

# Vulva morphogenesis involves attraction of plexin 1-expressing primordial vulva cells to semaphorin 1a sequentially expressed at the vulva midline

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Accepted 23 December 2004

Development 132, 1387–1400

Published by The Company of Biologists 2005

doi:10.1242/dev.01694

## Summary

Vulva development in *C. elegans* involves cell fate specification followed by a morphogenesis phase in which homologous mirror image pairs within a linear array of primordial vulva cells form a crescent shape as they move sequentially towards a midline position within the array. The homologous pairs from opposite half vulvae in fixed sequence fuse with one another at their leading tips to form ring-shaped (toroidal) cells stacked in precise alignment one atop the other. Here, we show that the semaphorin 1a SMP-1, and its plexin receptor PLX-1, are required for the movement of homologous pairs of vulva cells towards this midline position. SMP-1 is upregulated on the lumen membrane of each primordial vulva cell as it enters the forming vulva and apparently attracts the next flanking

homologous PLX-1-expressing vulva cells towards the lumen surface of the ring. Consequently, a new ring-shaped cell forms immediately ventral to the previously formed ring. This *smp-1*- and *plx-1*-dependent process repeats until seven rings are stacked along the dorsoventral axis, creating a common vulva lumen. Ectopic expression of SMP-1 suggests it has an instructive role in vulva cell migration. At least two parallel acting pathways are required for vulva formation: one requires SMP-1, PLX-1 and CED-10; and another requires the MIG-2 Rac GTPase and its putative activator UNC-73.

Key words: *C. elegans*, SMP-1, PLX-1, Morphogenesis, Guided migration

## Introduction

Morphogenesis is a process by which cells that acquire a specific fate, change their shape, migrate to form new cellular interactions and adopt a new spatial plan. In so doing, these cells collectively form tissues and organs.

Vulva morphogenesis in *C. elegans* is a process that depends on tight control over cell lineage and fate (Sternberg and Horvitz, 1986; Sulston and Horvitz, 1981), as well as stereotypical patterns of cell shape changes and movements (Sharma-Kishore et al., 1999; Sulston and Horvitz, 1977). Vulva formation in *C. elegans* encompasses many aspects of morphogenesis observed in animal development, and therefore is likely to embody molecular aspects of organ formation conserved throughout evolution.

Although genetic approaches have revealed major molecular mechanisms that underlie vulva cell fate determination (Greenwald and Rubin, 1992; Horvitz and Sternberg, 1991), little is known about the molecular mechanisms involved in vulva cell shape changes and movements that form the vulva proper. Fortunately, the sequence of cellular events taking place during vulva morphogenesis have been described in detail (Sharma-Kishore et al., 1999), and this description provides a blueprint for using genetics to understand vulva morphogenesis at a molecular level.

The *C. elegans* vulva comprises seven ring-shaped cells stacked precisely one on top of the other. Vulva development

begins during larval stage 3 (L3) when three vulva precursor cells (VPCs), P5.p, P6.p and P7.p, are induced by a somatic gonad cell, the anchor cell (AC) (normally located immediately dorsal to P6.p), to divide by mirror image sublineages (Greenwald and Rubin, 1992; Kimble, 1981; Sternberg and Horvitz, 1989; Sulston and White, 1980). These sublineages ultimately form a longitudinally oriented row of 22 ventral midline epithelial cells comprising the primordial vulva. These are arranged in a palindrome of homologous cell types (vulA, vulB1, vulB2, vulC, vulD, vulE, vulF, vulF', vulE', vulD', vulC', vulB2', vulB1' and vulA') (Greenwald and Rubin, 1992; Sternberg and Horvitz, 1989). The position between F and F' in this sequence represents the position of the vulva midline, the future position of the vulva lumen surrounded by ring-shaped vulva cells.

Based on laser-ablation and genetic studies, it has been shown that vulva morphogenesis can occur independently for each anteroposterior mirror-image vulva half (Sharma-Kishore et al., 1999), suggesting that the guidance mechanisms used to position the ring-forming homologs function autonomously from within each half palindrome. Each half ring may comprise one or two cells, depending on the ring (Fig. 1A). Each homologous opposite half ring undergoes similar mirror image shape changes and movements. In the first step of vulva morphogenesis during early L3, the four midline flanking cells (daughters of P6.pap and P6.ppa) first arrange as a four-cell

rectangle with the anchor cell nestled into a pocket in the middle (Fig. 1A). The anchor cell later breaks through the center of the rectangle and opens a pore that comprises the most dorsal part of the vulva lumen as the four cells fuse to form the vulF ring (Sharma-Kishore et al., 1999).

The first obvious shape change of the other primordial vulva half-rings is to extend two processes, one from each lateral surface (i.e. facing the left and right sides of the epidermis of the animal) towards the vulva midline. Each presumptive half ring thereby forms a crescent shape with edges of the crescent pointing towards the position of the forming vulva.

Eventually, the two extending processes of each crescent shaped half ring (Fig. 1A) meet and adhere to similar processes from mirror-image homologs projecting towards the presumptive vulva midline from the opposite half palindrome. As the lateral extensions insert between the hypodermis and the previously formed ring, the cells entering the vulva tend to migrate beneath their inner neighbors and push them upwards (in a dorsal direction, see Fig. 1A). The adherence of opposite extensions from mirror image half-ring homologs forms a full ring of cells, which encircle the vulva lumen. Ultimately, these adhering homologs fuse (with the exception of vulB1 and vulB2) to form a mature vulva.

As the lateral processes extend towards the vulva midline, the primordial vulva cell bodies also begin to move towards the vulva midline. Because the shape changes and extensions of lateral processes from vulva halves resemble cell extensions that cause cell movements and because the presumptive vulva cell bodies become displaced toward the vulva midline, we collectively refer to these shape changes and movements as vulva cell migrations.

The first cells to form extensions and to move are the precursors of vulE (daughters of P6.paa and P6.ppp). Although the lateral vulva cell extensions lead the way, the concave surface of these crescent-shaped cells also seems to actively migrate towards the midline of the vulva. By moving along the ventral membrane of the vulF ring, the four vulE cells (two cells per half vulva) eventually form a ring of four cells connected to each other by adherens junctions and align precisely along the DV axis attached to the ventral surface of the vulF ring. In a similar fashion, more concentric rings of vulva cells are formed by sequential recruitment of the next outer group of mirror image homologous half rings to the vulva midline until seven precisely stacked rings of cells have formed the vulva (Sharma-Kishore et al., 1999).

Normally during this process, all primordial vulva cells appear contiguously connected to one another through adherens junctions that are constantly remodeled as primordial vulva cells change shape and move. At no stage does a primordial cell normally become obviously dissociated from its neighbor(s).

During *C. elegans* development, different cell shape changes and movements require a combination of Rac GTPases MIG-2 and CED-10, and their GEF activator (UNC-73) (Lundquist et al., 2001; Spencer et al., 2001; Steven et al., 1998; Wu et al., 2002), suggesting that Rac signaling could be required downstream of different tissue-specific guidance receptors for controlling cellular movements. Vulva morphogenesis has been shown to require the activity of MIG-2, CED-10 and UNC-73. These GTPases are required primarily for shape changes and movements of these cells during vulva morphogenesis

(Kishore and Sundaram, 2002), although they also redundantly regulate, to a minor extent, the axis of cell divisions that form the primordial vulva cells. These results suggest that Rac signaling promotes rearrangement of the cytoskeleton required for primordial vulva cell migration.

Here, we provide evidence for a primordial vulva cell migration system in which PLX-1-expressing vulva cells that are poised to enter the forming stack of vulva rings are attracted towards SMP-1 expressed on the surface of their inner neighbors that have already entered the stack. SMP-1 expression occurs in a sequence that progresses from cells of ring 1 to ring 7. Using this model, the sequential expression of SMP-1 in each vulva cell as it forms a vulva ring explains the sequential attraction of outer neighbors towards inner neighbors and the orderly formation and alignment of concentric rings of cells that comprise the mature vulva.

Although the absence of SMP-1 and PLX-1 signaling causes vulva cell migration defects, the defects are not fully penetrant. This indicates that other mechanisms act in parallel with SMP-1 and PLX-1 to guide primordial cell migrations. The genetic data presented here suggest that CED-10 acts in the same pathway as SMP-1 and PLX-1, and that MIG-2 and UNC-73 act in a parallel pathway for vulva morphogenesis.

## Materials and methods

### Nematode culture

General procedures used for the culture, maintenance and storage of *C. elegans* can be found elsewhere (Wood, 1988). Mutant strains used in this study were: Linkage Group X (LGX), *mig-2(mu28)* (Zipkin et al., 1997); LGI, *smp-1(ev715)* (Ginzburg et al., 2002), *unc-73(ev509)* (Steven et al., 1998), *unc-73(e936)* (Desai et al., 1988), *unc-73(rh40)* (Steven et al., 1998) and *smp-2(ev709)* (Ginzburg et al., 2002); LGIV, *ced-10(n1993)* (Ellis et al., 1991), *plx-1(ev724)* (Dalpe et al., 2004), *plx-1(nc37)* (Fujii et al., 2002) and *let-60(n1046)* (Ferguson and Horvitz, 1985); and LGV, *him-5(e1490)* (Hodgkin et al., 1979). Strains not isolated in our laboratory were obtained from the *C. elegans* Genetics Center, courtesy of T. Stiernagle (The University of Minnesota).

### Microscopy and vulva morphogenesis observation

Vulva morphogenesis defects were scored by mounting 50 mM sodium azide-treated animals on 2% agarose pads for observation using DIC and fluorescence optics. Young adult hermaphrodites carrying the *ajm-1::GFP* reporter for adherens junctions (Simske and Hardin, 2001) were scored for vulva precursor cell body migration defects and for vulva ring formation defects (see Results and Tables). The *ajm-1::GFP* translational reporter was visualized with a Leica DMRXA microscope to assess epithelial cell morphologies. Confocal microscopy was performed using a Leica DMFLS laser confocal microscope equipped with a 63× PC APO CS lens (1.40-0.60). Serial optical sections in the z-axis were collected every 0.15 μm. Three-dimensional image reconstructions were obtained by processing confocal z-axis series using Volocity (Improvision, version 2.6.1) or the Leica Confocal software (version 11.04). Cell fate analysis was carried out with an *egl-17p::gfp (ayls4)* reporter (Burdine et al., 1998).

Standard errors for percentages of vulva defects were calculated assuming a binomial distribution with the observed percentage value and the actual sample size. Statistical tests were carried out using a standard (two-tailed) comparison of two proportions (Moore and McCabe, 1998). All *P* values represent the probability that the measured penetrance of the phenotype is significantly different between two strains. A *P* value of less than 0.05 is considered to be significant.

## Molecular biology

Standard molecular biology methods (Sambrook et al., 1989) were used unless otherwise noted.

## Transgenic constructs

The transcriptional and translational reporters of *plx-1* (*plx-1::gfp* and *plx-1p::PLX-1::GFP*, respectively), the *plx-1(+)* minigene, the *plx-1p::UNC-73(+)* minigene, the *smp-1p::gfp* and the *smp-1::GFP* (here referred to as *smp-1p::SMP-1::GFP* reporters) have been described previously (Dalpe et al., 2004; Ginzburg et al., 2002).

For ectopic expression of SMP-1, we amplified a cDNA encoding the extracellular and transmembrane regions of SMP-1 and subcloned it into the *SaII/PstI* cut pPD95\_77cplx plasmid (Dalpe et al., 2004). The resulting plasmid, pECTSMP\_plx-1p::SMP-1(+), encodes a functional SMP-1 protein (as demonstrated in the Results), encompassing amino acids 1-616 (deleted for a portion of the cytoplasmic domain), under the control of the *plx-1* 5' regulatory region.

To make a construct encoding PLX-1 with its cytoplasmic region deleted (*plx-1p::PLX-1delC::GFP*), we used modified PCR primers to amplify a 670 bp fragment of the *plx-1(+)* rescuing minigene (Dalpe et al., 2004), digested it with *SphI* and *KpnI* and, ligated the fragment into the original *plx-1* minigene plasmid cut with the same enzymes. The resulting plasmid encodes the extracellular and the TM domains of PLX-1, encompassing amino acids 1-1317 inclusively and an in-frame GFP.

## Germline transformation

Transgenic strains were as follows:

- evIs140* [*pPD95\_77cplx plx-1::gfp*; *rol-6(su1006)*] (*plx-1* transcriptional reporter);
- evEx162* [*pZH127 plx-1p::PLX-1(+)*; *rol-6(su1006)*]; (cDNA rescues *plx-1* mutant);
- evIs162* [*pZH127 plx-1p::PLX-1(+)*; *rol-6(su1006)*] (cDNA rescues *plx-1* mutant);
- evEx168* [*pZH163 plx-1p::UNC-73(+)*; *rol-6(su1006)*] (*unc-73* expressed by *plx-1* 5' regulatory region);
- evEx169* [*pZH157 plx-1p::PLX-1(+):GFP*; *rol-6(su1006)*] (functional *plx-1* translational reporter);
- evEx170* [*pVGS1a smp-1p::SMP-1delC(+):GFP*; *rol-6(su1006)*] (functional *smp-1* translational reporter);
- evEx183* [*pECTSMP\_plx-1p::SMP-1*; *rol-6(su1006)*] (ectopically expressed *smp-1* cDNA); and
- evEx184* [*plx-1p::PLX-1(+):delC::GFP*; *rol-6(su1006)*] (*plx-1* minigene deleted for cytoplasmic domain).

Transgenic strains were generated by co-microinjection of the DNA mix into the distal gonad arms of N2 or *him-5(e1490)* hermaphrodites (Mello and Fire, 1995). DNA mixes consisted of a test construct at a concentration of 50 µg/µl or 30 µg/ml, and a co-injection marker to create a final DNA concentration of 100 µg/µl. Transgenic extra-chromosomal arrays were integrated using a UV irradiation based method (Mitani, 1995). Integrated alleles were backcrossed five times to N2 Bristol (wild type) before phenotypic analysis.

## Results

### Abnormal vulva ring formation in *plexin-1* (*plx-1*) and *semaphorin-1a* (*smp-1*) mutants

We used the *AJM-1::GFP* reporter (Simske and Hardin, 2001), an adherens junction marker expressed on the apical side of epithelial cells (Francis and Waterston, 1985), to highlight epithelial cell outlines. This marker allowed us to characterize both the position and morphology of vulva cells and of their extensions in the wild type (Sharma-Kishore et al., 1999) and in mutants (see below).

In wild-type animals carrying the *AJM-1::GFP* reporter, the intermediate steps of ring formation during vulva morphogenesis are readily observed (in temporal order: early, intermediate and completed morphogenesis in Fig. 1G,H,I, respectively). During the third larval stage of wild-type animals, lateral processes from crescent shaped half-vulvae extend underneath the lateral edges of the next innermost primordial vulva cells (Fig. 1A,B). In *smp-1(ev715)*, *plx-1(ev724)* and *plx-1(nc37)* mutants of the same stage, cells are occasionally found detached and mispositioned within the array of primordial cells (Fig. 1C). In wild-type adult hermaphrodites, the vulva appears as seven concentric rings (Fig. 1D). In adult *smp-1* and *plx-1* mutants, the primordial vulva cells frequently fail to assume a crescent shape. Instead, *smp-1* and *plx-1* mutant vulva cells either stay round or abnormally change their shape without generating lateral processes that extend towards the vulva midline (Fig. 1E,F,J). We observe cells that are detached and others that are still positioned as a contiguous row of abnormally shaped cells, flanking a vulva with abnormally shaped rings in adult *smp-1* and *plx-1* mutants (Fig. 1E,F,J). These defects are variably observed on only one (Fig. 1E,F) or on both sides (Fig. 1J) of the mutant vulvae.

In *smp-1* and *plx-1* mutants, not all guidance functions are absent, as exemplified by migration defects that affect only one half of the vulva (Fig. 1E,F; below). In addition, when mutant vulva cells (i.e. *smp-1* and *plx-1* mutants) from one half of the vulva fail to generate two normally migrating lateral extensions, these cells rarely, if ever, meet the extensions from their mirror-image homologs in the opposite half of the vulva. However, vulva cells that fail to contact their appropriate homologs at the vulva midline do not fuse with non-homologous cells of another cell fate (see different examples in Fig. 1E,F,J) (see Shemer et al., 2000). These results suggest that the mechanisms of target recognition for homotypic cell fusion are still intact in *plx-1* and *smp-1* mutants, but fusion is prevented because process extensions are not appropriately guided for homologs to make contact.

The vulva cell migration and spreading defects of *smp-1* and *plx-1* mutants typically involve the most external cells (i.e. vulA, B1, B2, C, D) (Fig. 1C,F), but at a lower frequency many vulva cells from an entire half vulva are involved (Fig. 1J). In the latter case, the vulva cells that are detached from the forming vulva sometimes form a separate invagination, because some maintain their own ability to form torroids (Fig. 1J, asterisks).

In principle, the vulva cell lineages could be modified in *plx-1* mutants, increasing or reducing the number of vulva cells and in this way perturb their normal migration pattern. To address this possibility, we carefully examined several larval stage 4 (L4) mutant animals without finding any alteration in the number of vulva cells (0%, *n*=52). However, we found occasional changes in the axis of cell division from longitudinal to transverse (4%, *n*=52).

To determine more precisely if primordial vulva cells are made in excess or if cell fates are being altered in the mutants in ways that may not affect axis of division, we examined the vulva cell reporter *egl-17::gfp* (Burdine et al., 1998) for dividing P6.p cells in early L3s. Dividing P6.p cells were readily observed in all of these strains and there was never an excess of P6.p-derived vulva cells (vulE and F). Only six out



**Fig. 1.** Vulva ring formation defects in *plexin 1* (*plx-1*) and *semaphorin 1a* (*smp-1*) mutants characterized by DIC and the *AJM-1::GFP* reporter (see Materials and methods).

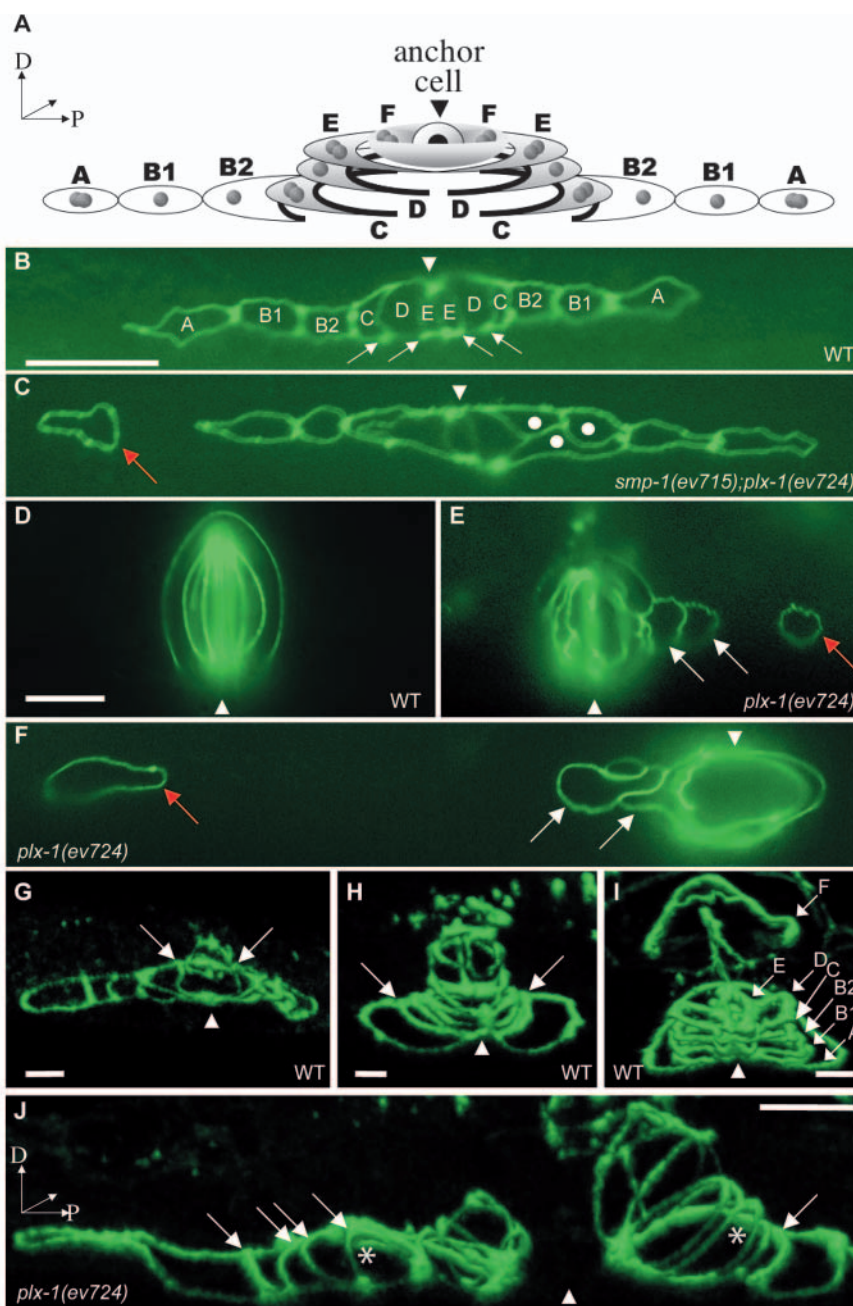
(A) Dorsolateral perspective; (B-F) ventral views; (G-J) dorsolateral projections of 3D confocal images. Arrowheads indicate the vulva midline (innermost position of the vulva primordium and site of the presumptive vulva lumen).

(A) Schematic showing an intermediate step in vulva morphogenesis (cell fate identities are indicated by letters). (B-F) Focal plane of F and other cells that have formed rings are out of focus. (B) At the beginning of larval stage 3, primordial vulva cells are linearly arranged in wild-type animals. Starting from the midline of the primordium, wild-type vulva cells sequentially modify their shape and generate processes (white arrows) that migrate around and under their inner neighbors. (C) In *smp-1(ev715)* and *plx-1(ev724)* single mutants and *smp-1(ev715); plx-1(ev724)* double mutants at the same stage, vulva cells are misguided (detached cells indicated by red arrow and mis-positioned cells indicated by white dots). (D) In wild-type adult hermaphrodites, the vulva appears as seven stacked concentric rings.

However, in *plx-1(ev724)* mutants (E,F), the ring structure is disorganized: some cells are detached from the primordium (red arrows) and vulva cell processes do not migrate correctly around their inner neighbors (white arrows).

(G-J) Fluorescence confocal microscopy and software-assisted 3D image reconstruction was also used to characterize vulva cell extensions and ring morphogenesis (see Materials and methods). Migrating cells send out processes ventrally around their inner neighbors within the vulva primordium in a sequential manner. Intermediate steps show the lumen-facing membrane of the migrating processes of the first (centrally positioned) cells during early stages (arrows in G) and, at a later stage, the processes of distally positioned cells (arrows in H). Seven stacked vulva rings (white arrows in I) signal the end of morphogenesis. In *plx-1(ev724)* mutants vulva (J), cells may fail to migrate towards the midline (shown by a gap) and frequently fail to extend processes around their inner neighbors (arrows showing the lumen-facing membrane). Mutant cells can adopt an abnormal crescent shape, which causes secondary invaginations (asterisks).

(A,G-J) The perspective orientation is indicated with the triple arrows (D, dorsal; P, posterior; unlabelled arrow indicates left-right depth). Scale bars: 25  $\mu$ m in B,C; 25  $\mu$ m in D-F; 3.8  $\mu$ m for G; 2.3  $\mu$ m for H; 3  $\mu$ m for I; 5.3  $\mu$ m for J.



of 90 *unc-73(rh40)* and four of 95 *plx-1(ev724)* animals were missing one or two *egl-17::gfp*-expressing cells. In late L3 early L4 larvae, *egl-17::gfp* expression decreases in P6.p-derived cells and increases dramatically in P5.p- and P7.p-derived cells (vulC and vulD). Only seven out of 105 *unc-73(rh40)* and six of 162 *plx-1(ev724)* animals were missing one or two *egl-17::gfp* late L3-expressing P5.p- or P7.p-derived cells. In the few animals that lacked *egl-17::gfp* expression in two cells, the two non-expressing cells were always from the same half of the vulva. Extra expressing cells were never observed in the mutants or wild type. Cell-fate defects (monitored by *egl-17::gfp*) are therefore minor compared with

primordial vulva cell migration defects, suggesting that the SMP-1, PLX-1 and UNC-73/Rac pathway are functioning primarily in guidance of these cells, rather than in determining their fates. Moreover, it is conceivable that an early migration defect in these strains [e.g. *plx-1(ev724)* and *unc-73(rh40)*] might disturb vulva cell fate specification [e.g. perturbing the LIN-3 availability for one vulva half or affecting LET-23 localization (Kim, 1997)].

Based on their highly related mutant vulva phenotypes, our data suggest that *smp-1* and *plx-1* are required for normally oriented vulva cell extension and stereotypical movements that take place during vulva morphogenesis. In *smp-1* and *plx-1*

presumptive null mutants, the fact that not all guidance functions are absent indicates that unidentified guidance mechanisms can sometimes compensate for the absence of SMP-1 and PLX-1 signaling within each half vulva.

### ***plx-1* and *smp-1* function in the same pathway for vulva morphogenesis**

Using the criteria defined above, we evaluated the penetrance of vulva ring formation defects in *plx-1* and *smp-1* mutants. The *plx-1(ev724)* mutant is predicted to encode a truncated receptor that is missing its transmembrane and cytoplasmic domains (Dalpe et al., 2004), and is therefore predicted to lack signaling activity. In young *plx-1(ev724)* mutant adults, we observe a penetrance of ~52% vulva defects (Table 1, row 2). For a previously described male tail phenotype, the *plx-1(ev724)* allele is genetically equivalent to another putative null deletion allele, *plx-1(nc37)*, in which the initiation codon and the first four exons have been deleted (Dalpe et al., 2004; Fujii et al., 2002). *plx-1(nc37)* has vulva morphogenesis defects essentially equivalent in penetrance and expressivity to those observed in *plx-1(ev724)*.

Interestingly, ~57% of *smp-1* [*smp-1(ev715)*] null mutant animals (Ginzburg et al., 2002) display vulva cell migration defects (Table 1, row 8). As both *plx-1* and *smp-1* mutants display an indistinguishable vulva phenotype, we predict they act in the same pathway. This was established by finding there is no significant enhancement of the vulva cell migration defects in *plx-1(ev724); smp-1(ev715)* double mutants (Table 1, row 11, 52%) when compared with each single *plx-1(ev724)* or *smp-1(ev715)* mutant (Table 1, row 8 and row 2; 57% and 52%, respectively). Furthermore, the vulva phenotypes of each single mutant and the double are qualitatively indistinguishable. This demonstrates that *plx-1* and *smp-1* act in the same pathway for vulva formation, rather than in parallel pathways. As SMP-1 has been shown to bind PLX-1 (Fujii et al., 2002), it is likely that SMP-1 acts as a ligand for PLX-1 that is used to regulate vulva cell shape changes and migrations that lead to normal vulva formation.

As some guidance functions are preserved in the absence of *smp-1* or *plx-1* function, or both, a vulva morphogenesis defect can occur on the anterior half of the vulva without affecting the posterior half, and vice versa. If both vulva halves act independently in response to *smp-1* and *plx-1* functions, the frequency of animals with defects in both vulva halves should roughly equal the product of the frequencies of animals with defects in one or the other vulva half. Indeed, we find that 19% of *plx-1(ev724)* mutant animals ( $n=124$ ) have a defect in the anterior half of the vulva, 21% have a defect in the posterior half and 7% have a defect in both halves. The observed 7% of animals with defects in both vulva halves is not significantly different from the expected frequency of 4% ( $P>0.05$ ), suggesting that *smp-1*- and *plx-1*-mediated morphogenesis functions operate autonomously within each half of the vulva.

The second *smp-1* gene in *C. elegans* [*semaphorin-1b* or *smp-2* (Ginzburg et al., 2002)] may play a very limited role in vulva morphogenesis as only ~3% of *smp-2(ev709)* mutant animals have vulva cell migration defects (Table 1, row 10), and *smp-1(ev715); smp-2(ev709)* double mutants do not display any enhancement of the *smp-1(ev715)* mutant penetrance (data not shown). This also corroborates the lack of *smp-2::gfp* transcriptional reporter expression in these cells

**Table 1. Vulva rings defects in plexin 1 and semaphorin 1a mutants**

Row	Genotype*	Ring defects (%) <sup>†</sup>	<i>n</i> <sup>‡</sup>
1	Wild type	0	111
2	<i>plx-1(ev724)</i>	52	217
3	<i>plx-1(ev724); evEx162[plx-1(+)]</i>	2	128
4	<i>plx-1(ev724); evIs162[plx-1(+)]</i>	10	148
5	<i>plx-1(ev724); evEx169[plx-1(+):GFP]</i>	15	130
6	<i>evEx184[plx-1p::PLX-1delC::GFP]</i>	0	<100
7	<i>plx-1(ev724); evEx184[plx-1p::PLX-1delC::GFP]</i>	78	119
8	<i>smp-1(ev715)</i>	57	228
9	<i>smp-1(ev715); evEx170[smp-1p::SMP-1delC(+):GFP]</i>	10	113
10	<i>smp-2(ev709)</i>	3	123
11	<i>smp-1(ev715); plx-1(ev724)</i>	52	211
12	<i>evEx183[plx-1p::SMP-1]</i>	11	35
13	<i>smp-1(ev715); evEx183[plx-1p::SMP-1]</i>	88	109

\*All strains have the *ajm-1p::AJM-1::GFP* reporter gene in the *him-5(e1490)* background. Animals were grown at 20°C.

<sup>†</sup>The frequency of vulva ring defects was determined as described in the Materials and methods. *n* represents the number of animals scored. Standard deviations (s.d.) were calculated assuming a binomial distribution with the observed percentage value and the actual sample size. For all comparisons described in the Results,  $P<0.05$  was considered to be a significant difference.

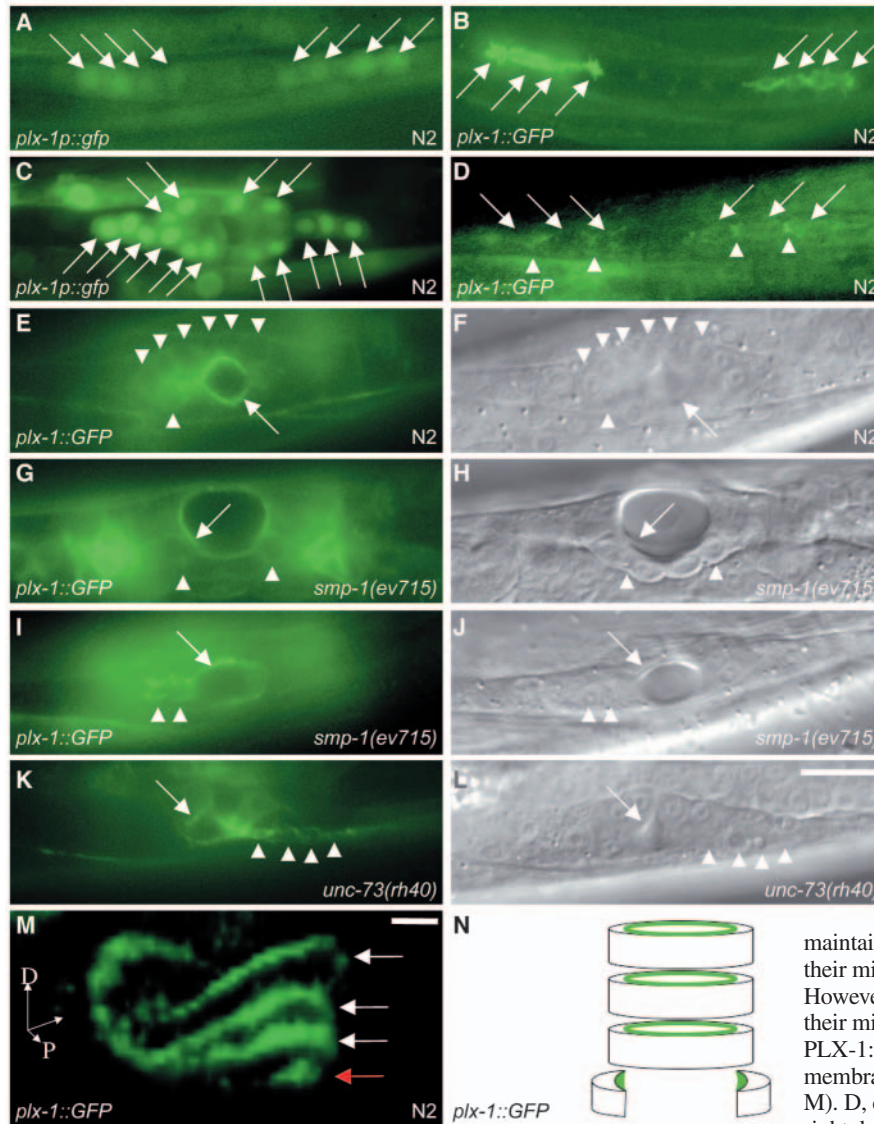
(Dalpe et al., 2004; Ginzburg et al., 2002). Thus, *plx-1* and *smp-1* largely function in the same pathway for the proper formation and guidance of vulva cell migrations, while SMP-2, another putative ligand for PLX-1 in other semaphorin-regulated mechanisms (Dalpe et al., 2004; Ginzburg et al., 2002), appears to play little, if any, role in vulva morphogenesis.

### **PLX-1 is expressed on the vulva midline-facing membrane in migrating vulva cells and on the lumen membrane within the forming vulva**

To determine in which cell types PLX-1 exerts its function, we used previously described transcriptional and rescuing translational reporters for *plx-1* (Dalpe et al., 2004) [e.g. *evIs140[plx-1p::gfp]* (transcriptional) and *evEx169[plx-1p::PLX-1(+):GFP]* (rescuing)]. Before the beginning of vulva morphogenesis, both reporters are expressed in all the descendants of P5.p and P7.p, and are expressed weakly in the descendants of P6.p (Fig. 2A,B). The GFP signal of the transcriptional *plx-1::gfp* reporter fills the cytoplasm and nuclei of expressing cells at this stage (Fig. 2A). However, the GFP signal of the *plx-1p::PLX-1::GFP* translational reporter is found predominantly at the cell membrane of the same cells, as expected if PLX-1 is a transmembrane receptor (Fig. 2B). The cell expression pattern (described below), is the same as the one observed for a previously described N-terminal translational reporter (*plx-1::egfp*) (Fujii et al., 2002) and is consistent with the expression of our *evIs140[plx-1p::gfp]* transcriptional reporter (data not shown).

The transgenic constructs expressing the full-length *plx-1(+)* cDNA minigene (Dalpe et al., 2004) (*evEx162* or *evIs162*) largely rescue the *plx-1(ev724)* vulva cell migration defects (2% and 10% versus 52% defects; Table 1, rows 3 and 4 versus row 2). Furthermore, the same cDNA fused in its C-terminal region to a GFP cassette (*plx-1p::PLX-1::GFP*) also rescues the *plx-1(ev724)* vulva cell migration defects (15% versus 52%





**Fig. 2.** The *plx-1::gfp* transcriptional reporter and the *plx-1p::PLX-1::GFP* translational reporter are expressed during vulva morphogenesis. In wild type (A-F), expression is predominant in P5.p and P7.p descendants and the reporters are widely expressed in most cells undergoing ring formation. The signal increases during later phases of ring formation. (A,C) Transcriptional reporter. (B,D) Translational reporter. The GFP signal of the *plx-1p::PLX-1::GFP* translational reporter is first (B) distributed on the whole cell membrane of early expressing cells and highlights filopodia-like extensions but later (D) begins to concentrate at the cell surface zone contacting neighboring cells (arrowheads). As vulva morphogenesis progresses (E,F), the PLX-1::GFP signal is widely expressed at the cell membrane of ring forming cells (arrowheads). The PLX-1::GFP signal is localized predominantly on the lumen side of vulva ring forming cells of wild-type animals (arrows in E,F). In *smp-1(ev715)* mutants (G,H), vulval cells that do not display a migration phenotype show the same pattern of expression as wild type. By contrast, cells displaying an aberrant migration phenotype in *smp-1(ev715)* and *unc-73(rh40)* mutants (arrowheads in I-L) often lose the predominant localization of the PLX-1::GFP signal at the lumen-facing membrane. F,H,J,L are DIC microscopic images of E,G,I,K, respectively. (M) Fluorescence confocal 3D microscopy revealed the PLX-1::GFP signal highlighting the lumen-facing surface of crescent-shaped cells forming vulva rings (M is a 3D confocal projection, N is a sketch of the staining in M with PLX-1 highlighted in green). The signal is maintained for a while in the vulva cells that have completed their migration and formed a ring (white arrows in M). However, as externally positioned cells are recruited to initiate their migration towards the presumptive vulva midline, the PLX-1::GFP signal becomes concentrated in cell surface membrane that is just entering the vulva proper (red arrow in M). D, dorsal; P, posterior; unlabelled arrow indicates left-right depth. Scale bars: 25  $\mu$ m in A-L; 1.8  $\mu$ m in M.

defects; Table 1, row 5 versus row 2). As the *plx-1p::PLX-1::GFP* reporter rescues the vulva cell migration defects of *plx-1(ev724)* mutants, we believe that the reporter represents a functional *plx-1* gene, strongly suggesting that its expression and localization patterns represent those of the endogenous protein.

At the beginning of vulva morphogenesis, a strong expression from the *plx-1::gfp* transcriptional reporter is found in all migrating vulva cells (Fig. 2C). As vulva morphogenesis progresses, expression from the *plx-1p::PLX-1::GFP* translational reporter increases at the plasma membrane of migrating vulva cell (Fig. 2B,D-F). However, although some signal is found on the entire cell membrane, PLX-1::GFP appears to be predominantly localized on the vulva center facing membrane (future lumen surface) of primordial vulva cells destined to enter the vulva proper (Fig. 2D,E).

Through analysis of reconstructed 3D confocal images, we observe that, at the end of morphogenesis, PLX-1::GFP is predominantly expressed in the most ventral vulva rings [vulA, vulB1, vulB2, vulC and vulD (which are P5.p and P7.p

derived)] and the signal is localized along the lumen formed by these cells (Fig. 2 M,N).

The subcellular localization of the PLX-1::GFP signal is surprisingly not concentrated at the tip of processes but is rather localized on a more central segment of the concave cell surface contacting its inner neighbor, this segment being shorter in cells poised to enter the vulva proper and longer in cells that have already entered the vulva stack (Fig. 2M,N). This suggests that the zone of PLX-1 localization increases as the zone of contact between a migrating cell and its inner neighbor increases.

### PLX-1 subcellular localization partially depends on SMP-1 and UNC-73

To evaluate whether PLX-1 subcellular localization is dependent upon its predicted ligand SMP-1, we introduced the PLX-1::GFP translational reporter into *smp-1(ev715)* mutants. In *smp-1(ev715)*, as in the wild type, the PLX-1::GFP signal is observed predominantly on the vulva midline-facing side of wild-type crescent shaped migrating primordial vulva cells (Fig.

2G,H). However, on vulva cells from *smp-1* mutants that display a non-crescent-shaped migration phenotype, *plx-1p::PLX-1::GFP* is more uniformly distributed on the whole cell membrane, rather than just the midline-facing side (Fig. 2I,J).

We also evaluated whether *unc-73*, which displays genetic interaction with *plx-1* (see results below), could affect the localization of PLX-1::GFP. In *unc-73(rh40)* (the strongest allele that does not display a severely lethal phenotype), we obtained results that were essentially identical to those observed in the *smp-1(ev715)* background (Fig. 2K,L). These results suggest that the specific subcellular localization of PLX-1 on the presumptive lumen of ring-forming vulva cells is partially dependent on SMP-1 and UNC-73, perhaps indicating that some PLX-1 clustering on the future lumen membrane may require SMP-1 ligand on an inner neighboring cell and a cell-autonomous intracellular polarizing function of UNC-73.

### SMP-1 is expressed sequentially in ring-forming vulva cells

The previously described transcriptional (*smp-1::gfp*) and genomic translational (*smp-1::GFP*; here referred to as *smp-1p::SMP-1::GFP*) reporter genes (Dalpe et al., 2004; Ginzburg et al., 2002) were used to evaluate *smp-1* expression during vulva development. At the beginning of the third larval stage, both types of reporters are expressed in dividing VPCs. The *smp-1::gfp* transcriptional reporter is predominantly expressed in P6.p-derived cells, and is more weakly expressed in P5.p and P7.p daughters. Before the beginning of morphogenesis, the expression in P6.p-derived cells diminishes over time, while the expression in P5.p and P7.p daughters increases and later decreases.

In order to follow SMP-1 protein expression during vulva morphogenesis, we focused on the translational *smp-1p::SMP-1::GFP* reporter (Dalpe et al., 2004; Ginzburg et al., 2002), because it encodes a functional SMP-1 protein with the ability to rescue the *smp-1(ev715)* vulva morphogenesis defect (see above; compare, rows 9 and 8 in Table 1). Expression from the *smp-1p::SMP-1::GFP* translational reporter is dynamic. Early during vulva morphogenesis, the protein is observed only in the first effective ring of cells (vulF) that in principle can serve as a template for aligning other cells that will form the next ring of the vulva proper. SMP-1::GFP signal appears localized to vulF cell membranes facing the anchor cell and also on their ventral surface (Fig. 3A,B). At this time, other primordial vulva cells, the processes of which have not completed their migration to the vulva midline, do not exhibit any detectable expression (Fig. 3A,B). Later on, vulva cells and their processes entering the forming vulva upregulate SMP-1::GFP on the lumen side of the newly forming ring-shaped cell (Fig. 3C-F), then attach to the ventral side of the previously formed vulva ring, pushing it upwards. This cycle of SMP-1 expression repeats until all 22 primordial cells have migrated, aligned and attached to one another to form the vulva proper (shown for the beginning, intermediate and late stages of vulva morphogenesis in Fig. 3A-J).

Interestingly, the SMP-1::GFP signal appears to be localized entirely on the lumen of the vulva rings (Fig. 3E-J,M-N). As vulva morphogenesis progresses, SMP-1::GFP expression is found on the ring cells being sequentially added ventrally (shown for early and later stages in Fig. 3M,N, respectively). This contrasts with PLX-1::GFP, which localizes largely to the

presumptive lumen-facing membrane of all presumptive vulva cells, but is also found at a uniformly lower level on the remaining cell membrane (Fig. 2E,F). Furthermore, the *SMP-1::GFP* signal appears to highlight membrane protrusions emanating from the ventral lumen side of each ring but extending outwards towards the newly docking primordial ring cells (Fig. 3I,J).

In principle, sequential expression of *smp-1* from cells already in or poised to enter the forming vulva could depend on a cell-autonomous or non-cell-autonomous program. In a cell-autonomous situation, vulva cells in *plx-1* mutants that fail to migrate towards the vulva midline express SMP-1::GFP at the same time as those that migrate normally. However, if SMP-1 expression is activated non-cell autonomously by a cell position-dependent mechanism (e.g. dependent on reaching and contacting the forming vulva or dependent on contiguous contacts between inner neighbors and the forming vulva, or dependent on a certain position within a morphogen gradient that radiates from the vulva midline), its expression should not be activated in mutant cells that fail to migrate towards the midline. In *plx-1(ev724)* mutants that show migration defects specific to one side of the presumptive vulva (i.e. anterior only), we observe a correlation between lack of SMP-1::GFP expression and cells that do not migrate properly towards the vulva midline, in contrast to cells that migrate correctly (Fig. 3K,L). These observations favor a position-dependent model of *smp-1* activation: those cells that do not migrate to the forming vulva are not autonomously programmed to express detectable levels of the SMP-1::GFP translational reporter.

Our results strongly support the idea that SMP-1 is induced in primordial vulva cells as they begin to form the vulva and this expression recruits (by attraction) the next outermost PLX-1-expressing cells into the vulva.

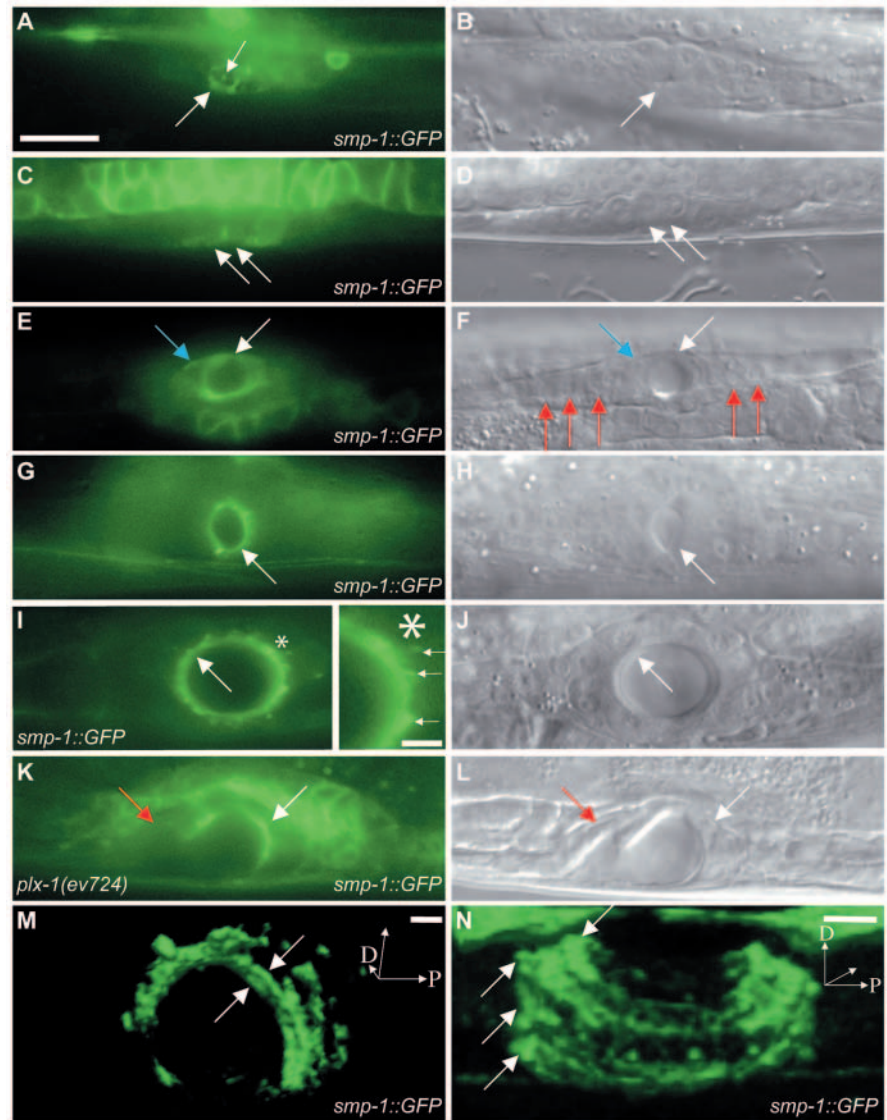
### A gain of function in Ras GTPase [*let-60(n1046gf)*] requires *plx-1* to form ectopic pseudovulvae and pseudovulvae express *smp-1* reporters

Gain-of-function mutations in Ras *let-60(1046gf)* cause VPCs that would normally adopt 3° fates to now adopt 2° and 1° fates. This produces ectopic pseudovulvae in addition to a largely normal vulva proper (multivulva phenotype or Muv) (Ferguson and Horvitz, 1985; Han and Sternberg, 1990). The number of cells in each pseudovulva varies. Some pseudovulvae have vulF cells as centers of attraction for neighboring cells, whereas others may lack vulE and vulF, in which case vulD could serve as a center of attraction for vul neighbors. In each case, the order of attraction appears preserved as in the wild type, so some pseudovulvae may have all the vul cell types (vulF, vulE, vulD, vulC, vulB, vulA in proper order) and others may only comprise rings vulD, vulC, vulB, vulA in proper order (Shemer et al., 2000). In light of our *smp-1*- and *plx-1*-mediated model of vulva morphogenesis, this suggests to us that the most dorsal vulva cell fate in a pseudovulva could function as an organizer by expressing SMP-1 to serve as an attractive guidance cue for neighboring PLX-1-expressing cells, just as happens in normal vulva formation.

This prompted us to examine the expression of the *smp-1::gfp* transcriptional reporter in *let-60(n1046gf)* strain. The *smp-1::gfp* is initially expressed in dividing P6.p cells in wild-type L3 hermaphrodites (see Fig. S1 in supplementary



**Fig. 3.** SMP-1 expressed on the lumen-facing side of vulva rings guides vulva cell migration. B,D,F,H,J,L are DIC microscopic images of A,C,E,G,I,K, respectively. The *SMP-1::GFP* translational reporter signal is expressed specifically on the vulva lumen membrane (white arrows in A-J) of vulva cells that have formed rings (shown at different chronological stages of vulva morphogenesis; at the beginning in A,B and nearing completion in I,J). (A,B) The lumen formed by both vulF (small arrow) and vulE (large arrow) rings show the *SMP-1::GFP* signal. (C-F) Wild-type vulva cells that do not change their shape or migration pattern do not obviously express the *SMP-1::GFP* signal (red arrows in F), with the exception of possibly one cell just joining the forming vulva proper (blue arrows in E,F). During the later stages of vulva morphogenesis (G-J), the *SMP-1::GFP* signal highlights cell membrane protrusions radiating out from the lumen (G,I; inset in I shows an enlargement of the region near the asterisk). (K,L) In *plx-1(ev724)* animals, mutant cells that fail to migrate properly towards the presumptive vulva midline fail to express *SMP-1::GFP* (red arrows) in comparison with cells that migrate correctly (white arrows). (M,N) Fluorescence confocal microscopy in accordance with the epifluorescence data (see A-J above) shows that the *SMP-1::GFP* reporter is sequentially turned on during vulva morphogenesis in cells that have finished (or have nearly finished) forming a vulva ring. As vulva morphogenesis progresses, the last formed vulva ring begins to show *SMP-1::GFP* on its lumen surface. (M) Only two vulva rings express the *SMP-1::GFP* reporter (white arrows), but later (N) two additional rings express the *SMP-1::GFP* signal. There is a strong gonadal and uterine *SMP-1::GFP* staining in A,C,N. M and N are dorsal and dorsolateral projections of 3D confocal images, respectively. D, dorsal; P, posterior; unlabelled arrow indicates left-right depth. Scale bars: in A, 25  $\mu$ m for A-L; 6.3  $\mu$ m for inset in I; 1.0  $\mu$ m in M; 2.7  $\mu$ m in N.



material). In L3 stage *let-60(n1046gf)* mutants, we observe what appears to be multiple vulva cell clusters that express the *smp-1::gfp*. The signal corresponds in intensity to the one we normally observe in P6.p-derived cells of wild-type hermaphrodites. Later on, we observe the *smp-1::gfp* expression in ring cells of pseudovulvae. This suggests that *smp-1* is expressed in cells that serve as centers of attraction for other cells that will form a vulva or a pseudovulva.

Torroids tend to form normally in a half vulva autonomous manner for both the pseudo and normal vulvae of *let-60(n1046gf)* mutants, with the exception of vulA cells that tend to be simultaneously attracted toward the midline of neighboring primordial clusters and therefore never enter either cluster because of inter-vulva competition (Shemer et al., 2000). However, we frequently observe severe torroid formation defects in both the pseudo and normal vulvae of *plx-1(ev724);let-60(n1046gf)* double mutants (see Fig. S1 in supplementary material). In *plx-1(ev724);let-60(n1046gf)*, torroid formation is dramatically impaired when compared with the control *let-60(n1046gf)* strain (a fusion between vulA

cells of two different vulvae was not considered to be a defect for this comparison). The type of vulva morphogenesis defects are similar to the ones we previously observed in *plx-1(ev724)* mutants.

All together, these results suggests that pseudovulvae form torroids by means of initiating sequential *smp-1::gfp* expression in cells of the most dorsal vulva cell fate in *let-60(n1046gf)* mutants. Not surprisingly, we also find a role for *plx-1* in guiding torroid formation in pseudovulvae. The fact that we also observe normal torroid development in *plx-1(ev724);let-60(n1046gf)* suggests that *plx-1* is not the only mechanism at work for proper vulva cell migration in pseudovulvae.

### SMP-1 expression is instructive and PLX-1 expression is permissive for guiding vulva cell movements during morphogenesis

If SMP-1 has an instructive guidance function, disturbing its precise temporal expression pattern during vulva morphogenesis should, in theory, misguide migrating



presumptive vulva cell extensions. By contrast, no effects would necessarily be expected if SMP-1 has a purely permissive role in vulva cell movements. To further examine these possibilities, we placed a functional *smp-1* cDNA (functional in spite of being truncated for its cytodomain-encoding portion, see Table 1, row 9 versus row 8) under the control of the *plx-1* 5' regulatory region to drive expression in all vulva cells with a predominant expression in P5.p- and P7.p-derived cells (see above). We observe that animals carrying this *plx-1p::SMP-1* transgene on an extra-chromosomal array show variable body morphology defects (data not shown), a result that is not surprising considering the previously described role for *smp-1* and *smp-2* in embryonic morphogenesis (Ginzburg et al., 2002). These transgenic animals also exhibit frequent vulva cell migration defects [Table 1, row 12; 11% at 20°C ( $n=35$ ) and 38% at 25°C ( $n=42$ )] like those observed in *plx-1* and *smp-1* mutants (Fig. 4A-C). The defects are frequently observed with vulA and vulB cells, consistent with the strong *plx-1* regulatory region activity in these cells (see PLX-1::GFP expression pattern above).

In theory, the vulva cell migration defects that are caused by the *plx-1p::SMP-1* transgene could result from a dominant-negative effect on the endogenous *smp-1* gene, thereby phenocopying the *smp-1* loss-of-function effect. If this is the case, then introducing the *smp-1*-null background into the transgenic line should not show a qualitative change in phenotype. However, if ectopic SMP-1 expression from the *plx-1* 5' regulatory region establishes ectopic attraction centers within the vulva primordium, introducing *smp-1(ev715)* into the *plx-1p::SMP-1* transgenic line could remove the competition with endogenously expressed SMP-1 (tending to cause normal morphogenesis). This could possibly cause an enhancement of the *smp-1* null mutant phenotype as well as a qualitative change in its manifestation. Consistent with the latter model, we observe a strong enhancement of vulva migration defects in *smp-1(ev715)* animals carrying the *plx-1p::SMP-1* transgene in comparison with the non-transgenic animals (88% versus 11%, Table 1, row 13 versus row 12). Moreover, multiple invaginations are more frequently observed in *plx-1p::SMP-1* transgenic *smp-1(ev715)* mutants (Fig.

4D,E), suggesting that the cells located externally in the vulva primordium become, in this context, a dominant source of an attractive guidance cue. Taken together with their similar mutant phenotypes, these results are more consistent with a guidance role rather than a permissive role for SMP-1 in the migration of primordial vulva cells.

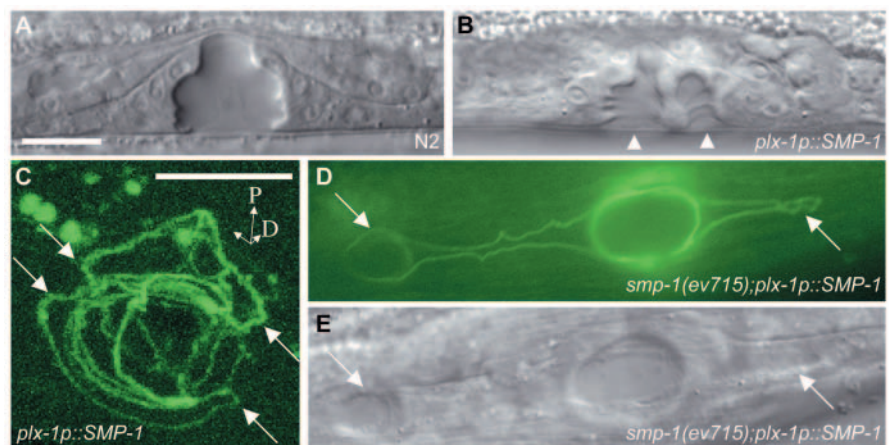
### ***C. elegans* Rac GTPases MIG-2 and CED-10, and their putative GEF activator UNC-73 function in a pathway parallel to *plx-1***

Vulva cell migration defects similar to those described in the *smp-1* and *plx-1* mutants have also been described in mutants for the *C. elegans* genes *mig-2* and *ced-10*, encoding homologs of mammalian Rac GTPases (Kishore and Sundaram, 2002; Lundquist et al., 2001; Zipkin et al., 1997). Similar defects have also been described for mutants of the *C. elegans* *unc-73* gene (Kishore and Sundaram, 2002), which encodes a guanine exchange factor that functions upstream of MIG-2 and CED-10 for many guided cell migrations (Lundquist et al., 2001; Steven et al., 1998; Wu et al., 2002).

We also recently described a role for *unc-73*, *mig-2* and *ced-10* in a pathway that functions in parallel to *smp-1* and *plx-1* for preventing anterior displacement of ray 1 cells during male tail development (Dalpe et al., 2004). This led us to examine whether *unc-73*, *mig-2* and *ced-10* might act in the same or in a pathway parallel to *smp-1* and *plx-1* for vulva morphogenesis. As for male ray 1 cell movements, both *mig-2* and *ced-10* single mutants display few vulva defects on their own (Table 2, rows 3,4) when compared with wild type (Table 2, row 1) or *plx-1* mutants (Table 2, row 2). However, as reported previously (Kishore and Sundaram, 2002), the *mig-2(mu28)*; *ced-10(n1993)* double mutants show a considerably enhanced expressivity and penetrance of vulva cell migration defects (Table 2, row 5) when compared with either single mutant. Although *ced-10(n1993)* is not a null allele (Lundquist et al., 2001), *mig-2(mu28)* is a null (Zipkin et al., 1997); therefore, these results strongly suggest that the two genes act in parallel to guide vulva cell migrations and positioning.

We observe a strong enhancement of vulva cell migration defects in the *plx-1(ev724)*; *mig-2(mu28)* double mutant, to a

**Fig. 4.** Vulva cell migration defects are caused by ectopic expression of *smp-1*. Observations use DIC microscopy (A,B,E) and the AJM-1::GFP reporter (C,D) (see Materials and methods). (A,B) Lateral views with anterior towards the left; (C) top-down projection from a 3D confocal image. D, dorsal; P, posterior; unlabelled arrow indicates left-right depth. (D,E) Ventral views, anterior towards the left. (A,B) Animals carrying the *plx-1p::SMP-1* transgene display vulva morphology defects (B) when compared with wild-type animals (A). In *plx-1p::SMP-1* transgenic animals, vulva cells from each side frequently fail to migrate towards the presumptive vulva midline, forming two invaginations (arrowheads in B) as seen in *smp-1* and *plx-1* mutants. (C) Three dimensional image reconstructions of the GFP signal from the AJM-1::GFP reporter in animals carrying the *plx-1p::SMP-1* transgene (see Materials and methods) reveal vulva cells from the anterior and posterior sides that do not complete their migration to form a vulva ring (arrows). (D,E) In *smp-1(ev715)* animals carrying the *plx-1p::SMP-1* transgene, frequent widely separated multiple invaginations are observed on both anterior and posterior sides of the presumptive vulva (arrows). Scale bars: in A, 25  $\mu$ m for A-B, D-E; in C, 8  $\mu$ m for C.



**Table 2. Vulva ring defects in *mig-2*, *ced-10* and *unc-73* mutants**

Row	Genotype*	Ring defects (%) <sup>†</sup>	n <sup>‡</sup>
1	Wild type <sup>‡</sup>	0	111
2	<i>plx-1(ev724)</i> <sup>‡</sup>	52	217
3	<i>mig-2(mu28)</i>	2	129
4	<i>ced-10(n1993)</i>	3	96
5	<i>ced-10(n1993);mig-2(mu28)</i>	77	82
6	<i>mig-2(mu28);plx-1(ev724)</i>	72	173
7	<i>ced-10(n1993);plx-1(ev724)</i>	50	164
8	<i>unc-73(rh40)</i>	54	163
9	<i>unc-73(e936)</i>	20	148
10	<i>unc-73(e936);plx-1(ev724)</i>	90	115
11	<i>unc-73(e936);evEx168[plx-1p::unc73(+)]</i>	0	145

\*All strains have the *ajm-1p::AJM-1::GFP* reporter gene in the *him-5(e1490)* background. Animals were grown at 20°C.

<sup>†</sup>The frequency of vulva ring defects was determined as described in Materials and methods. *n* represents the number of animals scored. Standard deviations (s.d.) were calculated assuming a binomial distribution with the observed percentage value and the actual sample size. For all comparisons described in the Results, *P* < 0.05 was considered to be a significant difference.

<sup>‡</sup>For comparison purposes, these numbers are included from Table 1.

penetrance approximating that of the *mig-2(mu28); ced-10(n1993)* double mutant (Table 2, row 6 versus row 5). By contrast, the penetrance of vulva defects observed in *plx-1(ev724); ced-10(n1993)* animals is not enhanced when compared with the *plx-1(ev724)* single mutant animals (Table 2, row 7 versus row 2), suggesting that *ced-10* may play a larger role in the semaphorin signaling pathway that regulates vulva morphogenesis than does *mig-2*, with the latter probably playing a larger role in parallel acting vulva cell migration mechanisms.

Two non-null mutant alleles of *unc-73* also show vulva morphogenesis defects like those observed in mutants of *plx-1* and *smp-1*. *unc-73(rh40)* behaved as expected for a strong loss-of-function allele for this phenotype compared with the weaker hypomorph *unc-73(e936)* (R. Steven, personal communication) (Table 2, row 8 versus row 9). Based on the enhancement phenotype observed between *plx-1(ev724)* and *mig-2(mu28)*, and on the likely possibility that *unc-73* functions upstream of both *mig-2* and *ced-10* in many cell migration processes (Lundquist et al., 2001; Wu et al., 2002), one would predict a strong enhancement in the *plx-1(ev724); unc-73(rh40)* double mutant compared with either single mutant. Unfortunately, this double mutant is embryonic lethal (Dalpe et al., 2004); however, *plx-1(ev724); unc-73(e936)* double mutants survive and show a synergistically enhanced vulva morphogenesis phenotype when compared with either *plx-1(ev724)* or *unc-73(e936)* single mutants (Table 2, row 10 versus rows 9 and 2). Taken together, our results suggest that *unc-73* and *mig-2* have functions that parallel *plx-1* functions during vulva formation. However, the genetic data do not exclude the possibility that *unc-73*, *mig-2* and *ced-10* also have a related function in the PLX-1 pathway (Fig. 5C).

### UNC-73 acts in PLX-1 expressing vulva cells

UNC-73 might function in parallel to PLX-1 by having a role in a cell type distinct from the one expressing PLX-1. However, the fact that *plx-1* is expressed ubiquitously in all vulva cells and that *mig-2*, which is known to function downstream of *unc-*

73, has been shown to act cell-autonomously in all vulva cells (Kishore and Sundaram, 2002) suggests they both might act in the same cell type. To address this, we put the full-length wild-type *unc-73(+)* cDNA under the control of the *plx-1* 5' regulatory region (Dalpe et al., 2004) and evaluated the rescue of the vulva phenotype in *unc-73* mutants. *unc-73(e936)* animals carrying the extra-chromosomal array containing the *plx-1p::UNC-73(+)* minigene are largely rescued for the vulva morphogenesis phenotype (Table 2, row 11 versus row 9), strongly suggesting that both *unc-73* and *plx-1* act cell autonomously in all vulva cells.

Interestingly, when the region of cDNA encoding the cytodomain of PLX-1 is deleted from *PLX-1::GFP* (*PLX-1delC::GFP*), the construct no longer rescues the *plx-1(ev724)* vulva cell defects (see results above), but enhances it considerably when compared with the null on its own (78% versus 52%, Table 1, row 7 versus row 2). The finding that *PLX-1delC::GFP* induces more severe defects than a putative *plx-1*-null allele suggests that in the absence of the endogenous *plx-1(+)*, the *PLX-1delC::GFP* functions as a dominant negative, possibly by interfering with a signaling component that takes part in a pathway functioning in parallel to PLX-1 for vulva morphogenesis.

## Discussion

### SMP-1 and PLX-1 have a role in guiding primordial vulva cell movements

We demonstrate that *smp-1* and *plx-1* genes are required for cell shape changes and guided movements involved in the complex process of vulva morphogenesis. In the wild type, primordial vulva cells form lateral processes that extend along the ventral edges of their inner crescent-shaped neighbors and end at the vulva midline just left and just right of the presumptive vulva lumen. Thus, four lateral extensions, two from anterior and two from posterior half-ring homologs surround the presumptive vulva lumen. Later, homologous processes from anterior and posterior fuse, thus forming ring-shaped cells that are precisely stacked along the dorsoventral axis. These cells comprise the vulva proper.

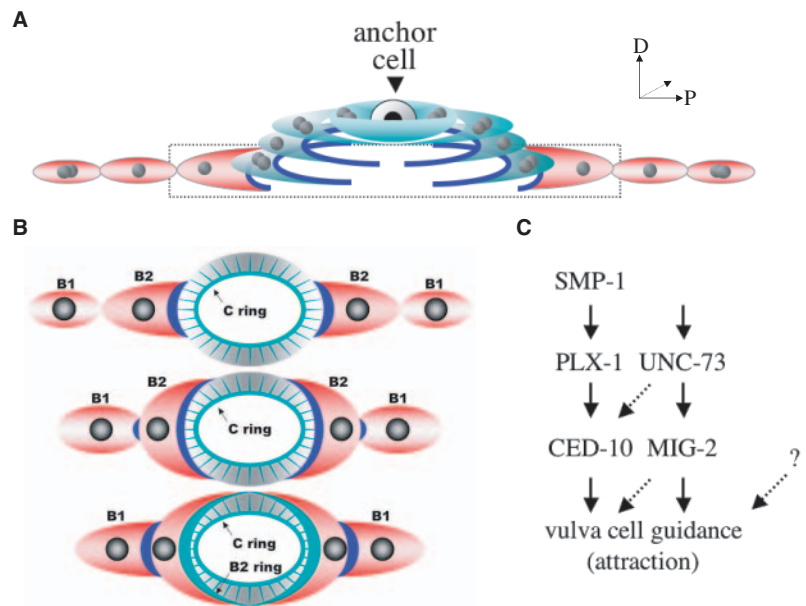
In *smp-1* and *plx-1* mutants, primordial vulva cells fail to extend two normal processes towards the vulva midline, suggesting that a mechanism guiding these processes is lost in the mutants, which prevents the formation of normally stacked vulva rings. Our genetic analyses show that both single *smp-1(ev715)* and *plx-1(ev724)* null mutants display approximately the same penetrance for this phenotype. The fact that a *smp-1(ev715); plx-1(ev724)* double mutants do not show any enhancement over either single mutant indicates that SMP-1 and PLX-1 function in the same pathway for vulva formation (Fig. 5C). This is entirely consistent with the finding that SMP-1 binds PLX-1 in vitro (Fujii et al., 2002).

### Rac GTPase- and UNC-73-dependant mechanisms of vulva cell migration

The incomplete penetrance of the null alleles and the lack of enhancement in double mutants indicates that there must be other mechanisms that act in parallel with SMP-1 signaling to regulate vulva cell migrations and morphogenesis. Similar vulva cell migration defects were previously reported for mutants of genes encoding *C. elegans* homologs of the Rac GTPases, MIG-



**Fig. 5.** A SMP-1 and PLX-1 based model of cell migration during vulva morphogenesis. (A) Lateral perspective; D, dorsal; P, posterior; unlabelled arrow indicates left-right depth. A series of short-range migrations is repeated for the formation of vulva rings. (B) Ventral perspective of the boxed area in A. Intermediate steps of the proposed model for vulva cell migration showing the PLX-1-expressing leading edge as a blue band on the future lumen side of cells poised to enter the forming vulva. The leading edge spreads ventrally under forming vulva rings expressing SMP-1 on their lumen surface (green spiked ring). SMP-1 expression increases dramatically in the newly formed ring, while the next external vulva half ring (not yet expressing SMP-1) initiates its migration by extending processes underneath it. (C) A genetically derived model of the molecular cascade regulating vulva morphogenesis. SMP-1, PLX-1 and CED-10 function in the same pathway when guiding vulva cells (attractive guidance to the vulva midline). UNC-73 and MIG-2 act in one or more parallel pathways whose function is similar to that of the SMP-1/PLX-1 signaling pathway. It is possible that MIG-2 and CED-10 also function downstream of PLX-1 (broken arrows from UNC-73 and MIG-2), as suggested by the literature, indicating that UNC-73 can activate MIG-2 and CED-10. Additional guidance mechanisms may function in parallel with PLX-1 and MIG-2 (broken arrows with question mark on the right).



2 and CED-10, and a gene encoding the Rac activator UNC-73. As observed by others (Kishore and Sundaram, 2002), we also find that loss-of-function mutations in *mig-2* and *ced-10* alone cause few vulva cell migration defects, but *mig-2(mu28); ced-10(n1993)* double mutants have highly penetrant vulva cell migration defects. This suggests that *mig-2* and *ced-10* function redundantly in vulva morphogenesis (Fig. 5C).

Partial loss-of-function mutations in *unc-73* also cause a significant penetrance of vulva cell migration defects, which is entirely consistent with the finding that this gene is required for the function of *mig-2*, *ced-10* and *rac-2* in other types of guided cell migrations (Lundquist et al., 2001; Wu et al., 2002). Our finding that mutations in *mig-2* and *unc-73* enhance the *smf-1* or *plx-1* null mutant vulva defects suggests that they function in a pathway parallel to *smf-1* and *plx-1* (Table 2; Fig. 5C). Mutations in *ced-10* are unable to enhance the *plx-1* mutant vulva defects, suggesting that CED-10 acts redundantly with MIG-2 because they act in parallel mechanisms (CED-10 in the PLX-1 mechanism and MIG-2 in an unknown mechanism). However, CED-10 is probably not the only pathway through which PLX-1 functions, because if it were, *plx-1* mutants are not expected to have a major vulva phenotype. Considering the synergistic enhancement of vulva morphogenesis defects we observe in the *mig-2(mu28); ced-10(n1993)* double mutants (77% Table 2, row 5), there is a high probability that MIG-2 and, by implication, UNC-73 also functions in the same pathway as PLX-1 (Fig. 5C). This interpretation takes into account that *ced-10(n1993)* and *unc-73(rh40)* are probably not null alleles as nulls of these genes are lethal (Lundquist et al., 2001; Steven et al., 1998).

The involvement of Rac GTPases in PLX-1 signaling is consistent with the classical biochemical view of plexin signaling in which activated Rac<sup>GTP</sup> binds the cytoplasmic portion of plexin receptors (Driessens et al., 2001; Hu et al., 2001; Pasterkamp and Kolodkin, 2003; Turner et al., 2004).

The nearly full enhancement (90% Table 2, row 10) of a *plx-1* putative null allele by a partial loss-of-function in *unc-73* indicates that PLX-1 and UNC-73 signaling mechanisms can account for most and possibly all of the guided cell migrations involved in vulva morphogenesis. However, as PLX-1 and UNC-73 act in parallel, it is possible that they act in different cell types. Our results show that *plx-1* mutant animals carrying a *plx-1p::PLX-1(+)* minigene and *unc-73* mutants carrying a *plx-1p::UNC-73(+)* minigene are both rescued for their respective vulva phenotypes. Thus, our results suggest that PLX-1 and UNC-73, and by implication the GTPases activated by UNC-73 (e.g. MIG-2 and CED-10) function cell-autonomously in all vulva cells to guide their movements.

The observed interference by the cytodomain-deleted PLX-1 transgene in a *plx-1* null mutant suggests that components acting in parallel to and redundantly with PLX-1 for vulva cell migration are probably interacting with the extracellular or transmembrane domains of PLX-1. Based on the intermediate penetrance of the *plx-1(ev724)* phenotype, this redundant mechanism must have at least one function that is independent of PLX-1. Nevertheless, the dominant-negative effect we observe with the cytodomain-deleted PLX-1 transgene indicates that components of this unknown parallel mechanism might take part in a protein complex that includes PLX-1. Plexins usually function in conjunction with co-receptors (Pasterkamp and Kolodkin, 2003). The proposed dominant-negative effects of *plx-1p::PLX-1delC::GFP* may result from an effect on the ability of a co-receptor to bind and be activated by a non-semaphorin ligand involved in vulva morphogenesis.

#### A model for vulva morphogenesis based on sequential SMP-1 expression

Seven vulva cell types are formed from 22 epithelial cells (primordial vulva cells) arranged in a longitudinal row along the ventral epidermis. During vulva formation, these cells are

sequentially recruited to the midline position of the primordial vulva, starting with cells closest to the center of the primordium and extending outwards. First the four innermost cells (vulF cells) are born surrounding the anchor cell at the midline of the primordial vulva and eventually fuse to form a single ring-shaped cell (vulF ring). Next, the four vulE cells (outer neighbors to vulF cells) are recruited to the vulva midline, align just ventral to the vulF ring and eventually fuse to each other to form the vulE ring. This process continues for vulD (two cells recruited), vulC (four cells recruited, two cells per half ring), vulB2 (two cells recruited), vulB1 (two cells recruited) and vulA cells (four cells recruited, two cells per half ring) until the longitudinal row of 22 primordial cells are converted into seven rings of cells aligned along the dorsoventral axis that comprise the mature vulva (Fig. 5A,B).

The spatial and temporal expression of *plx-1* and *smp-1* in the primordial vulva cells is consistent with a role for these proteins in mediating orderly attraction of these cells to the midline of the forming vulva and can also explain their precise alignment to form a contiguous vulva lumen. As observed with our *PLX-1::GFP* translational reporter, PLX-1 is found at the cell membrane of vulva cells undergoing morphogenesis, with a much greater localization on membrane facing the vulva midline (the presumptive lumen membrane of the vulva). The *SMP-1::GFP* reporter is also highly expressed on vulva cell membrane facing the midline but this expression is dynamic. *SMP-1::GFP* expression is first observed on the cell membranes of each presumptive vulF cell on the side that faces the anchor cell (Fig. 3), which marks the vulva center and presumptive vulva lumen. Expression on the other primordial vulva cells is first evident on the midline facing membrane (presumptive lumen side) of these cells as they acquire a ring shape and as they dock onto the ventral end of the forming vulva cell stack. Expression continues on the lumen membrane of these cells even after they have formed a ring and become an integral part of the forming vulva. Sequentially, the next set of vulva cells start expressing SMP-1 on their presumptive lumen membrane as they form a ring ventral to the previously formed ring. This process repeats until the seven precisely stacked vulva rings are formed (Fig. 5A,B).

SMP-1 is involved in lateral process extension and migration of primordial vulva cells to the vulva midline, and of positioning vulva rings precisely one on top of the other. Theoretically, SMP-1 could function as a diffusible cue that emanates from the vulva midline and attracts PLX-1-expressing vulva cell processes; however, this is unlikely given that SMP-1 is a predicted transmembrane protein. We propose that SMP-1 guides vulva cell morphogenesis by means of its precise spatiotemporal expression pattern. In so doing, poised PLX-1-expressing vulva cells that are adjacent to a SMP-1 expressing half ring, extend lateral processes that crawl underneath the half ring, presumably to reach high concentration of SMP-1 present at the ring lumen membrane of its inner neighbor.

The sequential expression of the SMP-1 on the lumen membrane of ring cells ensures that only the PLX-1-expressing outer neighbors will extend processes and move towards the midline before other primordial vulva cells even further away from the midline extend lateral processes in the same direction. This is most probably what accounts for the ordered stacking of vulva cell types during vulva morphogenesis. Second, sequential SMP-1 expression from ring shaped cells of the vulva proper

dictates a polarity of migration, ensuring that cell processes from vulva cells neighboring a SMP-1 expressing ring cell (in the vulva proper) extend towards the forming vulva lumen. Third, the processes of forming half rings that spread underneath the previously formed half ring and follow the outline of the presumptive vulva lumen ensures that each new ring aligns itself according to the shape of the previously formed ring.

In principle, if a precise SMP-1 expression pattern is necessary for the orderly migration of vulva cell processes, then disturbing the spatiotemporal sequence should greatly affect the polarity of migration within the vulva primordium. Consistent with this idea, a more ubiquitous SMP-1 expression, which is driven by the *plx-1* 5' regulatory region (with higher expression from the P5.p- and P7.p-derived cells), causes vulva migration defects even when competing with the endogenous *smp-1* gene. Furthermore, we observe a dramatic enhancement of vulva cell migration defects when ectopic SMP-1 expression operates in the absence of all endogenous *smp-1*(+) function [i.e. *plx-1p::SMP-1* in a *smp-1(ev715)* background], indicating that new centers of vulva cell recruitment can be created by inducing SMP-1 expression in different vulva cells. By implication and based on the *smp-1* loss-of-function phenotype, SMP-1 expression in the wild type initiated at the midline has an instructive role for the orderly recruitment of vulva cells (Fig. 5A,B).

Interestingly, we observe cell detachment from externally positioned vulva primordium cells in *smp-1* and *plx-1* mutants as they begin vulva morphogenesis. This suggests that low levels of SMP-1, undetectable by the *smp-1::GFP* translational reporter, might also guide these cells in a cell-autonomous or non-autonomous manner. This interpretation would be consistent with our observation that P5.p- and P7.p-derived cells initially express low levels of the *smp-1::gfp* transcriptional reporter.

Interestingly, PLX-1 localizes to the midline-facing side of the crescent-shaped migrating vulva cells at first to a patch at the region that first enters the vulva proper then spreads along the entire leading concave edge as the cell aligns with the previously formed vulva ring. This suggests that the vulva cells use this entire leading edge for transducing the SMP-1 signaling into a migration and adhesion response. This adds to the concept that the tips of lateral cell extensions constitute the only motile subdomains of vulva cells that guide their migration, as suggested by direct microscopic observation (Sharma-Kishore et al., 1999). Based on the SMP-1- and PLX-1-dependent guidance function, we propose that the leading edge (i.e. the midline-facing membrane expressing PLX-1) of vulva cells senses SMP-1 on the lumen of the neighboring ring cell, then modifies the shape of that leading edge to spread and adhere to the lumen surface. PLX-1 might even guide migration by means of a spreading mechanism using the well-known adhesion functions of activated Rac signaling (Luo, 2000; Mueller, 1999; Suter and Forscher, 1998; Yuan et al., 2003), which is required in a cell-autonomous manner for vulva morphogenesis (Kishore and Sundaram, 2002).

PLX-1 localization to the lumen-facing side suggests that migrating vulva cells could use this PLX-1 subcellular domain as a structure that leads their migrations the same way axons use growth cones or the gonad primordium in *C. elegans* uses distal tip cells (Hedgecock et al., 1987). Consistent with this, we have observed the accumulation of actin at the leading edge of



migrating vulva cells is correlated with the greater accumulation of PLX-1 at the leading edge (data not shown).

Observations of abnormally migrating cells demonstrate that the subcellular localization of PLX-1 partially depends on SMP-1 and UNC-73, suggesting that PLX-1 is probably recruited to the midline facing side of the migrating cell by the ligand it recognizes and by intracellular signaling events triggered by UNC-73, which could involve docking of PLX-1 to activated Rac (e.g. activated MIG-2 and CED-10 could be localized to the leading edge). Particularly, UNC-73 has been shown to be required for polarizing neuroblast migration in *C. elegans*, along with UNC-40/DCC and DPY-19 (Honigberg and Kenyon, 2000). Other unknown factors may also help localize PLX-1 to this membrane compartment.

### Homotypic recognition between homologous vulva cells is not affected by SMP-1 or PLX-1 signaling

The tips of misguided cell processes do not fuse in a heterotypical manner in an *smp-1* or a *plx-1* mutant, indicating that recognition of the homolog target processes is independent of the *plx-1* and *smp-1* functions. Correspondingly, Sharma-Kishore and colleagues (Sharma-Kishore et al., 1999) have shown that, in laser-ablation experiments targeting only one vulva half, the cells from the non-ablated half send out processes that migrate correctly, but instead of meeting their contralateral homolog target pairs, they meet processes from the same half ring at the presumptive vulva midline (Sharma-Kishore et al., 1999). Taken together, these findings suggest that homologous target cell recognition is a specific event that normally occurs in a homotypical manner and is independent of PLX-1 signaling. Considering the fact that each vulva half begins morphogenesis non-simultaneously (Sharma-Kishore et al., 1999), these findings also indicate that the migration guidance mechanisms are autonomous within each vulva half. Moreover, pseudovulvae from the *let-60(n1046)* Muv mutant lacking half of the primordial cells form torroids autonomously. Characterization of many pseudovulvae in these Muv mutants revealed asymmetry between vulva halves, evidence for the model in which vulva halves develop autonomously (Shemer et al., 2000).

The independence of each vulva half could arise by two separate signals that regulate individually the independent formation of the anterior and posterior half vulva. However, our data showing that the effects of PLX-1 are independent for each half vulva indicate that SMP-1 and PLX-1 can function independently in each half vulva. Thus, a single signal, SMP-1, possibly emanating from a single midline source, can have independent effects on cells destined to form each vulva half.

### The dorsal vulva ring organizer initiates SMP-1 expression

VPC-derived cells destined for a primary fate make vulF cells, which appear programmed to express SMP-1 as they form the first ring during normal vulva development. In *let-60(gf)* Muv mutants, there are cell fate changes such that not only P6.p but also other VPCs can form primordial vulva cells developing into pseudovulvae. The cells forming the most dorsal ring in pseudovulvae, whether they are of vulF or vulD fate, act as organizer for torroid formation and are able to express the SMP-1, which presumably attracts PLX-1-expressing neighbors almost to the same level as vulF-fated cells of the normal vulva. As *let-60(gf)* mutations effect complex fate changes among the

VPCs that allow them to bypass the anchor cell mediated induction, we believe it likely that pseudovulva midline cells that serve as an organizer for torroid formation may adopt some, but not all, of the same properties of vulF cells from the normal vulva. One of these properties is the ability to express SMP-1 and thereby attract PLX-1 expressing vulva primordial cells to form torroids.

Whether an inductive signal is involved in inducing SMP-1 expression in the normal vulva remains to be examined. However, the correlation we observe between cells that did not migrate towards the midline in *plx-1* mutants and the lack of SMP-1 expression suggests that there might be a position-dependent stimulation of *smp-1* expression. In this manner, cells that reach their normal position and are about to enter the vulva proper are instructed to express SMP-1 at the lumen membrane, and, by so doing, a cell with proper shape and position serves as template for the next round of vulva cell migration. This way of establishing sequential semaphorin expression would also ensure that *smp-1*- and *plx-1*-dependent guidance functions are autonomous within each half of the vulva.

SMP-1 appears to be localized in an active subcellular region of the cell membrane of ring cells. These ring cell protrusions express membrane-anchored SMP-1 and contain higher concentrations of actin (data not shown). They extend away from the vulva lumen, suggesting that cells expressing PLX-1 that are poised to enter the vulva proper could sense a low SMP-1 concentration on the thin membrane protrusions and initiate migration towards a higher SMP-1 concentration on the lumen side of the forming vulva. Likewise, in *Drosophila*, long-range cell-cell signaling can be established between developing wing imaginal disc cells and signaling centers through actin-based extensions called cytonemes that project from disc cells (Ramirez-Weber and Kornberg, 1999), indicating that both morphogens and guidance molecules could, in this way, increase their range of action.

### Summary

Multicellular tube morphogenesis, such as the development of the *Drosophila* tracheal system and the vascular system in vertebrates, requires regulatory mechanisms coordinating the complex cell shape changes and movements involved (Nelson, 2003). The stereotypical series of cellular shape changes and movements taking place during *C. elegans* vulva morphogenesis makes it a powerful model system for a genetic approach to understanding these processes. Following a cell differentiation phase, linearly arranged primordial vulva cells tend to send extensions and move in a mirror image fashion toward the vulva midline, and in so doing form an aligned stack of vulva ring cells. We propose that sequential SMP-1 expression from the ring cells formed at the vulva midline ensures coherent movements of the PLX-1-expressing cells as they enter the vulva proper. The apparent long-range attraction of some of the outer primordial vulva cells can therefore be explained by a series of short-range attractions involving the sequential establishment of local semaphorin gradients. Interestingly, the molecular pathways involving semaphorin signaling and Rac function are required in a strikingly similar manner for guided cell migrations taking place in the *C. elegans* male tail (Dalpe et al., 2004) and in hermaphrodite vulva morphogenesis. Conceivably, the two *C. elegans* sexual organs could have co-evolved a common mechanism for their morphogenesis.

We thank members of the Culotti Laboratory, especially Naomi Levy-Strumpf and Takehiro Kawano, for comments on the manuscript. We also thank Dr Shin Takagi for providing the *plx-1(nc37)* mutant strain and the Caenorhabditis Genetics Center (which is supported by the NIH Center for Research Resources) for providing other strains. This work was supported by a F.C.A.R. postdoctoral fellowship to G.D. and by grants to J.G.C. from the Canadian Institutes of Health Research (MT9990 and 13207) and the N.I.H. (NS41397).

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