary consequences of the arrangement defects of VPCs. Conversely, we found that the arrangement defects in the vulval primordium can be generated although VPCs appeared to be specified normally. It is the proper arrangement of VPCs that seems to be critical for proper morphogenesis of the vulval primordium.

It has been reported that cell–cell interactions play important roles during later development of the vulval primordium (Hanna-Rose and Han, 2002; Wang and Sternberg, 1999, 2000). The aberrant arrangements of VPCs in plx-1 mutants led to the aberrant arrangements of its descendants. It is likely that these arrangement defects directly affect cell–cell interactions among vulval cells required for their proper morphogenesis.

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**References**


