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Pristionchus pacificus vulva formation: polarized division, cell migration, cell fusion, and evolution of invagination ☆

Irina Kolotuev and Benjamin Podbilewicz*

Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel

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Abstract

Tube formation is a widespread process during organogenesis. Specific cellular behaviors participate in the invagination of epithelial monolayers that form tubes. However, little is known about the evolutionary mechanisms of cell assembly into tubes during development. In *Caenorhabditis elegans*, the detailed step-to-step process of vulva formation has been studied in wild type and in several mutants. Here we show that cellular processes during vulva development, which involve toroidal cell formation and stacking of rings, are conserved between *C. elegans* and *Pristionchus pacificus*, two species of nematodes that diverged approximately 100 million years ago. These cellular behaviors are divided into phases of cell proliferation, short-range migration, and cell fusion that are temporally distinct in *C. elegans* but not in *P. pacificus*. Thus, we identify heterochronic changes in the cellular events of vulva development between these two species. We find that alterations in the division axes of two equivalent vulval cells from Left–Right cleavage in *C. elegans* to Anterior–Posterior division in *P. pacificus* can cause the formation of an additional eighth ring. Thus, orthogonal changes in cell division axes with alterations in the number and sequence of cell fusion events result in dramatic differences in vulval shape and in the number of rings in the species studied. Our characterization of vulva formation in *P. pacificus* compared to *C. elegans* provides an evolutionary-developmental foundation for molecular genetic analyses of organogenesis in different species within the phylum Nematoda.

Keywords: Cell fusion; Body patterning; Organogenesis; *Pristionchus pacificus*; *Caenorhabditis elegans*; Epithelial cells; Vulva development; Morphogenesis

Introduction

Invagination is a conserved cellular process required for developmental events involving rearrangements of cells to form three-dimensional tubes that originate from two-dimensional sheets of cells (Lubarsky and Krasnow, 2003; Trinkaus, 1984). Classic morphogenetic processes where invagination takes place are gastrulation, neurulation, and vulva formation. One of the few organs that is highly characterized at the molecular, cellular, and structural levels is the vulva (vagina) of the nematode *Caenorhabditis elegans* (reviewed in (Greenwald, 1997; Kornfeld, 1997; Shemer and Podbilewicz, 2000; Wang and Sternberg, 2001). However, little is known about the cellular mechanisms of evolutionary modifications of the vulva or any other invaginated organ between related species. Though the study of single model organisms is important to understand specific processes, having a system of several related organisms can aid in understanding the evolutionary mechanisms of developmental, genetic, and molecular variation (Blaxter et al., 1998). Detailed description and comparison between the representatives of closely related families may help to better understand single organ evolution (Emmons, 1997; Fitch and Emmons, 1995; Sommer, 2000; Sommer and Sternberg, 1994). An example of such a comparison between two species is the nongonadal cell lineage of *Panagrellus redivivus* compared with the lineage of *C. elegans* (Sternberg and Horvitz, 1982). There are numerous works that give comparisons between vulval lineages and induction mechanisms (Dichtel et al., 2001; Eizinger and Sommer, 1997; Felix and Sternberg, 1997, 1998; Felix et al., 2000; Jungblut and Sommer, 1998; Louvet-Vallee et al., 2003; Sommer and Sternberg, 1994, 1996b; Sommer et al., 1998), but little is known about the cellular events that follow vulval cell proliferation in species related to *C. elegans*.

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* Corresponding author. LCMB—Laboratory of Cellular and Molecular Biophysics, NICHD, NIH, Building 10/Room 10D10, 10 Center Drive, Bethesda, MD 20892-1855. Fax: +1-301-594-0813.
E-mail address: podbilew@tx.technion.ac.il (B. Podbilewicz).

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The vulva is the nematode copulatory and egg-laying organ that is initially generated from 16 to 26 epithelial cells in different species. Recently, two major nematode genetic systems were established for comparison with *C. elegans*: *Pristionchus pacificus* and *Oscheius* sp.1 CEW1 (Dichtel et al., 2001; Sommer and Sternberg, 1996b). In *P. pacificus* (a representative of the Diplogastridae family), the complete vulval cell lineage and induction pathway of vulval cells was described both in wild-type and in mutant animals (Eizinger and Sommer, 1997; Jungblut and Sommer, 1998; Sigrist and Sommer, 1999; Sommer and Sternberg, 1994, 1996b; Sommer et al., 1998).

Nomarski microscopy on live *P. pacificus* animals allows cell lineage experiments as in *C. elegans*. To study the progression and timing of different events during development, we follow single nuclei divisions and shape changes. We are interested in characterizing organogenesis of the vulva of *P. pacificus* to compare it to the known processes in *C. elegans* to study how the cellular mechanisms of vulva formation have changed during the evolutionary separation

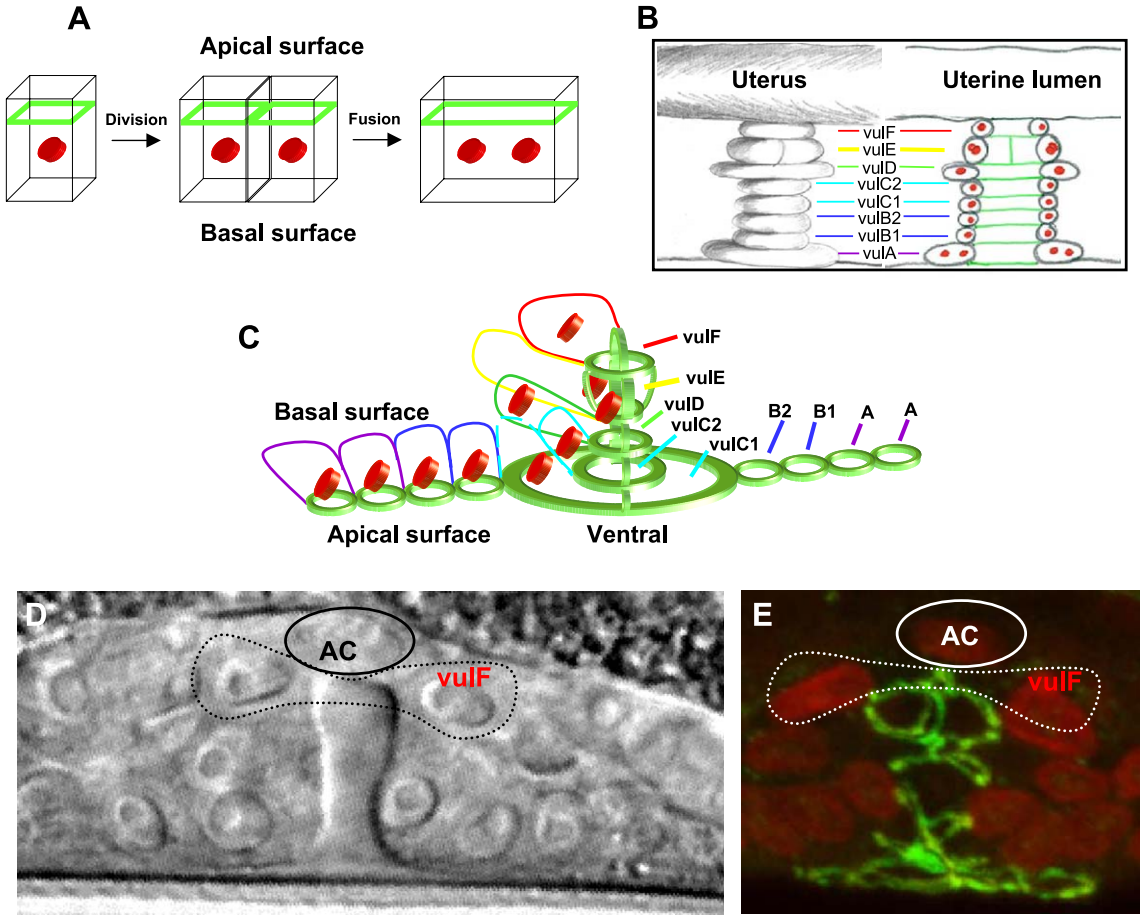

Fig. 1. Changes in the morphology of adherens junctions (AJ) reveal cell behavior during vulva formation. The monoclonal antibody MH27 stains AJs that represent the apical borders of epithelial cells including all vulval and hypodermal cells. Nuclear staining is used to confirm our observations concerning the junctions. We show negative micrographs of the junctions and supply schematic drawings based on detailed analysis of rotations and 3D views of the particular image. Note that the apical side of the vulva primordium is facing the ventral region of the animal and during morphogenesis the apical adherens junctions face the lumen of the vulva. (A) Graphical representation of immunofluorescent staining in epithelial cells. Green represents the adherens junction (AJ) belt; red cylinders represent nuclei in cells. We present a very simplified model of the fusion process at the border between two neighboring cells. Though we cannot see the disappearance of the cell membrane, the disappearance of the AJ borders represents the fusion process based on EM and other studies (Podbilewicz and White, 1994; Sharma-Kishore et al., 1999). (B) Stack of vulva with eight toroidal cells (right, **VulA**–**VulF**) and the cross section through the vulva (left). The vulva is from a late J3 stage hermaphrodite after completion of invagination and intratoroidal fusions. Green lines represent AJs and red are nuclei. (C) Graphical representation of the images presented in this work. A 20-cell stage vulva primordium after completion of cell proliferation but before initiation of cell fusion events. Five toroids in the center are already formed. In the left side of the picture, we show the staining of AJs, the nuclei, and the basolateral domains based on Nomarski images. The colors are according to the color code that we use for the different vulval cells. Right side shows only the apical AJs without the nuclei and the estimated positions of the basolateral borders. (D) Nomarski image of vulva from middle J3 stage. AC—anchor cell. The dotted line represents the position of the **vulF** ring containing two nuclei and their nucleoli. The stage of vulva development in this micrograph corresponds almost exactly to the stage of vulva in panel E. Unlike the Christmas tree invagination shape of *C. elegans* vulva, in *P. pacificus* the vulva has a bell shape. (E) Double-stained image of vulva middle J3 stage. Green lines represent AJ and red are nuclei. Dotted line sketches estimated position of the **vulF** toroid based on many observations including Nomarski optics as shown in panel D. AC—anchor cell.

of these two species. However, it is difficult to study all the cellular dynamic changes using light microscopy without staining. To overcome this problem, we followed cellular markers that localize to the adherens junctions using the monoclonal antibody MH27 (Francis and Waterston, 1991; Podbilewicz and White, 1994) (Fig. 1A). Using this marker, it is possible to visualize cellular processes and structural modifications during tubulogenesis (Figs. 1B,C) (Hanna-Rose and Han, 1999; Hurd and Kemphues, 2003; Podbilewicz and White, 1994; Sharma-Kishore et al., 1999; Shemer et al., 2000).

In this work, we determined the sequence of events during organogenesis of the vulva in *P. pacificus* and compared it to *C. elegans*. We found that the basic cellular mechanisms such as cell migration, ring formation, and cell fusion are conserved. However, our results reveal that there are significant differences in the pattern of the division, migration, and fusion of equivalent cells: (1) Cell migration in *P. pacificus* begins during the cell proliferation stage and not after its completion as in *C. elegans*. (2) The vulva of *P. pacificus* is formed from eight, instead of seven rings in *C. elegans*. (3) The additional ring in *P. pacificus* is formed as a result of a longitudinal division instead of a transverse division with no further cell fusion events as in *C. elegans*.

Background: vulva formation

In *C. elegans*, 12 ectoblastic cells (P1.p through P12.p) are generated after hatching and are evenly distributed in a

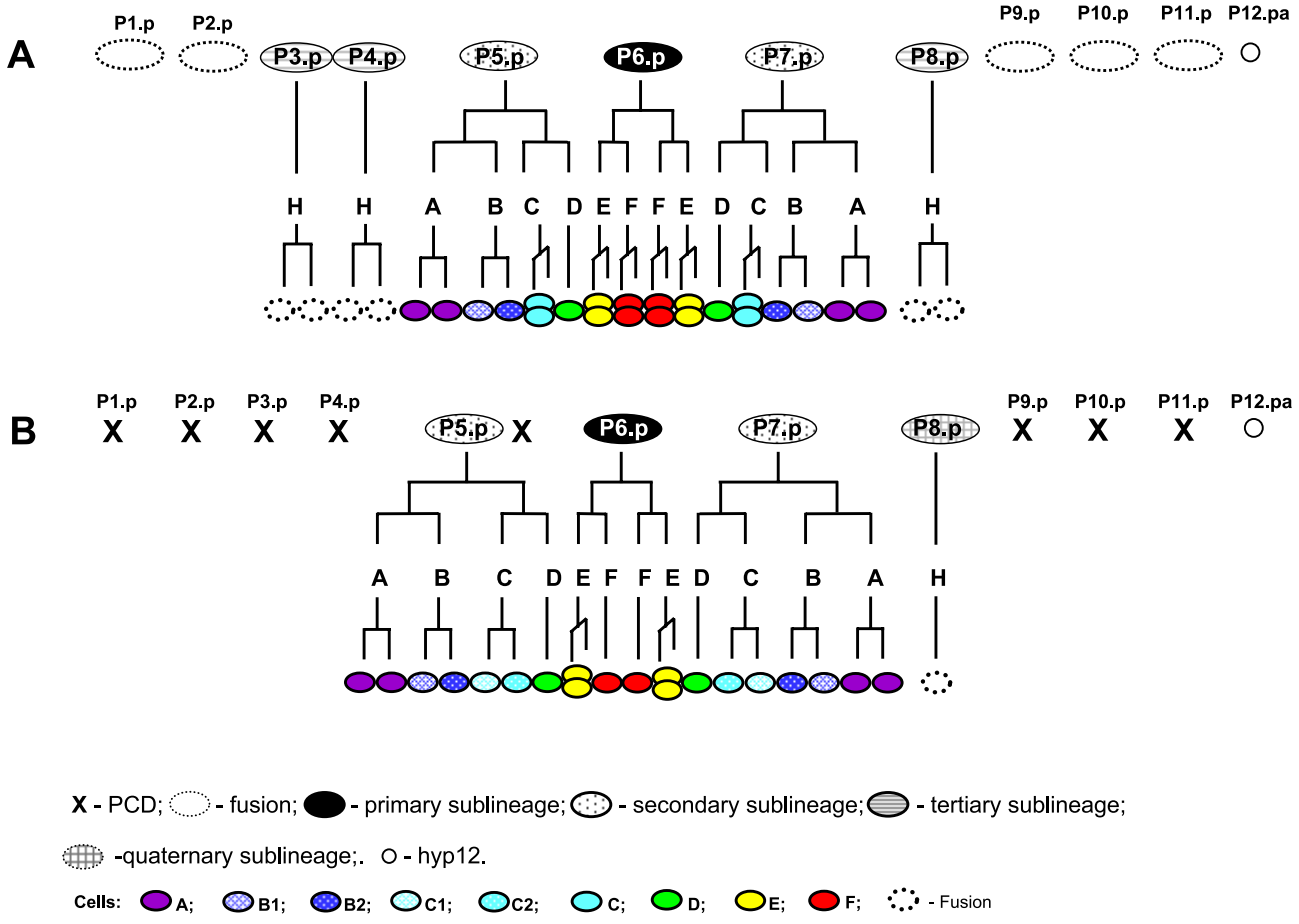

Fig. 2. Pn.p ectoblast sublineages and cell fates in *P. pacificus* and *C. elegans*. The pattern of VPCs divisions (sublineage 1°, 2°, 3°, 4°) is represented in the upper part of each panel in black and white. Bars represent the pattern of cell divisions. Cell fates (**A**–**F** and **H**) are color-coded. (A) *C. elegans*. P1.p, P2.p, P9.p–P11.p fuse to hyp7 early in L1. P12.pa forms hyp12 that participates in the formation of the anus. The VPCs (P3.p–P8.p) represent the vulva equivalence group in *C. elegans*. P3.p, P4.p., and P8.p divide once and fuse to the syncytial cell hyp7 (3° sublineage). These cells are capable to switch their pattern of cell fates when one or all of P5.p–P7.p are ablated. P6.p gives rise to eight cells (1° sublineage); P5.p and P7.p give rise to seven cells each (2° sublineage). Cells **C**, **E**, and **F** divide transversely (left–right). Cells **D** do not divide and all other divisions are longitudinal (anterior–posterior). Twenty-two cells generate the vulva in *C. elegans* (Sharma-Kishore et al., 1999; Sulston and Horvitz, 1977). (B) *P. pacificus*. Cells P1.p–P4.p, P9.p–P11.p die by programmed cell death (PCD) early in J1. P12.p divides to give P12.pp that also dies by PCD and P12.pa that fuses to give hyp12. P8.p does not participate in vulva formation and fuses to epidermal syncytial cell hyp7 (4° sublineage, **H** cell fate). P5.p–P7.p are the VPCs that divide in a stereotypic manner and give rise to 20 cells that will form the adult vulva. P6.p gives rise to six cells (1° sublineage); P5.p and P7.p give rise to seven cells each (2° sublineage). Cells **E** divide transversely, **D** and **F** do not divide, and all other divisions are longitudinal. Twenty cells generate the vulva in *P. pacificus* (Sommer and Sternberg, 1996b). As in *C. elegans*, the cell fates are **A**–**F** and **H**.

row alongside the ventral midline of the animal from anterior to posterior. During the L1 and early L2 stages, P(1, 2, 9–11).p fuse to hyp7 (the largest hypodermal syncytial cell). The most posterior P12.p cell divides once and P12.pa becomes part of the preanal hypodermis (hyp12; Sulston and Horvitz, 1977). Six unfused cells P(3–8).p represent a group of vulval precursor cells (VPCs) and acquire one of three possible sublineages (1°, 2°, 3°; Fig. 2A). At least four signaling pathways and numerous molecules including EGF/RTK/RAS/MAPK,

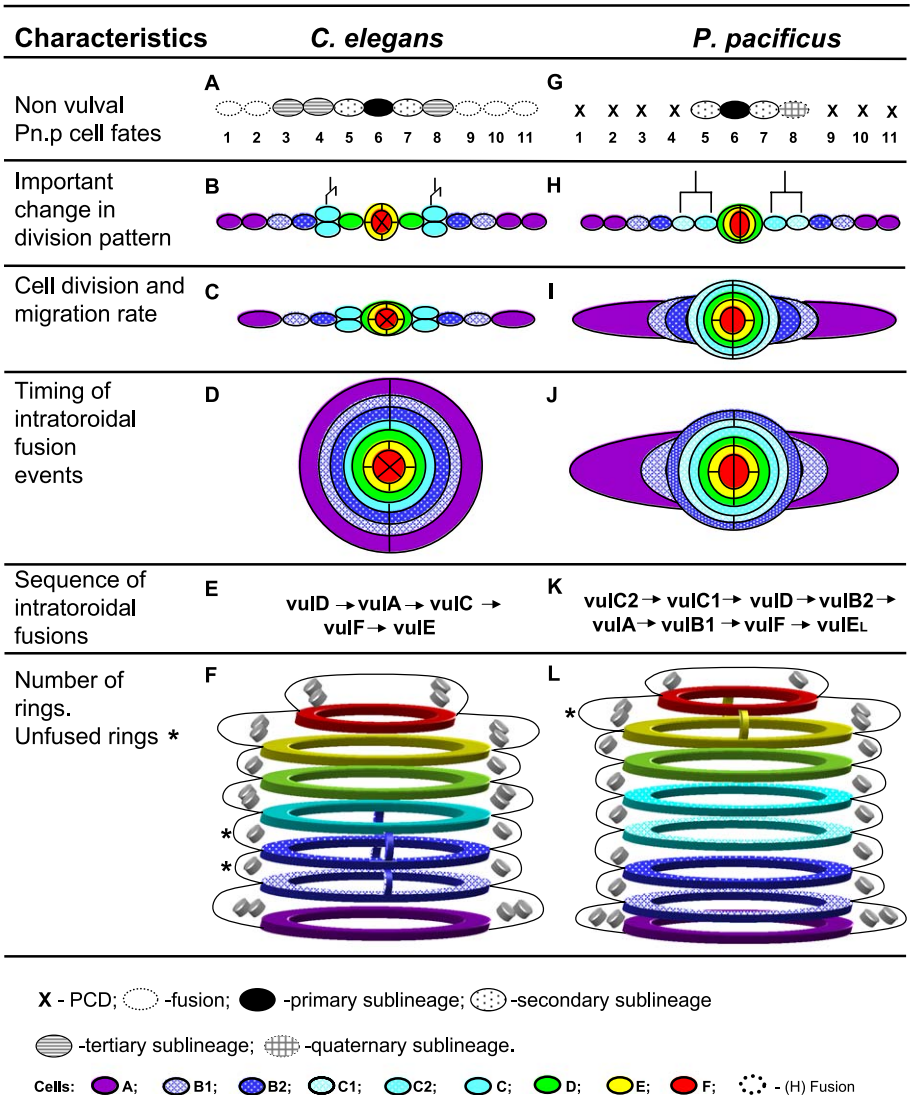

Fig. 3. Summary of the differences in vulva formation between *C. elegans* (left A–F) and *P. pacificus* (right G–L). Pictures represent lateral (F, L) or dorsal views (others) on vulva. (A) Sublineages of Pn.p cells. P(1–4, 9–11).p fuse to hyp7 during L1. The daughters of P(3,4,8).p fuse to hyp7 at mid-L3 and do not participate in vulva formation. In 50% of the wild-type animals, P3.p adopts the 4° sublineage and fuses to hyp7 at late-L2. P(5–7).p cells represent the vulva equivalence group. (B) P(5–7).p divide three times and start to migrate to the center. **C** cells divided transversely (shown by the diagram above). The central rings **vulF** and **vulE** are shown in the center. (C) The vulval cells migrate to the center and form the rings **vulF**, **vulE**, and **vulD**. **A** cells have fused pair wise in the anterior (left) and posterior (right) before migration. (D) After the migration of all vulval cells to the middle and the formation of all seven vulval rings, the process of intratoroidal fusion begins. The first event of intratoroidal fusion is **vulD** (green). (E) Sequence of vulval rings fusion. (F) Vulval structure at the end of vulva formation before eversion. All the rings are fused, except for **vulB1** and **vulB2** (marked with asterisks; Sharma-Kishore et al., 1999). The colored circles represent the apical adherens junctions of the rings, the small gray discs are the nuclei and the black lines are the basolateral cell borders. (G) Sublineages of Pn.p cells. P(1–4, 9–11).p die by PCD during embryogenesis. P8.p fuses to hyp7 during J1 and does not participate in vulva formation. The vulva equivalence group is represented by P(5–7).p. (H) P(5–7).p divide twice forming the cells **A**–**FF**–**A**. **C** cells divide longitudinally (shown by the diagram above). Rings **vulF**–**vulD** are already formed. **E** cells did not divide yet. (I) The vulval cells continue to migrate to the center to form the rings **vulC1** and **vulC2**. **E** cells divide transversally during this stage. **A** cells from both sides have fused between themselves to give rise to two binucleate cells. (J) The vulval cells are still migrating to form the ring **vulB1**. In this stage, the first intratoroidal fusion of **vulC2** takes place. (K) The sequence of vulval rings fusion is invariant. **T** in $\mathbf{vulE_T}$ indicates for longitudinal (left–right) fusion. (L) Vulval structure at the end of the process of vulva formation before eversion. All the rings are fused, except for $\mathbf{vulE_L}$ (**L** for longitudinal; marked with asterisk). The colored circles represent the apical adherens junctions of the rings, the small gray discs are the nuclei, and the black lines are the basolateral cell borders.

WNT/ARM/HOX, LIN-12/NOTCH, and LIN-15/RB/NURD participate in the induction of the different vulval and nonvulval cell fates (Gleason et al., 2002; Greenwald, 1997; Horvitz and Sternberg, 1991; Kornfeld, 1997; Solari and Ahringer, 2000; Wang and Sternberg, 2001). In wild-type animals, the gonad anchor cell (AC) induces P(5–7).p cells. P6.p adopts the 1° sublineage dividing longitudinally twice and the resulting row of four granddaughters named **EFFE** divide transversely in a final TTTT division orientation giving rise to eight vulval cells that will form the central and most dorsal rings called **vulF** and **vulE** (T—transverse, left–right orientation; Fig. 2A). P5.p and P7.p adopt the 2° sublineage by dividing twice and producing granddaughter cells named **ABCD** and **DCBA** that divide with a final LLTN and NTLL division orientation, respectively (L—longitudinal, anterior–posterior orientation; N—no division; Fig. 2A) and give rise to 14 cells that will form the toroidal/ring-shaped cells named **vulD**, **vulC**, **vulB1**, **vulB2**, and **vulA** (Sharma-Kishore et al., 1999). The 22 great-granddaughters of P(5–7).p cells form the vulva at the end of L4. The other VPCs [P(3,4,8).p] adopt 3° sublineage usually dividing once and fusing to the hypodermis (H fate) in a process dependent on the activity of *eff-1* (Mohler et al., 2002). Cell ablation experiments show that P(3,4,8).p have the potential to participate in vulva formation (Fig. 2A; Kimble, 1981; Sulston and White, 1980). In wild-type animals, P(3–8).p in the L1 and P(5–7).p in the L3 avoid fusion to hyp7 (H fate) as a result of *lin-39*/HOX repression of EFF-1-dependent cell fusion (Shemer and Podbilewicz, 2002).

Thus, vulval primordial cells divide twice and are induced to a particular cell fate that relates to the final vulval structure and function according to the invariant position and the expression of a combination of molecular markers within seven rings (Burdine et al., 1998; Chang et al., 1999; Hanna-Rose and Han, 1999; Hurd and Kemphues, 2003; Inoue et al., 2002; Pettitt et al., 1996; Sharma-Kishore et al., 1999; Shemer et al., 2000; Wang and Sternberg, 2000). From anterior to posterior, these vulval cell fates have been classified as: **ABCDEFFEDCBA** (Fig. 2A). Morphogenesis of the vulva takes place in two phases: First, the cell migration phase in which outer cells send apical extensions to the middle elevating the inner cells dorsally (Figs. 3B,C). In a similar way, a cylinder of stacked rings is formed as the next cells send extensions acquiring the ring shape (Figs. 3B–F). Two cell fusion events occur before ring formation when the daughters of **A** cells fuse longitudinally before migration (Fig. 3C) and the daughters of **C** cells fuse transversely during cell migration (Sharma-Kishore et al., 1999).

The second phase of morphogenesis is the fusion between the cells that form each ring. The sequence of the rings that fuse is invariant: **vulD**–**vulA**–**vulC**–**vulF**–**vulE**. The rings **vulB1** and **vulB2** remain unfused (Figs. 3E,F). The formation of the vulva is complete after connection to the uterus and eversion (Sharma-Kishore et al., 1999).

In *P. pacificus* as in *C. elegans*, the vulva is in the ventral midbody and is formed from the descendants of three of 12 P ectoblast cells. In contrast to that found in *C. elegans*, in *P. pacificus*, the vulva equivalence group is reduced and cells that do not participate in vulva formation undergo apoptosis rather than cell fusion (Sommer and Sternberg, 1996b). The gene *lin-39* of *P. pacificus* is responsible for the protection of the VPCs P(5–8).p from the apoptotic fate (Eizinger and Sommer, 1997). The vulva equivalence group in *P. pacificus* is composed of P(5–7).p that acquire 2°–1°–2° sublineages, respectively. In general, P8.p is incompetent to adopt vulval fates and fuses to the hypodermis (4° sublineage) during J1 stage (Fig. 2B). However, after ablation of P7.p in the first few hours after hatching, P8.p is competent to adopt a vulval fate (Sommer, 1997).

Unlike *C. elegans*, which has four juvenile stages (L1–L4), *P. pacificus* only has three juvenile stages (J1–J3; Felix et al., 1999). In contrast to *C. elegans*, the inductive signal that specifies the vulval fates in *P. pacificus* is continuously provided by the gonad, from early J1 until the birth of the AC (Sigrist and Sommer, 1999). The 1° sublineage in *P. pacificus* is expressed as: TNNT (P6.p) and the 2° sublineage as LLLN or NLLL (P5.p and P7.p, respectively). The result is that 20 cells form the vulva in *P. pacificus* (Sommer and Sternberg, 1996b; Fig. 2B).

Materials and methods

Nematode strains and culture

The strains used are *C. elegans* wild-type N2 (Brenner, 1974) and *P. pacificus* wild-type PS312 var. California (Sommer et al., 1996). All the strains were cultured at 20°C using standard procedures (Brenner, 1974; Sommer et al., 1996).

Permeabilization, fixation, and immunofluorescence staining of worms

Two different techniques were used to fix and permeabilize the worms. The Finney–Ruvkun protocol (Finney and Ruvkun, 1990) was used for *C. elegans* with paraformaldehyde (Fluka) at a final concentration of 2%. The methanol–acetone protocol (Podbilewicz, 1996; Podbilewicz and White, 1994; Sharma-Kishore et al., 1999) was used to fix and permeabilize *P. pacificus* because this species has a particularly thick cuticle. Though both Finney–Ruvkun and methanol–acetone protocols were excellent for *C. elegans* strains, *P. pacificus* fixation gave better results with methanol–acetone, or the so-called 'mixed' protocol.

The 'mixed' protocol combines both mentioned techniques. The first part is the same as in methanol–acetone. The worms are attached to a polylysine-coated glass slide, frozen on dry ice, and the coverslip is slipped off. The slides are

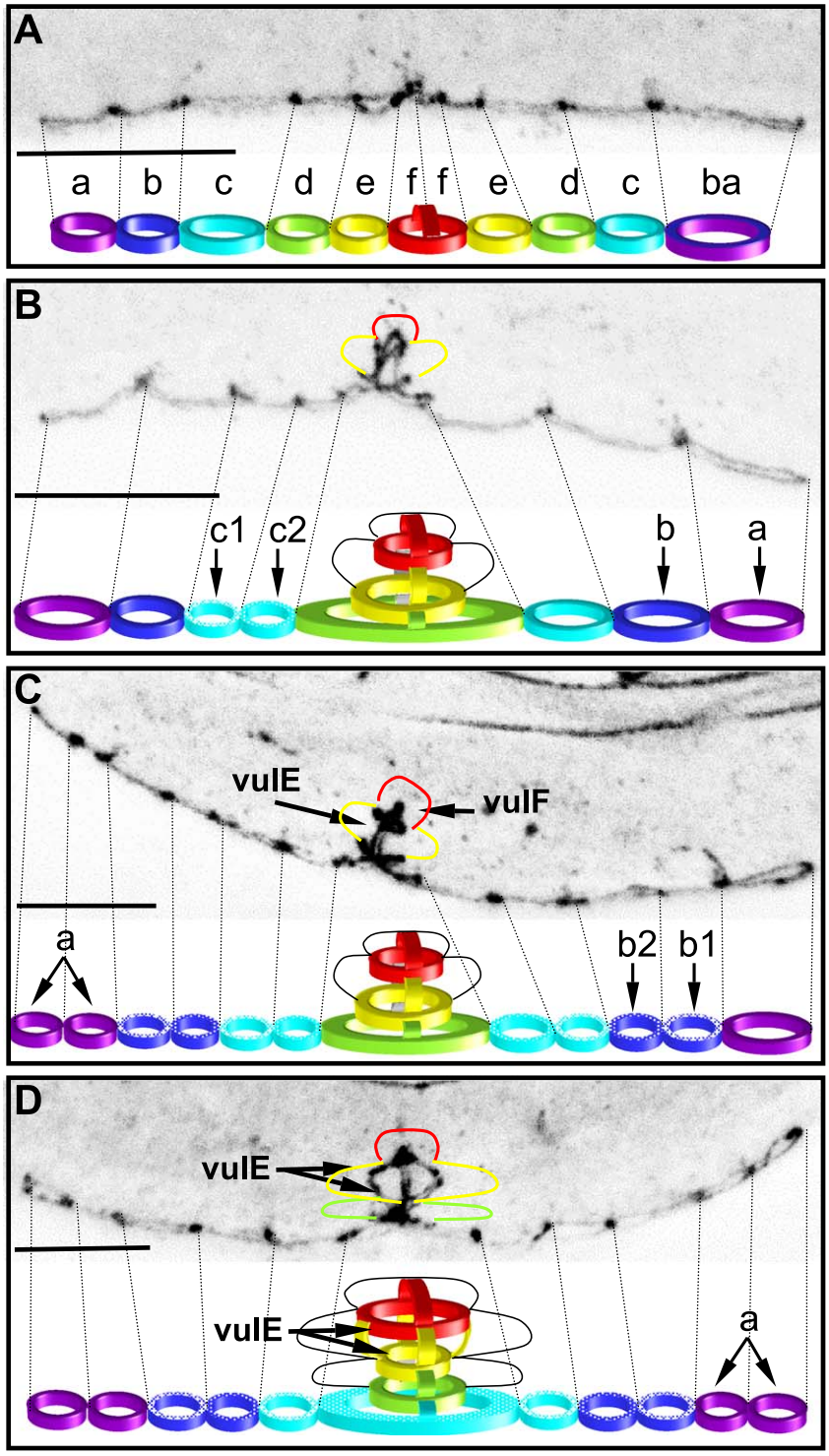

Fig. 4. *P. pacificus* vulval cells division and early ring formation. Each panel shows a negative confocal micrograph image of fluorescently labeled adherens junctions of vulval cells (mAb MH27; Francis and Waterston, 1991) and a graphical diagram describing the corresponding micrograph. Dotted lines connect between the micrographs and graphic diagrams that are drawn after the examination of the rotation projections. Colored drawings on the micrographs show the predicted shape of the rings of the same color code. Each micrograph is a projection of 5–15 confocal sections of 0.4–0.5 μm. The letters and arrows relate to the cells and their divisions. Anterior is to the left; all the micrographs are lateral views. Scale bar 10 μm. (A) Eleven-cell stage. P5.p–P7.p descendants have completed two rounds of divisions, except for the most posterior cell that is marked as **BA**. At this stage, no invagination can be seen ($n = 15$). (B) Thirteen-cell stage. Posterior **BA** cell has divided to give rise to **B** and **A** cells. Anterior **C** cell has divided to give rise to **C1** and **C2**. This micrograph clearly shows that the invagination of the vulval cells and initial ring formation (three stacked toroids) begins even before the generation of all vulval cells ($n = 6$; see rotation of this vulva in Supplemental Movie 1A). (C) Seventeen-cell stage. **B** cells from both sides and anterior **A** cell have divided to give rise to **B1**, **B2**, and two **A** cells, respectively. Three stacked rings **vulF**, **vulE**, and **vulD** can be seen ($n = 9$). (D) Twenty-cell stage. All the cells have completed their proliferation. The transverse division of **E** cells takes place when at least four rings **vulF**, **vulE**, **vulD**, and **vulC2** have formed ($n = 4$).

placed horizontally in a humidity chamber and are incubated for 5 min in methanol and then for 5 min in acetone, both at −20°C. Air-dried slides are transferred to the incubation buffers of Finney–Ruvkun protocol and all the staining part is as described in this protocol using buffers A and B at room temperature (Finney and Ruvkun, 1990).

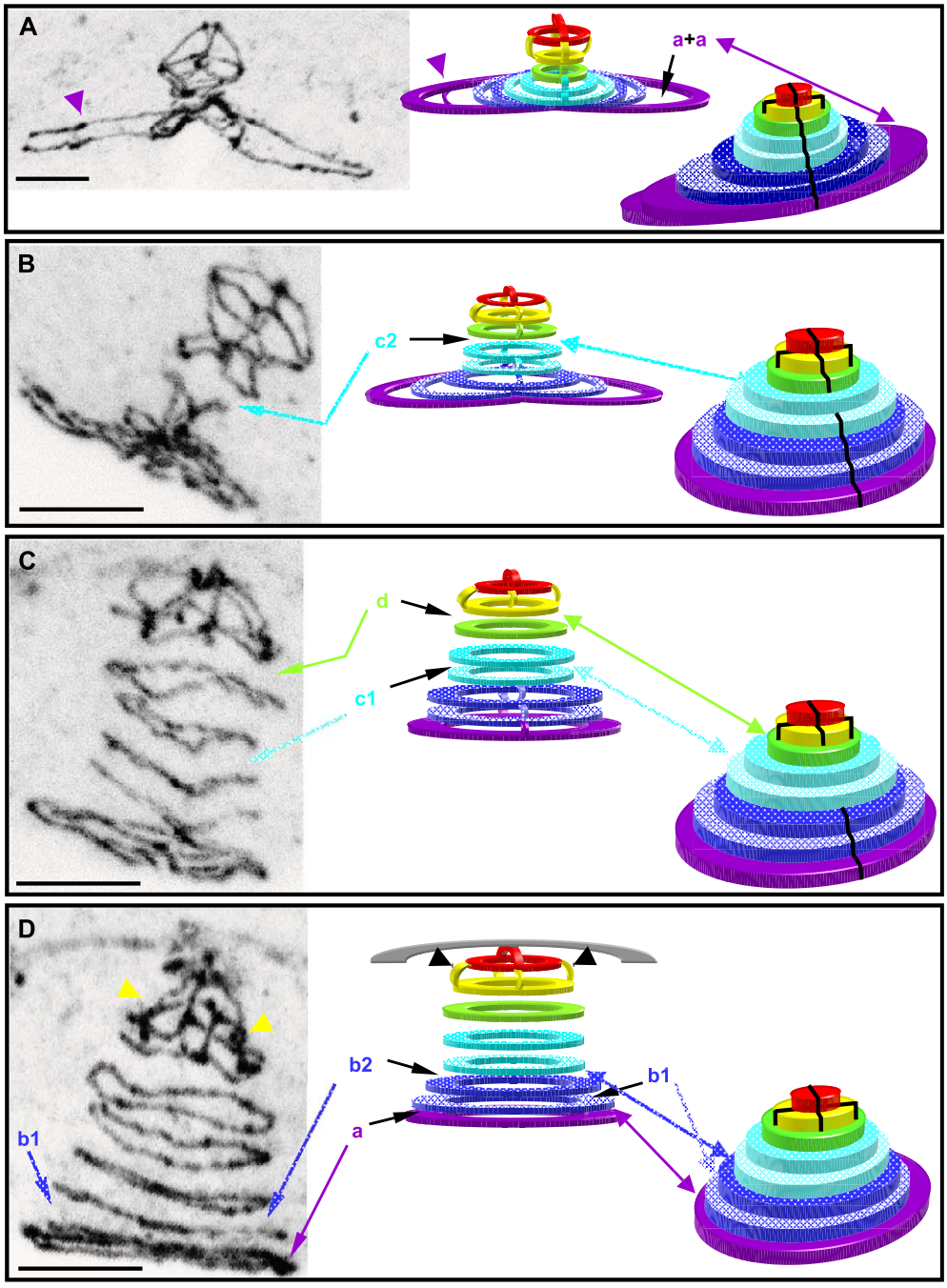

Fig. 5. Late vulval cell migrations and intratoroidal fusions in *P. pacificus*. Each panel represents a negative image of a confocal micrograph of fluorescently labeled adherens junctions of the vulval cells (mAb MH27) and a graphical interpretation of the corresponding micrograph. Graphical interpretation is divided into two parts: close graphical version of junctions as they appear on micrographs and the corresponding drawing of a representation of the entire vulva showing the stack of vulval rings (see Fig. 1 for more details). The letters and arrows relate to fused vulval cells. Arrowheads point to the unfused junctions. Each micrograph is a projection of 15–25 confocal sections of 0.4–0.5 μm. Anterior is to the left; all the micrographs represent lateral views. Scale bar 5 μm. (A) Migration of vulval cells. All 20 cells have been generated. Six rings have been completed: **vulF**–**vulB2**. First fusion event in vulva formation takes place between the two posterior **A** cells (arrow; $n = 3$); note that the anterior **A** cells have not fused (arrowhead). (B) First intratoroidal fusion. The two **C2** cells that formed the ring **vulC2** fused to give a binucleate toroid. Seven rings have been completed and **vulA** is in late stages of migration toward the center becoming the eighth most ventral toroid in the stack of rings ($n = 30$). (C) Intratoroidal fusion progression within **vulC1** and **vulD**. A stack of eight toroids is clearly forming a cylinder ($n = 7$). (D) Fusions within the **vulB2**, **vulA**, and **vulB1** rings. Only **vulE** and **vulF** are unfused; arrowheads mark the left–right adherens junctions in **vulE** ($n = 9$).

The immunofluorescence staining of the epithelial cell adherens junctions was done with the monoclonal antibody MH27 (Francis and Waterston, 1991). Nuclei were stained with propidium iodide (Podbilewicz, 1996; Podbilewicz and White, 1994; Sharma-Kishore et al., 1999). All the procedures were performed in 1.5-ml eppendorf tubes. The aliquots of worms for every tube were obtained from agar plates with unstarved worms of the desired stage (Shemer et al., 2000).

Microscopy and image analysis

The stained worms were analyzed using an MRC-1024 laser confocal scanning microscope (Bio-Rad, Hempstead, UK) with the objective Nikon Plan Apo 60X/1.40 (Sharma-Kishore et al., 1999). The projection pictures were obtained from 5 to 25 serial optical sections, collected every 0.4–0.5 μm, in the *z*-axis. For a more precise visualization of the details, tilt and rotation projections were performed using the Laser Sharp Acquisition program of Bio-Rad (see Supplemental Movies). Cell fusion was detected by the disappearance of the adherens junctions boundaries as determined by the monoclonal antibody MH27. In cases where this assay did not yield clear results, the analysis was also based on nuclear score (Fig. 1).

In our micrographs, we present the projection of all sections. Sometimes, we add the graphic interpretation that was based on the rotation movies (Figs. 4 and 5; Supplemental Movie 1). In addition to the drawings of AJs, we also present pyramidal models of all vulval cells in Fig. 5.

Results

Cellular mechanisms of vulva invagination are conserved

To study the intermediates during vulva organogenesis in *P. pacificus*, we used the monoclonal antibody MH27 to visualize the cell borders and propidium iodide for nuclei staining, as described in Materials and methods (see Fig. 1). To trace the precise sequence of events, we analyzed worms from late J2 larvae till young adult stages. We found three smoothly transitioning stages during vulva formation that had been previously observed in *C. elegans* (Figs. 2 and 3): (1) generation of vulval cells; (2) migration of vulval cells toward the middle to form a stack of rings; (3) intra-ring cell fusion. Figs. 4 and 5 show that in *P. pacificus*, but not in *C. elegans*, these stages in vulva formation are overlapping.

To study the process of vulva invagination, we monitored three-dimensional confocally reconstructed intermediates showing primordia that contain early invaginations of the ventral epidermis by analyzing the apical adherens junctions of the vulval cells and their contacts with the surrounding hypodermis. We found that concerted cell migrations result in the formation of ring structures.

Cell division, cell migration, and initial ring formation (steps 1 and 2)

The first phase of vulva formation in *P. pacificus* covers the proliferation of the vulval precursor cells (VPCs) to the conclusion of the vulval lineages overlapping with the initiation of ring (toroidal cell) formation. Three divisions of the VPCs P5.p–P7.p give rise to 20 vulval cells in *P. pacificus* (Fig. 2B; Sommer and Sternberg, 1996a). Fig. 4 shows that during the last round of division (at the 12-cell stage), vulval cells start the migration toward the center of the vulva primordium, which results in invagination. We found that concerted cell migration results in the formation of ring structures (Figs. 4B–D; see also Supplemental Movies). Ring formation follows a precise order starting from the **E** cells that are adjacent to the central **F** cells, followed by **D**, **C2**, **C1**, **B2**, **B1**, and **A** (Figs. 4 and 5). The anterior vulval cells send filopodia to the midline to meet the corresponding cells from the posterior and form a ring or toroid (Figs. 3H–J and 4B; see also Supplemental Movie 1A). As the inner cells meet and form a ring, the adjacent outer cells migrate toward the middle leading to elevation of the central **vulF** and **vulE** rings dorsally. During ring formation, some rearrangements of apical domain junctions take place. Even during early stages, it was found that in *P. pacificus*, some vulval cells are still proliferating while others are already migrating and forming an invagination (Figs. 3H and 4B–D). This is in contrast to *C. elegans* where ring formation begins only after all vulval cells (except **F**) have been generated (Fig. 3B).

To learn the sequence of the generation of vulval cells and ring formation, we followed intermediates between early J3 and young adult stages. The final divisions of the vulval cells in *P. pacificus* occur in the following order: **C**, **B**, **A**, and **E** (Figs. 4B–D). P6.paa and P6.ppp divide to give the four daughters of vulval cells **E** forming the ring **vulE**. This is the last and only transverse division during the generation of *P. pacificus* vulval cells (Sommer et al., 1996). This division is completed after the invagination of four dorsal vulval rings has already occurred (Fig. 4D). This is in contrast to *C. elegans* where no rings are formed before all the vulval stem cells (except **F**) have completed their proliferation.

Throughout the proliferation of vulval cells, some random antero-posterior asymmetry in the division timing was noticed. For example, the division of **C** cells is not simultaneous in the posterior and anterior sides (Fig. 4B; see Supplemental Movie 1A).

To investigate the timing of cell fusion with respect to the early cellular events in vulva formation, we looked for cell fusion events in vulva primordia during the last cell division cycle and the initial stages of ring formation. We found that in primordia that contained 20 cells and had formed four rings, the first part of vulva formation was completed before the beginning of intratoroidal fusions. The transition point between cell proliferation and intratoroidal fusion stages is the fusion between the daughters of **A** primordial cells (n = 3) on the anterior and posterior ends of the vulva (P5.paaa +

P5.paap and P7.pppp + P7.pppa, respectively; Fig. 2B). These pairs of cells fuse during the migration stage to give rise to two binuclear cells (Fig. 5A).

In summary, the invagination of the vulva in *P. pacificus* as in *C. elegans* involves the formation of rings derived from the descendants of three VPCs. While in *C. elegans*, organogenesis starts only after the vulval cells have been generated, in *P. pacificus* short-range cell migration, ring formation, and invagination occur during cell proliferation. In *P. pacificus*, the cell fusion events between **vulA** precursors occur during the migration of the central cells, but only after five rings have been formed and stacked, while in *C. elegans* **A** + **A** fuse before the onset of cell migration and ring formation.

Ring formation, intratoroidal fusions, and vulva shaping (steps 2 and 3)

To study the second phase of vulva formation in *P. pacificus*, we followed the apical domain rearrangements that reflect the fusion events within toroids. These intratoroidal fusions result in the formation of syncytial ring-shaped cells that form the final cylindrical vulval structure. Since the course of invagination and fusion events in *C. elegans* is known from previous studies (Sharma-Kishore et al., 1999), we were interested in the characterization of this process in *P. pacificus*.

We found that intratoroidal fusions in *P. pacificus* start before all cells have reached the center and formed an eight-ring cylindrical structure. In *P. pacificus*, **vulC2** is the first ring that undergoes longitudinal intratoroidal fusion (Figs. 3J, 5B, and 6A) (n = 30) when **A**–**C1** cells have not fully migrated to the middle. Late intermediates can be seen only after all vulval cells have finished their migration.

vulC1 is the next ring that fuses longitudinally (n = 14) (see the complete pathway in Fig. 6). It is followed by **vulD** longitudinal fusion (n = 7). The next fusion events are: **vulB2** (n = 6), **vulA** (n = 6), **vulB1** (n =11), and **vulF** (n = 4), which show longitudinal fusion. **vulE$_T$** (T for transverse; n = 5) is the last fusion event in *P. pacificus* vulva formation and **vulE$_L$** (L for longitudinal) is the only junction that remains unfused. In this way, a stack of eight toroids is generated (Fig. 3L). As in *C. elegans*, the anchor cell (**AC**) fuses to a large uterine syncytium (**utse**) to allow the connection between the vulva and the uterus necessary for egg-laying through the vulval tube. While in *C. elegans*, the **AC** fuses to the **utse** between the intratoroidal fusions of **vulA** and **vulC** (Sharma-Kishore et al., 1999), in *P. pacificus* this equivalent heterotypic fusion occurs sometime between **vulB1** and **vulF** intratoroidal fusion events.

Thus, although the general process of ring formation is similar in *C. elegans* and *P. pacificus*, there are differences

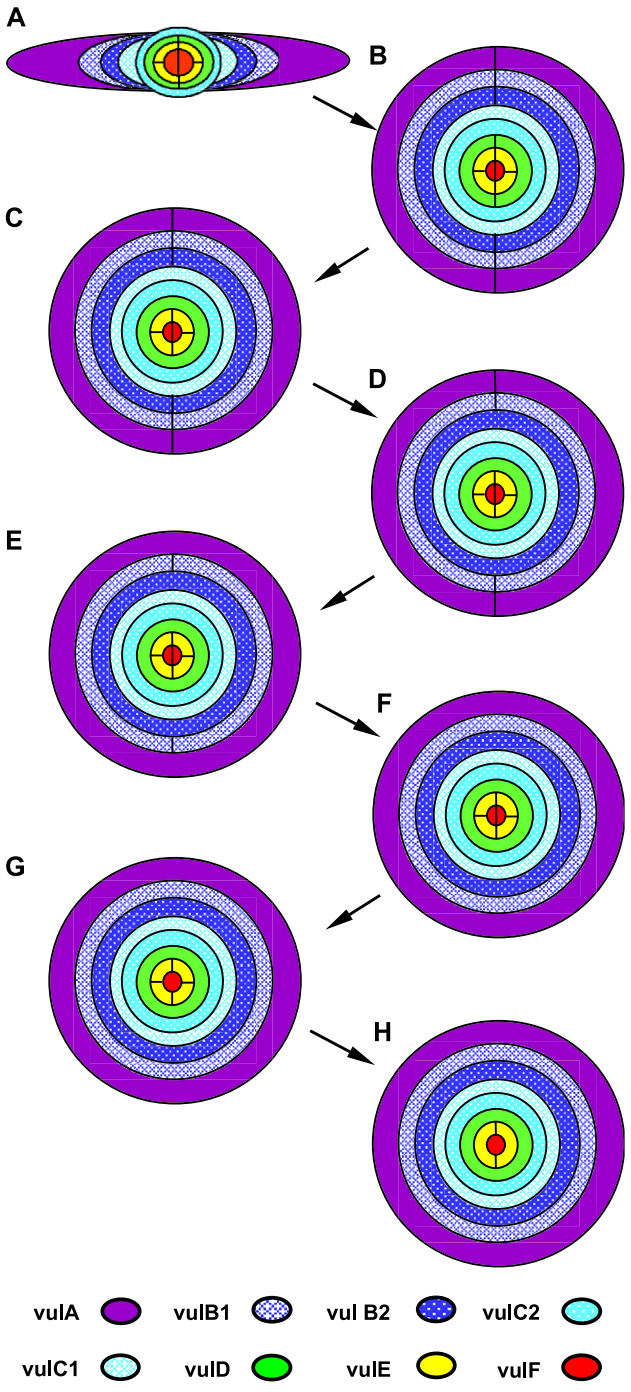

Fig. 6. Summary of temporal sequence of intratoroidal fusions during *P. pacificus* vulva formation. The colored concentric circles show different rings according to the color code. Horizontal and vertical bars within circles represent unfused junctions between cells of one ring and they disappear when cells fuse. The order of fusions is: (A) **vulC2** fusion (n = 30). This is the first intratoroidal fusion during vulva formation in *P. pacificus* that occurs while the migration of the more ventral rings toward the middle is not completed. (B) **vulC1** fusion (n = 14). (C) **vulD** fusion (n = 7). (D) **vulB2** fusion (n = 6). (E) **vulA** fusion (n = 6). The two halves of the **vulA** ring are binuclear and the origin is in earlier intercellular fusion of the **A** cells. (F) **vulB1** fusion (n = 11). (G) **vulF** fusion (n = 4). During this fusion or later, the connection of the vulva to the uterus occurs, though the event was not specifically scored. (H) **vulE$_T$** fusion (n = 5). This is the last fusion event in vulva morphogenesis. Both **vulE$_L$** junctions are the only ones that appear unfused even in the adults. **vulA** is a tetranuclear toroid, while all the other rings are binuclear. Figures are oriented with the anterior of the worm on the left-hand side of the page, and the projections are dorsal views.

between vulva organogenesis in these species (summarized in Fig. 3). In *C. elegans*, there are no intratoroidal fusions before the end of the cell migration toward the center. The sequence and the number of intratoroidal fusions are different in these two species, and the number of toroids is eight in *P. pacificus* vs. seven in *C. elegans*. The additional eighth ring in *P. pacificus* is a direct consequence of a change in the division axes of two equivalent vulval **C** cells from transverse (left–right) division in *C. elegans* to longitudinal (anterior–posterior) cleavage in *P. pacificus*. Furthermore, the unfused rings are also different: **vulB1** and **vulB2** in *C. elegans* vs. $\mathbf{vulE_L}$ in *P. pacificus*.

The last stage of vulva formation is the eversion of the vulva during the last larval molt, which takes place after the connection of the vulva to the uterus. This last event is followed by the initiation of egg laying in the mature animal.

In summary, 20 cells form the vulva in the wild-type hermaphrodite in *P. pacificus*. As in *C. elegans*, three VPCs divide, migrate, and form rings and later on fuse in an invariant pathway. Unlike *C. elegans*, in *P. pacificus*, all these events overlap. Finally, in *P. pacificus*, eight syncytial toroidal cells form a cylindrical structure where only one ring does not undergo complete intratoroidal fusion and remains composed of two binucleate cells. The vulva in *C. elegans* is composed of seven rings and two of them remain unfused (Fig. 3). An additional structural rearrangement during eversion finishes the formation of the functional vulva in both species.

Discussion

In this study, we show that a novel cellular mechanism of invagination initially described for *C. elegans* is conserved during evolution. *P. pacificus* is gradually becoming an integral satellite model organism to *C. elegans.* There is a great deal of information concerning vulval lineages, induction pathways, and HOX-mediated body plan determinations in this nematode species (Eizinger and Sommer, 1997; Grandien and Sommer, 2001; Gutierrez et al., 2003; Jungblut and Sommer, 1998; Sommer and Sternberg, 1994, 1996b; Sommer et al., 1998) (I. Kolotuev, unpublished). Here we introduce additional criteria for the comparison between these two organisms: the cellular events during invagination of the vulva.

The cellular mechanism of invagination is conserved in evolution

We hypothesized that the main events of organ formation may be conserved for two representatives of the same phylum. We found that vulva formation in *P. pacificus* involves a plan very similar to *C. elegans*. In both species, the vulval cells migrate to the center, the outer vulval cells send apical extensions pushing the inner cells dorsally. In this way, a hollow tube composed of syncytial rings is formed, connecting the uterus to the outside, which is essential for egg laying and mating.

Heterochrony in cellular events during vulval invagination

We found differences in vulva formation between the two species studied. The main alteration is heterochrony in morphogenetic processes (McKinney and McNamara, 1991). The rates and timing of cell division, cell migration, and intratoroidal fusion in *P. pacificus* are different than in *C. elegans*. While in *P. pacificus*, these processes occur simultaneously, in *C. elegans* they are temporally distinct (compare Figs. 3B and C with H and I). In *C. elegans*, the events of cell proliferation, cell migration, and intratoroidal cell fusion occur in a precise sequence. For example, in *C. elegans*, all vulval cells (except **F**) divide before the beginning of cell migration. In *P. pacificus*, some vulval cells are still dividing at the same time as other cells are migrating to the center. The migration of the cells to the center and the sequence of intratoroidal fusions show a similar picture: In *P. pacificus*, the outer cells are still migrating, while some inner rings are already formed and have even undergone cell fusion. The process of intratoroidal cell fusion in *C. elegans* begins only after the end of vulval cells migration to the center. Thus, in *C. elegans*, there is a temporal sequence that divides vulva development into three phases: first, cell proliferation followed by short range-migrations; second, cell fusions within the vulval rings; and third, eversion (the vulva turns inside out). In *P. pacificus*, ring formation by cell migration overlaps with cell proliferation and intratoroidal cell fusion.

Heterochrony is a common alteration in the evolution of development. Mutations in heterochronic genes in *C. elegans* result in changes in the timing of developmental events (Ambros and Moss, 1994; Slack and Ruvkun, 1997). Precocious or retarded cell division cycles, cell migration, and cell fusion are some of the cellular events affected in heterochronic mutants (Euling and Ambros, 1996; Newman et al., 2000; Rougvie and Ambros, 1995). It is conceivable that evolutionary changes in heterochronic gene activities may have resulted in the observed differences in the cellular events between *C. elegans* and *P. pacificus*.

A single change in cell division axis can reduce the number of rings

The total number of vulval rings and the sequence in which the cells that form each ring fuse are different in *P. pacificus* and *C. elegans* (compare Figs. 3E and F with K and L). The initial number of vulval cells in *P. pacificus* is 20 while in *C. elegans* it is 22; the final number of rings is eight and seven, respectively. The additional ring in the *P. pacificus* vulva results from one change in the division axis of specific vulval cells. In *P. pacificus*, the **C** cell divides in anterior–posterior manner (A–P; longitudinally); in *C. elegans*, the equivalent **C** cell divides left–right (L–R; transversely) (Figs. 3B,H). As a result of the change in division axis

without cell fusion in *P. pacificus*, two rings are generated: **vulC1** and **vulC2** (Fig. 3). Thus, specific cellular changes in the orientation of a cell division lead to a modification in organ structure. In vulva formation, these alterations result in either the addition or reduction of a ring. In all cases where the division is transverse, a single ring forms, whereas two rings may form in the case of longitudinal division.

At the genetic level, the modification could be explained by changes in the activities of at least two genes. First, a change in a *par* gene may cause an orthogonal change in the position of the centrosomes and thus a change in the position of the spindle apparatus resulting in a 90° rotation and a different division axis (Kemphues, 2000). This first genetic change may be followed by a change in the activity of a fusogen that in *C. elegans* causes the fusion between the two halves of **vulC**, while in *P. pacificus* lack of this activity may result in the formation of two independent rings **vulC1** and **vulC2**. It has recently been shown that *par-1* is active in morphogenesis of vulval cells (Hurd and Kemphues, 2003). In addition, *eff-1*, a candidate cell-to-cell fusion gene, is essential for intratoroidal vulval cell fusion including **vulC** in *C. elegans* (Mohler et al., 2002; Shemer, 2002; Shemer and Podbilewicz, 2002, 2003). Thus, developmental differences in the cellular activities of polarity and partition *par* genes and cell fusion genes such as the *eff-1* gene may account for evolutionary changes in the number of rings and shape of the vulva.

Evolution of vulva formation

Under laboratory conditions, the cellular and morphological changes between the vulvae of *C. elegans* and *P. pacificus* do not seem to influence their function. Thus, these modifications can be explained as neutral changes in organ formation.

We have recently studied the general outline of vulva formation in the nematode satellite species—*Oscheius* sp. 1 CEW1 (Louvet-Vallee et al., 2003). The vulva in *Oscheius* sp. 1 CEW1 is derived from P5.p, P6.p, and P7.p that acquire 2°–1°–2° sublineages and divide in the following manner: NNNN–TTTT–NNNN (Dichtel et al., 2001). We found that the 16 vulval cells in *Oscheius* sp. 1 CEW1 generate six rings. As in *C. elegans*, the cell divisions are completed before the stage of short-range migrations (Louvet-Vallee et al., 2003). Thus, based on the characterization of vulva formation in three species, we can now predict how the orientation of cell divisions at the lineage level and the presence or absence of cell fusion can cause alterations in the final number of vulval rings in nematodes with central vulva. For example, cells from the same origin that divide transversely usually result in one ring and cells that divide longitudinally have the potential to form two separate rings. This prediction has been confirmed by all rings in three species except for the outermost vulval cell longitudinal division (**A** cell in *C. elegans* and *P. pacificus*) that most likely results in intercellular fusion and generation of large binucleate cells. This observation, together with lineage studies, show that the number of vulval rings in different nematode species may vary at least from six to eight.

To get a broad perspective of the process of vulva evolution, we will need to perform a wide-range characterization of vulva morphogenesis in additional species. In nematodes, the vulva can be located either in the middle of the body—central type (*C. elegans*, *P. pacificus*), or close to the anus—posterior type (*Panagrellus redivivus*, *Cruznema tripartitum*). Interestingly, posterior vulva type in the studied species is generated from the same set of cells as central vulvae. The difference is that after the generation of VPCs, the cells migrate from their place of origin in the middle of the body to their posterior location (Sommer et al., 1994; Sternberg and Horvitz, 1982). Using comparative developmental studies, we will ask how this early cell migration of vulval stem cells influences the later process of vulva morphogenesis. The precise characterization of the process of vulva morphogenesis in *P. pacificus*, *C. elegans*, and *Oscheius* sp. 1 CEW1 will allow molecular genetic analyses of the evolution of epithelial invagination and tube formation.

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