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sem-4/spalt and *egl-17*/FGF have a conserved role in sex myoblast specification and migration in *P. pacificus* and *C. elegans*

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Abstract

Evolutionary comparisons between *Caenorhabditis elegans* and the satellite organism *Pristionchus pacificus* revealed major differences in the regulation of nematode vulva development. For example, Wnt signaling is part of a negative signaling system that prevents vulva formation in *P. pacificus*, whereas it plays a positive role in *C. elegans*. We wondered if the genetic control of the second major part of the nematode egg-laying system, the sex muscles, has diverged similarly between *P. pacificus* and *C. elegans*. The sex muscles derive from the mesoblast M, which has an identical lineage in both species. Here, we describe a large-scale mutagenesis screen for mutations that disrupt the M lineage and the sex myoblast (SM) sublineage. We isolated and characterized mutations that result in a failure of proper SM fate specification and SM migration and showed that the corresponding genes encode *Ppa-sem-4* and *Ppa-egl-17*, respectively. *Ppa-sem-4* mutants have additional defects in the specification of the vulva precursor cells P(5, 7).p and experimental studies in the *Ppa-egl-17* mutant background indicate a complex set of gonad-dependent and gonad-independent mechanisms required for SM migration. Mutations in *Cel-sem-4* and *Cel-egl-17* cause similar defects. Thus, the molecular mechanisms of SM cell specification and migration are conserved between *P. pacificus* and *C. elegans*.

Keywords: *Pristionchus pacificus*; Sex muscles; *sem-4*; *egl-17*/FGF; *Caenorhabditis elegans*

Introduction

Evolutionary developmental biology tries to elucidate the origin of developmental novelty by comparing developmental processes in related organisms. Nematodes are particularly suited for comparative developmental studies because the model organism *Caenorhabditis elegans* belongs to this phylum and provides a molecular paradigm. In addition, many nematode species can be cultured in the laboratory and developmental processes can be studied with single cell resolution (Sommer, 2001). One particular species, *Pristionchus pacificus*, has been developed as a satellite system in evolutionary developmental biology (Sommer, 2001, 2005; Sommer and Sternberg, 1996a, b). *P. pacificus* propagates as self-fertilizing hermaphrodite, has a 4-day life cycle (20°) and is amenable to forward and reverse genetic analysis. An integrated genome map of *P. pacificus* contains a genetic linkage map of more than 400 molecular markers and a physical map of nearly 10,000 fingerprinted BAC clones (Srinivasan et al., 2002, 2003). A whole-genome sequencing project is currently ongoing (http://www.nhgri.nih.gov/12511858). *P. pacificus* and *C. elegans* shared a last common ancestor about 200–300 million years ago (Pires-daSilva and Sommer, 2004).

The developmental process that has so far been studied in greatest detail in *P. pacificus* is the formation of the vulva, which is part of the egg-laying apparatus of nematodes. The vulva is a derivative of the ventral epidermis, which consists of 12 precursor cells, called P(1–12).p, in all studied nematodes, including *P. pacificus* and *C. elegans*. Vulva formation in *P. pacificus* differs in various aspects from the corresponding process in *C. elegans* (Sommer and Sternberg, 1996a). For example, the fate of the non-vulval cells changed during nematode evolution. Non-vulval cells in *P. pacificus*, P(1–4, 9–11).p, die of programmed cell death, whereas in *C. elegans*, the corresponding cells, P(1,2,9–11).p, have an epidermal fate and fuse with the

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hypodermal syncytium *hyp7*. The vulva is formed by the progeny of P(5–7).p, which adopt a 2°–1°–2° pattern in both, *P. pacificus* and *C. elegans* (Figs. 1A and dB). However, vulva induction is a continuous process in *P. pacificus* that requires several cells of the somatic gonad and occurs over more than 10 h of larval development (Fig. 1B) (Sigrist and Sommer, 1999). In *C. elegans*, vulva induction by the AC results from a short-time interaction with the VPCs (Fig. 1A) (Kimble, 1981). Finally, a negative signal in *P. pacificus* counteracts vulva induction and prevents inappropriate vulva differentiation. We have previously shown that negative signaling involves a Wnt pathway (Zheng et al., 2005). For example, mutations in *Ppa-lin-17*/Frizzled and

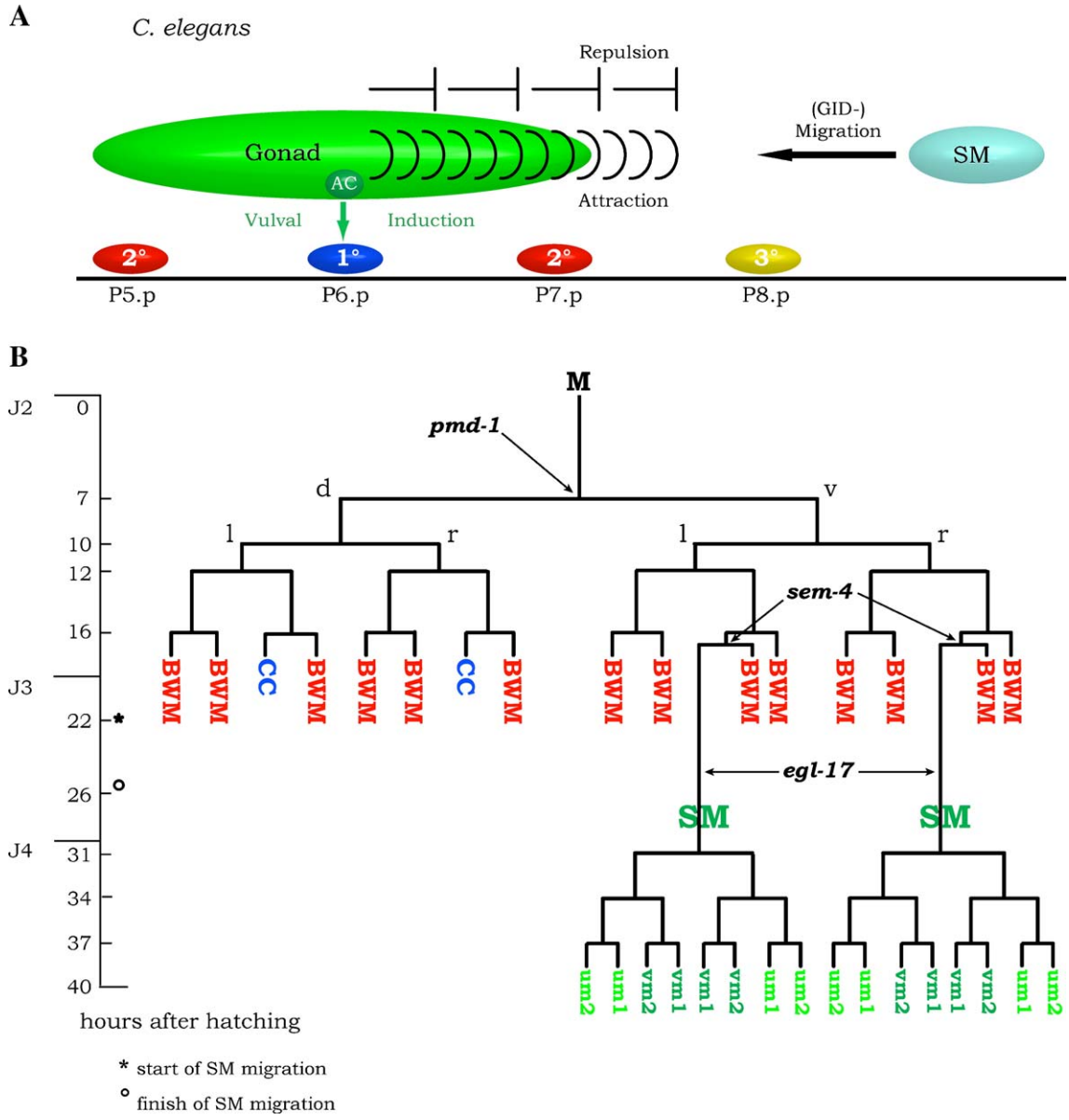


Fig. 1. Architecture of the nematode egg-laying system in *C. elegans* and *P. pacificus* and M cell lineage and misspecification in *P. pacificus* mutants. (A) The *C. elegans* egg-laying system consists of the gonad (green), the sex-myoblast (SM, light blue), and the epidermal vulva. The vulva is formed by the progeny of P(5–7).p. P(5,7).p (red ovals) have a 2° fate, generate seven progeny each and form the outer part of the vulva. P6.p (dark blue oval) has the 1° fate, generates eight progeny and forms the central part of the structure. Vulva induction requires a signal from the gonadal anchor cell (AC) (green arrow from to P6.p). P8.p (yellow oval) remains epidermal and has a 3° fate. The two SM cells migrate during the J3 stage towards the central body region and generate eight vulval and uterine muscles, respectively. SM cell migration depends on a complex set of interactions. A gonad-dependent attraction propels the SM cells towards the anterior. Detailed mutant analysis indicated that a gonad-independent (GID) attraction and a gonad-dependent repulsion contribute to SM cell positioning. In *P. pacificus*, the egg-laying system consists of the same three major components, gonad, vulva, and sex muscles. (B) M cell lineage in *P. pacificus*. The M cell divides during postembryonic development and gives rise to 14 body wall muscles (bm), two coelomocytes (cc) and two sex myoblasts (SM). The two SM cells give rise to 16 progeny, the uterine muscles (um) and vulval muscles (vm), respectively. Cells in the M lineage divide along the anterior–posterior axis, except for the first two divisions. The first division occurs along the dorsoventral axis and the second one along the left–right axis. The arrows point to the defects in the *pmd* mutants. In *Ppa-mab-5* and *pmd-1*, the lineage orientation and division properties of the M cell are affected. *pmd-2* affects SM cell fate specification and *pmd-3* SM cell migration. Dorsal (d), ventral (v), left (l), right (r) descendants in the M lineage. Cell division, which are not specifically labeled occur along the anterior–posterior axis.

morpholino knockdowns of *Ppa-bar-1*/β-catenin result in gonad-independent vulva differentiation and a multivulva phenotype (Zheng et al., 2005). In *C. elegans* in contrast, the AC releases the EGF-like molecule LIN-3, a signal that is transduced within the vulval precusor cells (VPCs) by an EGFR/RAS/MAPK pathway (Sternberg, 2005). *lin-12*/Notch signaling and Wnt signaling have been shown to function with EGF during vulval cell fate specification and mutations in *Cel-bar-1*/β-catenin result in a hypoinduced vulva phenotype (Eisenmann et al., 1998; Greenwald, 2005). Thus, the genetic control of vulva formation differs between nematode species and Wnt signaling has opposite functions in *P. pacificus* and *C. elegans*.

We wanted to know if these fundamental differences in the genetic and molecular control of vulva development are specific to the vulva, or if other parts of the egg-laying apparatus show similar alterations between *P. pacificus* and *C. elegans*. In general, the nematode egg-laying system consists of multiple cells and tissues that communicate by numerous cell–cell interactions to form a functional unit (Fig. 1A). Besides the epidermal vulva and different sets of neurons, the gonad and the sex muscles are essential for egg-laying activity. In *C. elegans*, various cell–cell interactions between groups of cells of the egg-laying system have been described, and several genetically identified components have been molecularly characterized. In particular, intensive studies over more than two decades revealed detailed insight into the development of the gonad (Kimble, 2005) and the migration of the sex myoblasts (Chen and Stern, 1998).

The *C. elegans* gonad consists of a four-cell primordium at hatching and develops into a two-armed structure during postembryonic development (Kimble and Hirsch, 1979). The *C. elegans* gonad is required for the proper positioning of the sex muscle system in the central body region. All postembryonic muscles cells are generated from a single mesoblast, the M cell (Sulston and Horvitz, 1977). The M cell is born midway between the future vulva in the central region and the rectum. During postembryonic development, the M cell gives rise to a total of 32 progeny, 14 body wall muscles, two coelomocytes, and two sex myoblasts (SM), the latter of which will generate eight progeny each (Fig. 1B). The two SM cells, one on each side of the animal, are also born midway between the developing gonad and the rectum (Fig. 1A). They both migrate towards the central body region before dividing into the 16 uterine and vulval muscles (Stern and Horvitz, 1991; Sulston and Horvitz, 1977). Genetic and experimental studies in *C. elegans* revealed a complex set of influences on SM migration (Fig. 1A) (Stern and Horvitz, 1991). Together, a gonad-dependent attraction, a gonad-dependent repulsion, and a gonad-independent attraction are involved in propelling the SM cells towards the future vulva (Fig. 1A) (Chen and Stern, 1998). In gonad ablated *C. elegans* hermaphrodites, the SM cells migrate anteriorly but are not precisely positioned indicating the existence of a gonad-dependent and a gonad-independent attraction. *Cel*-EGL-17 was shown to act as the gonad-dependent attractant and multiple cells of the somatic gonad express *Cel*-EGL-17 (Branda and Stern, 2000), recruiting the SM cells that respond using an FGF-receptor encoded by the gene *egl-15* (DeVore et al., 1995). However, SM cells remain in the posterior body region in *Cel-egl-17*, whereas they migrate anteriorly in gonad-ablated wild-type animals. If the gonad is ablated in *Cel-egl-17* mutants, the SM cells migrate anteriorly similar as in gonad ablated wild-type animals indicating that a gonad-dependent repulsion underlies the gonad-dependent attraction (Chen and Stern, 1998).

Previous studies have shown substantial differences in the regulation of the early development of the M lineage in *P. pacificus* (Jungblut and Sommer, 2001). Although the M cell lineage is identical between *C. elegans* and *P. pacificus*, that is, the final number of progeny and cell types is similar, the genetic analysis of the *Antennapedia*-like Hox gene *Ppa-mab-5* indicated an essential role of this gene in *P. pacificus* M cell development (Fig. 1B). In *Ppa-mab-5* mutants, the M cell itself and its two progeny divide along the anteroposterior axis resulting in four intermediate precursor cells, all of which are located in the same quadrant of the animal (Fig. 1B). In contrast in wild-type animals, the M cell divides dorsoventrally, and the two progeny divide along the left–right axis resulting in four progeny that occupy similar positions of the four quadrants of the animal (Fig. 1B). As a result of the misspecification in *Ppa-mab-5* mutant animals, the subsequent development of the postembryonic muscles is severely disrupted and animals form a tumor of unspecified cells (Jungblut and Sommer, 2000). These unspecified mesodermal cells cause an ectopic vulva differentiation of the epidermal cell P8.p (Jungblut and Sommer, 2000, 2001). The phenotype of *Ppa-mab-5* is much stronger than the phenotype of *Cel-mab-5* in the M cell lineage. Early aspects of M cell development are mostly normal in *Cel-mab-5* mutations (Harfe et al., 1998; Kenyon, 1986; Liu and Fire, 2000). Interestingly, *Cel-twist* mutants have an M cell lineage defect that resembles in large parts the defects seen in *Ppa-mab-5* (Corsi et al., 2000).

To obtain insight into the genetic and molecular control of SM cell development in *P. pacificus*, we initiated a large-scale mutagenesis screen for M and SM cell-defective mutants. Surprisingly, these studies revealed striking similarities in the regulation of sex myoblasts. We describe the genetic and molecular characterization of mutations in *Ppa-sem-4* and *Ppa-egl-17*. Mutations in *Ppa-sem-4* result in SM cell specification and vulva defects. Mutations in *Ppa-egl-17* prevent the proper migration of the SM cells towards the developing vulva, and cell ablation experiments in the *Ppa-egl-17* background indicate that a set of cell–cell interactions similar to the ones described in *C. elegans* regulate SM migration in *P. pacificus*. Thus, in contrast to vulva formation and early aspects of M cell lineage specification, the genetic control of SM cell fate specification and migration are conserved between *P. pacificus* and *C. elegans*.

Materials and methods

Nematode strains and cultures

Worms were grown on 5 cm NG agar plates seeded with OP50, a uracil requiring mutant of *E. coli* (Sommer and Sternberg, 1996b). Worms for daily use were grown at 20°C, and cultures were maintained by transferring several hermaphrodites. The following strains were used in this study: *P. pacificus* PS312 (the wild-type strain) is a derivative of an isolate from Pasadena, California; *P.*

pacificus PS1843, isolated from Port Angeles, Washington (Srinivasan et al., 2001).

Cell ablation experiments

Animals were picked into M9 buffer placed on a pad of 5% agar in water containing 10 mM sodium azide as anaesthetic. All ablation experiments were carried out 0–1 h after hatching of the larvae (20°C) and were carried out as described elsewhere (Jungblut and Sommer, 2000).

Mutagenesis

Mixed stage animals were washed off the plates in M9 buffer and ethyl methanesulphonate (EMS) added to a final concentration of 50 mM for 4h at 20°C. The suspension was washed in M9 five times, and the worms were spotted onto the surface of NG plates. After 1 h, excess liquid had been absorbed, and individual motile J4 hermaphrodites were picked individually to plates. In the F2 generation, egg-laying defective mutants were isolated, and their progeny were reanalyzed for vulva defects using Nomarski microscopy. Mutant hermaphrodites were backcrossed multiple times using wild-type males. Complementation tests were carried out using morphological markers (Kenning et al., 2004).

PCR cloning of Ppa-sem-4 and mutant sequencing

To clone the 5′and 3′region of *Ppa-sem-4*, we used RACE (*R*apid *A*mplification of *c*DNA *E*nds) experiments (Frohman, 1994; Ranasinghe and Hobbs, 1998). Full-length cDNA clones and genomic fragments were sequenced using the following PCR primers for sequence analysis:

AG 5813 5′-AGCACTTCTCCTCGTCGTC,
AG 5814 5′-CTCTCCAGATCCACATGC,
AG 5815 5′-TTCAGATTGCCGCGAGTG,
AG 5816 5′-ACACTTGAACGGCTTGTC,
AG 5911 5′-TTGAGATGACATCACTGTCG,
AG 5912 5′-ACTGTCGTCTCGTCGTAAAC,
AF 6755 5′-ATCTTCCGTTGGTGCAGAGA,
AF 6756 5′-GTGAACGCTCGTTGACAGAT,
AF 6757 5′-ACGGTTCCTCAACTGCAATC,
AF 6877 5′-TGAAAAGAGACGCCGGTTAC,
AF 6878 5′-TACGGTAGCAAGCAGGGAAG,
AF 7440 5′-ATCTGTCAACGAGCGTTCAC,
AF 7441 5′-GATTGCAGTTGAGGAACCGT

Mutant alleles were analyzed by PCR: DNA was isolated from three independent batches of animals, amplified by independent PCR reactions and sequenced. PCR reactions typically consisted of 10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl2, 200 mM dNTP, 1 U *Taq* polymerase, and 1 μM of each primer. Thermocycling was done in a Perkin Elmer (Norwalk, CT) Gene Amp 9700 PCR machine under conditions consisting of an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final incubation at 72°C for 7 min. The GenBank accession number for the *Ppa-sem-4* sequence is DQ270213.

PCR cloning of Ppa-egl-17 and mutant sequencing

To clone *Ppa-egl-17* and to carry out mutant sequence analysis, we used similar experiments as described above for *Ppa-sem-4*. The following PCR primers were used for sequence analysis:

AF 9221 5′-AGACTGCAACATGCACTGACAAAG,
AF 9222 5′-CTTTGTCAGTGCATGTTGCAGTCT,
AF 9223 5′-GACAAAGAGATATTTGTGTATG,
AF 9224 5′-CATACACAAATATCTCTTTGTC,
AF 9361 5′-GTCGAGTACAAAGTGAAATACAACC,
AF 9362 5′-CTTCAAGCGGAATTTATTGATGTCG,
AF 10346 5′-ATCACGCGAAAATAACTTTGCTTTAACC

The GenBank accession number for the *Ppa-egl-17* sequence is DQ270212.

Mapping and SSCP detection

For mapping mutant hermaphrodites in the California background were crossed with males of the Washington strain. To extract genomic DNA, individual F3 or F4 mutant animals were picked to single tubes containing 2.5 μl of lysis buffer (50 mM KCl; 10 mM Tris–HCl pH 8.3; 2.5 mM $MgCl_2$; 0.45% NP-40; 0.45% Tween; 0.01% gelatin; 5 μg/ml Proteinase K) and incubated for 1 h at 65°C, followed by inactivation of the Proteinase K at 95°C for 10 min. To assign linkage of a mutation to a certain chromosome, two representative SSCP markers per chromosome were tested against 21 Washington-backcrossed mutant animals. We used the following SSCP markers for chromosome assignment: chromosome I: S28, S31; chromosome II: S38, S77; chromosome III: S52, S55; chromosome IV: S34, S148; chromosome V: S106, S142 and chromosome X: S105, S140. For SSCP detection, PCR samples were diluted 1:1 in denaturing solution (95% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue), denatured at 95°C for 5 min, and loaded onto a GeneGel Excel prepoured 6% acrylamide gel (PharmaciaBiotech, Piscataway, NJ). Gels were fixed and silver stained to detect the DNA.

Phalloidin staining

The phalloidin staining was performed as described (Sasson and Stern, 2004). In short, young adults were washed three times in M9 and fixed in 3.7% formaldehyde in 0.1 M Na_2HPO_4 for 3 h with gentle rocking. Animals were washed three times in PBS, dehydrated in ice-cold acetone for 2 min, and washed again three times in PBS. Packed worms (30 μl) were then stained with Alexa488-conjugated phalloidin (Molecular Probes) at 1:50 dilution for 3 hours at 20°C in the dark. Worms were repeatedly washed for 30 min in PBS.

Photomicroscopy

Confocal images were taken using a Zeiss Axioplan 2. Images were processed using MetaMorph software (universal imaging corporation provided by Visitron System GmbH).

Results

The Pristionchus vulval muscle fiber arrangement differs from C. elegans

The M cell lineage in *P. pacificus* hermaphrodites is identical to the corresponding lineage in *C. elegans*, including the SM lineage (Fig. 1B) (Jungblut and Sommer, 2001). However, scanning electron microscopy of the vulva reveals substantial differences in the final architecture (Fig. 2). Whereas the *C. elegans* vulva has a slit-like opening, the *P. pacificus* vulva opening forms a pore-like structure (Figs. 2A and B).

To determine how the differences in vulva architecture affect vulval muscle arrangement, we used phalloidin staining of the actin fiber network. In *C. elegans*, the eight vulval muscle cells align as four pairs of cells with nearly parallel muscle fibers (Fig. 2D). In contrast, phalloidin staining in *P. pacificus* indicates a star-like arrangement of the eight vulval muscle fibers around the central vulval opening (Fig. 2C). Thus, the morphology of the vulva and the vulval muscle arrangement differ between *P. pacificus* and *C. elegans*.

A screen for Pristionchus M cell-defective mutants identified three classes of genes

To reveal the genetic and molecular mechanisms of M and SM cell specification in *P. pacificus*, we carried out large-scale

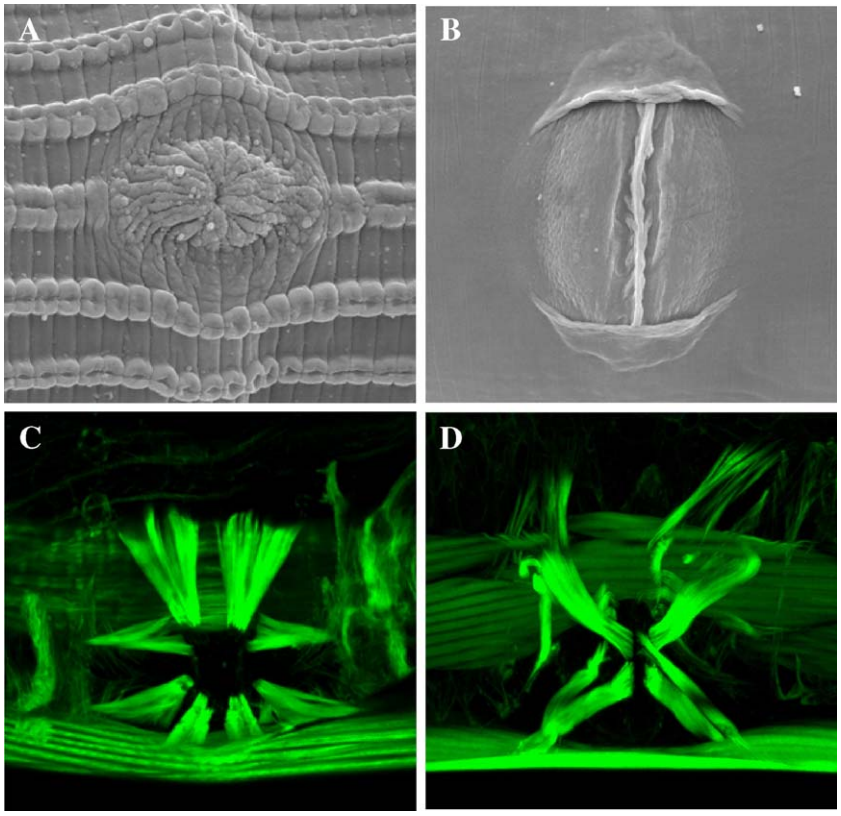


Fig. 2. Scanning electron micrographs (A, B) and phalloidin staining (C, D) of the *P. pacificus* (A, C), and *C. elegans* (B, D) vulva and muscles fibers. (A, B) The *P. pacificus* vulva has a pore-like opening, whereas the *C. elegans* opening is a slit. (C, D) Phalloidin staining of *P. pacificus* vulval muscle fibers shows a nearly symmetrical arrangement of the eight fibers around the pore opening of the vulva, resulting in a star-like fiber structure. In *C. elegans*, the eight fibers arrange in four pairs. *P. pacificus* (*Ppa*), *C. elegans* (*Cel*).

mutagenesis screens for egg-laying defective mutants. Using previously defined protocols (Eizinger and Sommer, 1997; Jungblut and Sommer, 1998; Zheng et al., 2005), egg-laying defective mutants were isolated in standard F2 screens, and homozygous mutant lines were analyzed by Nomarski microscopy in the F3 or F4 generation. We screened a total of 14,400 gametes and isolated 11 mutants with M or SM cell lineage defects (Fig. 3). Based on the particular cell lineage phenotype and complementation studies (see Materials and methods for details), the original 11 mutations fell into three complementation groups. We use a *Pristionchus*-specific provisional nomenclature for these mutants, where "*pmd*" stands for *Pristionchus M cell-defective*. As for vulva-defective mutants, we plan to rename mutants in agreement with the *C. elegans* nomenclature after the molecular nature of the corresponding genes have been identified. Mutations in *pmd-1* affect the early aspects of the M cell lineage and will be described elsewhere. In contrast, *pmd-2* and *pmd-3* specifically affect the SM cell and are described in detail below.

The SM cell fate specification mutant pmd-2 encodes a sem-4/spalt-like gene

In the mutagenesis screen for M lineage defects, we isolated three mutants that result in the absence of SM founder cells as revealed by Nomarski observation (Figs. 3A and B). Complementation tests indicated that all of these mutants are alleles of the same gene, which was named *pmd-2*. More detailed analysis by phalloidin staining of adult *pmd-2(tu352)* and *pmd-2(tu350)* mutant worms revealed that 0% (n = 120) and 16% (34 out of 211 animals) had proper sex muscles, respectively. In most *pmd-2* mutant animals presumptive SM cells have a body-wall muscle phenotype instead. Thus, *pmd-2* mutants show a strong reduction in SM cell fate specification. *pmd-2* mutant worms show no phenotype in the gonad or other major tissues except for the vulva (see detailed description below).

To identify the molecular nature of *pmd-2*, we mapped the locus in the polymorphic strain *P. pacificus* var. Washington (Srinivasan et al., 2002). *pmd-2* maps to chromosome V between the single-stranded conformational polymorphism (SSCP) markers S492 and S240, the same chromosome as *Ppa-lin-17*/Frizzled (Zheng et al., 2005) (Fig. 4A). Detailed analysis of various genes located on chromosome V indicates that this chromosome corresponds in large parts to *C. elegans* chromosome I (Zheng and Sommer, unpublished observation). Interestingly, the *spalt*-like transcription factor *Cel-sem-4* maps to chromosome I (Basson and Horvitz, 1996). Mutations in *Cel-sem-4* result in a similar phenotype as *pmd-2*, making this gene a likely candidate for the *pmd-2* locus (Basson and Horvitz, 1996). We cloned a fragment of *Ppa-sem-4* by PCR

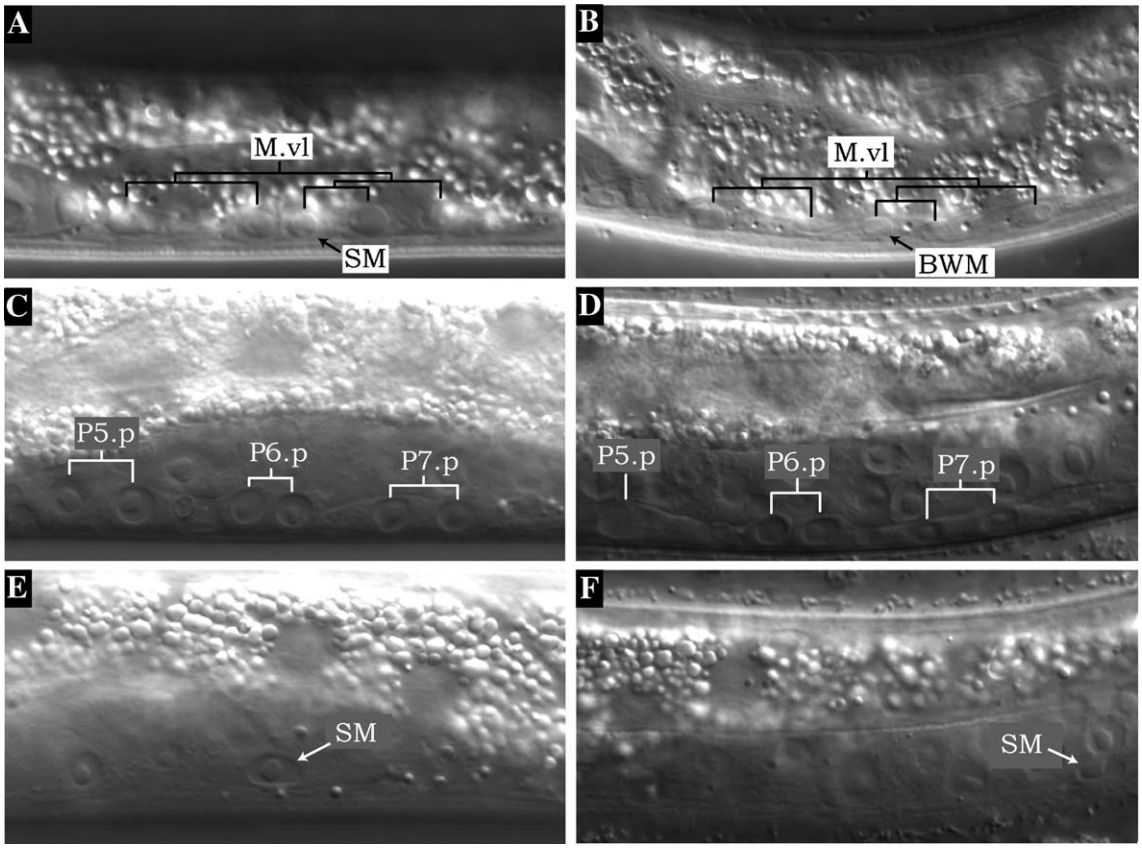


Fig. 3. Nomarski photomicrographs of various stages of the M lineage and SM fate specification and migration in *P. pacificus* wild-type (A, C, E) and *Ppa-sem-4* (B) and *Ppa-egl-17* (D, F) mutant animals. (A) Wild-type animal 19 h after hatching, showing the five progeny of M.vl. The SM cell (arrow) is morphologically distinct from the four body wall muscles. (B) *Ppa-sem-4(tu352)* mutant animal 19 h after hatching. The SM founder cell is misspecified and adopts body wall muscle (BWM) morphology (arrow). (C) Wild-type animal 26 h after hatching, showing the daughters of P(5–7).p after one round of cell divisions. (D) *Ppa-egl-17(tu312)* mutant animal 26 h after hatching. P6.p and P7.p already divided, P5.p is in division. (E) Wild-type animal 26 h after hatching, same animal as in panel C. In this plane of focus, the SM cell (arrow) is visible in its final position laterally to P6.p and its progeny. (F) *Ppa-egl-17(tu312)* mutant animal 26 h after hatching, same animal as in panel D. The SM cell (arrow) did not migrate properly and rests in a position between P8.p and P7.pp.

and generated an associated SSCP marker S236 in this gene. Further mapping experiments indicated that *pmd-2* and *Ppa-sem-4* map to the same position of the *P. pacificus* genome (Fig. 4A).

Next, we cloned a full-length cDNA and the genomic locus of *Ppa-sem-4* (see Materials and methods for details) (Figs. 4B–D). To determine if *Ppa-sem-4* corresponds to *pmd-2*, we sequenced the *Ppa-sem-4* gene in the *pmd-2* alleles and identified mutations in all of them (Fig. 4B). *tu314* and *tu350* result in a splice donor and splice acceptor mutation of intron 12 and 7, respectively. *tu352* causes a C to T transition resulting in a nonsense mutation (Fig. 4B). Taken together, these results indicate that *Ppa-sem-4* is identical to *pmd-2*. In agreement with our nomenclature rules, we renamed *pmd-2* to *Ppa-sem-4*.

Ppa-SEM-4 and *Cel*-SEM-4 show a similar domain structure and have an amino acid identity of 75% in the zinc finger domains (Fig. 4D). However, outside of the DNA-binding domain sequence similar is very low. Interestingly, the spacing of the seven zinc finger domains of *sem-4* is similar in *P. pacificus* and *C. elegans* (Fig. 4C). The only small difference is seen in the distance between the closely spaced zinc finger domains 2–4 and 5–7 (Fig. 4C). Most importantly, however, zinc fingers 2 and 3 and 5 and 6 are closely spaced in both nematodes and are 100% and 90% conserved at the amino acid level, respectively (Figs. 4C and D). This suggests that these zinc fingers are crucial for SEM-4 function. In contrast, the spacing of the finger domains differs between the nematode SEM-4 proteins and the *Drosophila* Spalt protein, which is the most similar protein encoded in the *Drosophila* genome (Basson and Horvitz, 1996; Kuhnlein et al., 1994).

Ppa-sem-4 has a dual function in SM and vulva formation

Next, we wanted to know if *Ppa-sem-4* has additional functions during postembryonic development. To determine if *Ppa-sem-4* is involved in vulva cell fate specification, we carried out cell lineage analysis of all three available *Ppa-sem-4* alleles (Table 1). We observed cell lineage defects of P5.p and P7.p in all three *Ppa-sem-4* alleles. In *Ppa-sem-4*, P(5,7).p often adopt an intermediate fate in that Pn.px (2.5° fate) or Pn.pxx (2.25° fate) cells remain undivided. The defect of the 2° lineages was more pronounced in P7.p than in P5.p. Specifically, P5.p had a 3° fate in 3 or 4% of *Ppa-sem-4* mutant animals, whereas P7.p had a 3° fate in up to 11% in *Ppa-sem-4(tu352)* (Table 1). The cell lineage of P6.p is unaffected in *Ppa-sem-4* mutant animals (data not shown). Later in development, vulval

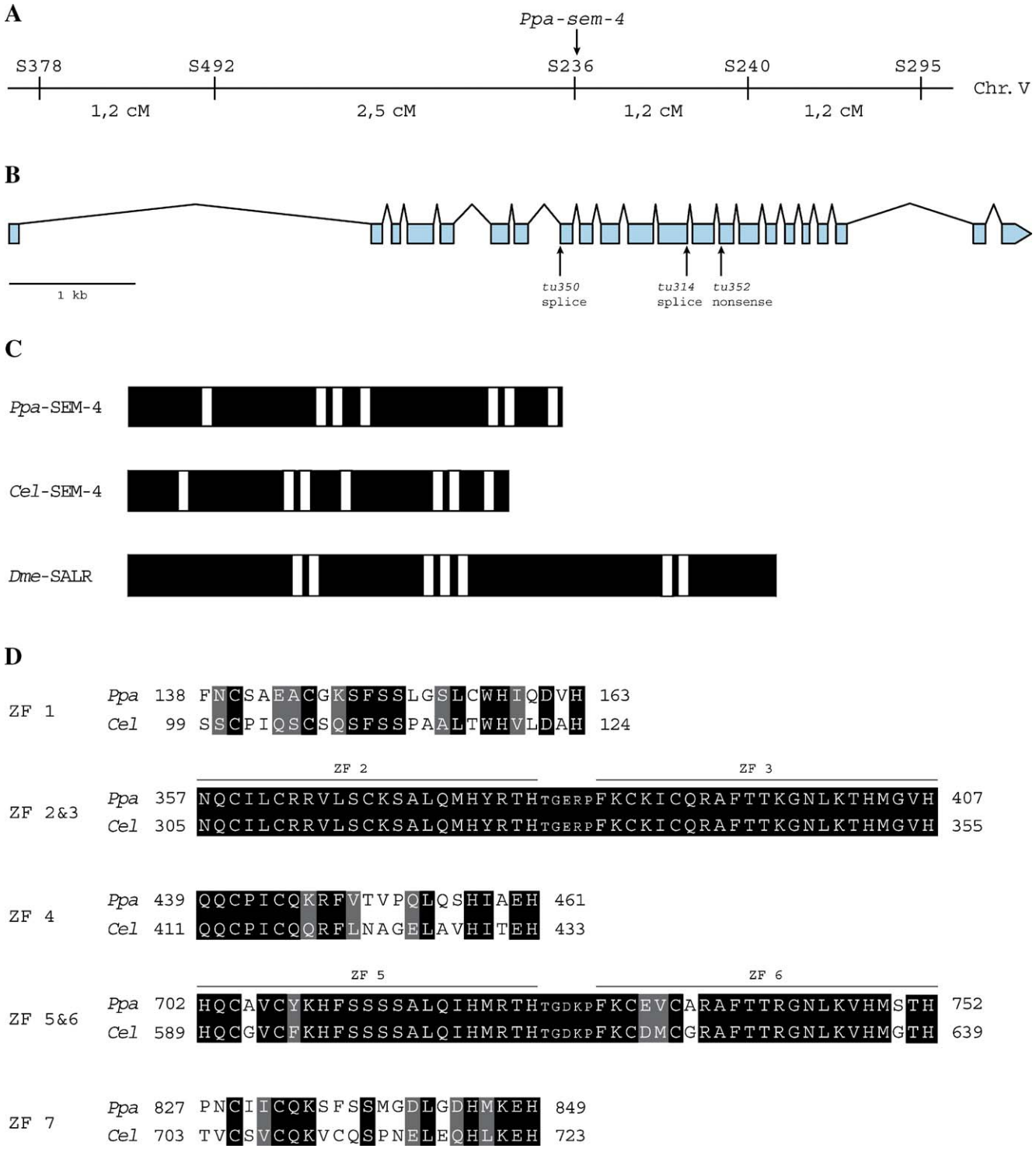


Fig. 4. Molecular cloning of *pmd-2/Ppa-sem-4*. (A) Map position of *Ppa-sem-4* on chromosome V. (B) Genomic structure of *Ppa-sem-4*. The gene spans a region of 9 kb and contains 21 introns. In comparison, *Cel-sem-4* contains only 9 introns. The position of the three mutations is indicated by arrows. (C) Comparison of the location of the seven zinc finger domains in *Ppa*-SEM-4, *Cel*-SEM-4, and *Drosophila* SPALT. The proteins from the two nematodes share a similar spacing of the zinc finger domains. (D) Amino acid sequence comparison of the seven zinc finger domains of SEM-4 between *P. pacificus* (*Ppa*) and *C. elegans* (*Cel*). Identical amino acids are shown in black, similar amino acids in grey. Sequence similarity is low outside of the zinc finger region (data not shown) but is high in the seven finger domains. In particular the two zinc fingers 2 and 3 have identical amino acid sequences in *P. pacificus* and *C. elegans*. The zinc fingers 2 and 3 and 5 and 6 are separated by a small spacer of five amino acids (smaller font of amino acids) that is highly conserved in sequence. ZF, zinc finger.

morphogenesis was aberrant in a number of animals leading to a protruding vulva phenotype. *Cel-sem-4* was also shown to have dual functions during SM cell specification and vulva formation (Grant et al., 2000). Thus, *Ppa-sem-4* and *Cel-sem-4* show similar patterning defects indicating that the dual function of this gene has been conserved during nematode evolution.

Table 1
Cell fate specification defects during vulva formation in *Ppa-sem-4* alleles

Allele	*n*	P5.p fate				P7.p fates		
		2°	2.5°	2.25°	3°	2°	2.5°	3°
Ppa-sem-4 (tu314)	123	99/123 (81%)	10/123 (8%)	10/123 (8%)	4/123 (3%)	101/123 (82%)	12/123 (10%)	10/123 (8%)
Ppa-sem-4 (tu350)	127	105/127 (83%)	10/127 (8%)	8/127 (6%)	4/127 (3%)	106/127 (83%)	8/127 (6%)	13/127 (10%)
Ppa-sem-4 (tu352)	126	92/126 (73%)	15/126 (12%)	14/126 (11%)	5/126 (4%)	96/126 (76%)	16/126 (13%)	14/126 (11%)
Cel-sem-4 (ku200)	159	115/159 (72%)	20/159 (12%)	–	24/159 (15%)	92/159 (58%)	13/159 (8%)	54/159 (34%)

Percentage of cells with indicated fates. 2° cells have a wild-type lineage and undergo three rounds of division generating seven progeny, all of which form part of the vulva. 2.5° fate includes Pn.p cells that undergo only incomplete divisions in that one Pn.px cell fuses with the hypodermis without forming part of the vulva. 2.25° fate includes Pn.p cells, in which one of the Pn.pxx progeny cells fuses with the hypodermis. 3° cells do not divide and fuse with the hypodermis.

The SM migration-defective mutant pmd-3 encodes an egl-17/FGF-like gene

We identified a single allele of an additional gene with an SM cell-specific defect. In *pmd-3(tu312)* mutant animals, the SM cells fail to migrate towards the central body region, whereas they migrate precisely underneath P6.p in wild-type animals (Figs. 3C–F). Specifically, phalloidin staining indicated that in 189 of 205 (92%) analyzed *pmd-3(tu312)* mutant animals the sex muscles were located in a posterior position between P8.p and the progeny of P7.p. In the remaining 16 (8%) mutant animals, SM cells migrated properly underneath the vulva. Later in development, SM cells of *pmd-3(tu312)* mutant animals divide similar to wild-type animals. However, the resulting sex muscle cells are not properly connected to the rest of the egg-laying system because of their posterior location.

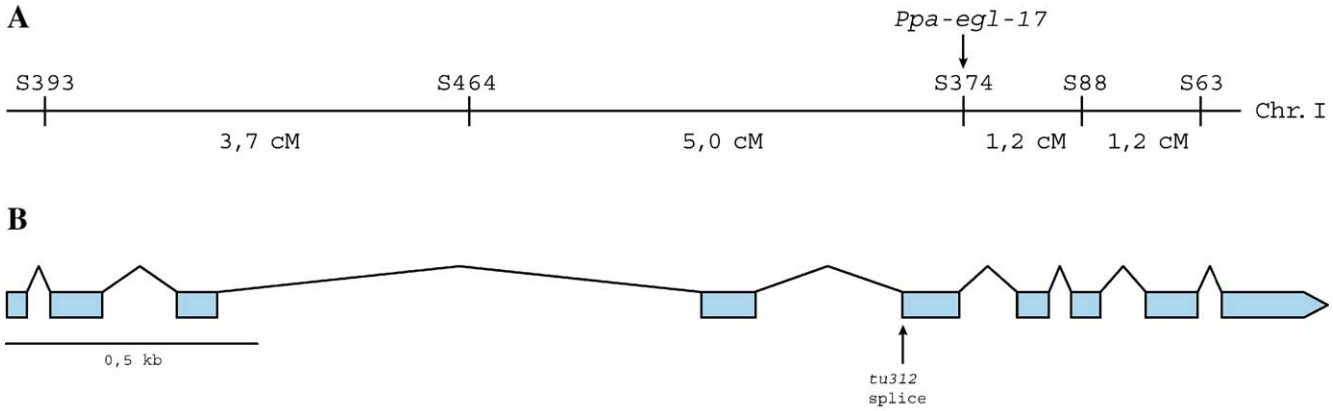
The *pmd-3* locus was mapped to chromosome I between the SSCP markers S464 and S88 using the polymorphic reference strain *P. pacificus* var. Washington (Fig. 5A). Chromosome I of *P. pacificus* corresponds largely to *C. elegans* chromosome V but contains on one arm multiple genes that are orthologous to *C. elegans* X chromosomal genes (Srinivasan, 2004). Interestingly, *Cel-egl-15*/FGFR and *Cel-egl-17*/FGF are located on the X chromosome and mutants in *Cel-egl-15* and *Cel-egl-17* have a phenotype that is strikingly similar to the *pmd-3* (Burdine et al., 1997; DeVore et al., 1995). Therefore, the *P. pacificus* orthologs of *egl-15* and *egl-17* are both candidates for the *pmd-3* mutant. Fragments of the *P. pacificus* orthologs of *egl-15* and *egl-17* are available from the whole genome-sequencing project. We generated associated SSCP markers in *Ppa-egl-15* and *Ppa-egl-17* and placed them on the genetic linkage map. The *Ppa-egl-15* ortholog we found maps to the X chromosome and can be ruled out as a candidate for *pmd-3* (data not shown). In contrast, S374 in *Ppa-egl-17* maps to the same position as *pmd-3* and is indeed a candidate for this genetic locus (Fig. 5A).

We cloned a full-length cDNA and the genomic locus of *Ppa-egl-17* (Figs. 5B–D). *Ppa-egl-17* encodes for a predicted protein of 265 amino acids and is comparable in size and structure to *Cel-egl-17* (Fig. 5D). Amino acid sequence comparison reveals that *Ppa*-EGL-17 and *Cel*-EGL-17 have an overall amino acid identity of 29% and similarity of 49% (Fig. 5D). When we sequenced the genomic locus of *Ppa-egl-17* in the *pmd-3* allele *tu312*, we identified a mutation in a splice acceptor site (Figs. 5B and C). In addition, we sequenced *Ppa-egl-17* cDNA fragments of *pmd-3* mutant and wild-type control animals. In *pmd-3* mutant animals, the *Ppa-egl-17* cDNA fragments were all wrongly spliced resulting in premature stop codons. In contrast, the *Ppa-egl-17* cDNA fragments of wild-type control animals were all properly spliced. Taken together, these results indicate that *Ppa-egl-17* is identical to *pmd-3*, which we now rename to *Ppa-egl-17*.

SM cell migration in P. pacificus relies on gonad-dependent attraction and repulsion

Does SM cell migration in *P. pacificus* rely on a similar set of attractive and repulsive signals from the gonad as in *C. elegans*? The available *Ppa-egl-17* mutant in combination with cell ablation experiments allows this question to be addressed experimentally (Fig. 6). Indeed our studies reveal that a similar logic underlies SM migration in *P. pacificus*. After gonad ablation in *P. pacificus* wild-type animals, the SM cells migrate towards anterior but are not properly positioned (Figs. 6A and B). In contrast, the SM cells remain in the posterior body region in *Ppa-egl-17(tu312)* mutant animals (Fig. 6C). If the gonad was ablated in the *Ppa-egl-17(tu312)* mutant background, SM cells migrated to the central body region but were not precisely positioned (Fig. 6D). This result is similar to gonad-ablated wild-type animals (Fig. 6B). Thus, the gonad provides an attractive cue for the SM cells that requires *Ppa*-EGL-17/FGF function. Furthermore, the gonad-dependent attraction, as revealed by gonad ablated wild-type animals, is accompanied by a gonad-dependent repulsion as seen from gonad-ablated *Ppa-egl-17* mutant animals.

Cel-egl-17 is most strongly expressed in P6.p although this is not the major source of *Cel*-EGL-17 affecting SM migration (Branda and Stern, 2000). We used mutant analysis to determine if P6.p or any of the VPCs in *P. pacificus* are of importance for SM migration. In *Ppa-lin-39* mutants, P(5–8).p die of programmed cell death, like their anterior and posterior lineage counterparts providing an easy system to study the influence of the VPCs on SM migration (Eizinger and Sommer, 1997). We studied SM cell migration in the allele *Ppa-lin-39 (tu2)*, in which 0.1 VPCs survive on average per animal (Eizinger and Sommer, 1997). SM cell migration was unaffected in *Ppa-lin-39(tu2)* mutant animals, and SM cells always migrated to their proper position close to the AC (Fig.



C

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  1 M  I  D  Y  R  V  P  L  N  G  L  I  R  N  V  V  L  L  L  L
  1 ATGATCGACTACAGAGTGCCACTGAACGGATTGATACGAAACGTCGTCCTTCTCCTCCTC
 21 T  L  L  P  T  V  S  P  G  M  T  E  L  R  G  Q  D  V  W  Q
 61 ACCCTCCTTCCTACCGTATCCCCTGGAATGACTGAACTCCGTGGACAAGATGTGTGGCAG
 41 L  C  N  I  C  S  D  H  C  L  A  L  E  H  G  S  T  R  I  H
121 CTCTGCAATATATGCAGTGATCACTGTCTCGCTCTTGAGCATGGAAGTACACGAATTCAT
 61 A  R  G  N  H  S  D  P  D  C  S  T  D  F  N  V  L  S  V  D
181 GCAAGAGGAAATCACTCAGATCCCGATTGCAGCACGGATTTCAATGTTCTCTCAGTCGAT
 81 F  G  V  I  R  L  Q  H  A  L  T  K  R  Y  L  C  M  S  S  K
241 TTCGGTGTGATCAGACTGCAACATGCACTGACAAAGAGATATTTGTGTATGAGTTCCAAG
101 K  K  L  T  T  K  L  N  G  G  G  D  R  C  A  F  V  E  H  M
301 AAGAAATTGACTACGAAGTTGAACGGAGGTGGAGATCGATGTGCCTTCGTTGAACACATG
121 D  E  M  H  Y  T  R  L  E  N  V  Q  F  H  G  T  Y  L  A  F
361 GATGAAATGCATTATACGAGACTCGAGAATGTTCAATTTCATGGAACTTATCTCGCATTC
141 N  R  K  G  R  F  L  A  N  A  S  T  N  Q  W  R  C  V  Q  F
421 AATCGGAAAGGTCGCTTTCTGGCGAATGCATCAACGAATCAATGGAGATGCGTCCAATTC
161 N  K  Y  Q  R  R  V  S  D  T  E  R  R  S  C  C  R  R  E  Q
481 AATAAGTATCAACGACGCGTTTCCGACACAGAGCGAAGGAGTTGCTGTAGGAGGGAACAA
181 R  C  R  V  D  R  E  A  G  V  E  P  P  K  K  E  E  E  P  P
541 AGATGTCGCGTTGATCGTGAGGCGGGTGTCGAACCTCCGAAGAAGGAAGAAGAGCCACCA
201 P  S  I  Q  D  H  L  T  R  I  T  D  D  L  I  L  K  S  L  L
601 CCAAGCATTCAAGATCATCTAACACGAATCACTGACGATCTAATCCTGAAATCGCTCCTT
221 A  D  S  N  D  L  L  D  P  Y  W  E  P  T  T  T  T  T  H  R
661 GCCGATTCAAACGATCTTCTCGATCCCTACTGGGAACCAACAACGACAACAACTCATCGT
241 P  F  D  Q  H  A  F  L  S  H  L  T  R  R  S  E  R  M  R  R
721 CCATTCGATCAACATGCATTCTTGAGTCATCTAACGAGAAGATCAGAGAGAATGCGAAGG
261 R  L  K  P  F  *
781 AGACTCAAACCATTCTGA
```

D

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Ppa-EGL-17      MIDYRVPLNGLIRNVVLLLLTLLPTVSPGMTELRGQDVWQLCNICSDHCLALEHGSTRIH 60
Cel-EGL-17      --MLKVLLT-LMLSTNFRNTCARFQIKPNVHYL-GETHWQLFNECSQGMLQSFLGS--LN 54

Ppa-EGL-17      ARGNHSDPDCSTDFNVLSVDFGVIRLQHALTKRYLCMSSKKKLTTKLNGGGDRCAFVEHM 120
Cel-EGL-17      TRG-YPDKHCLTDWNVVGEWDGKFRLQHAQSRKFLCFNKRARITLRFNGSDAKCTFIEEV 113

Ppa-EGL-17      DEMHYTRLENVQFHGTYLAFNRKGRFLANASTN-QWRCVQFNKYQRRVSDTERRSCCRRE 179
Cel-EGL-17      RDNGFSRLRSSWKPELYLCFNGRGRFQNPLSYHLKPRCFDWIKLVRYVAESEKSVCS--- 170

Ppa-EGL-17      QRCRVDREAGVEPPKKEEEPPPSIQDHLTRITDDLILKSLLADSNDLLDPYWEPTTTTTH 239
Cel-EGL-17      -----------TPPKPKLSPSP--LEHSS-FVHHAVRSNFLKKVSATHDSLYRTMKSRKS 216

Ppa-EGL-17      RPFDQHAFLSHLTRRSERMRRRLKPF 265
Cel-EGL-17      -------------------------
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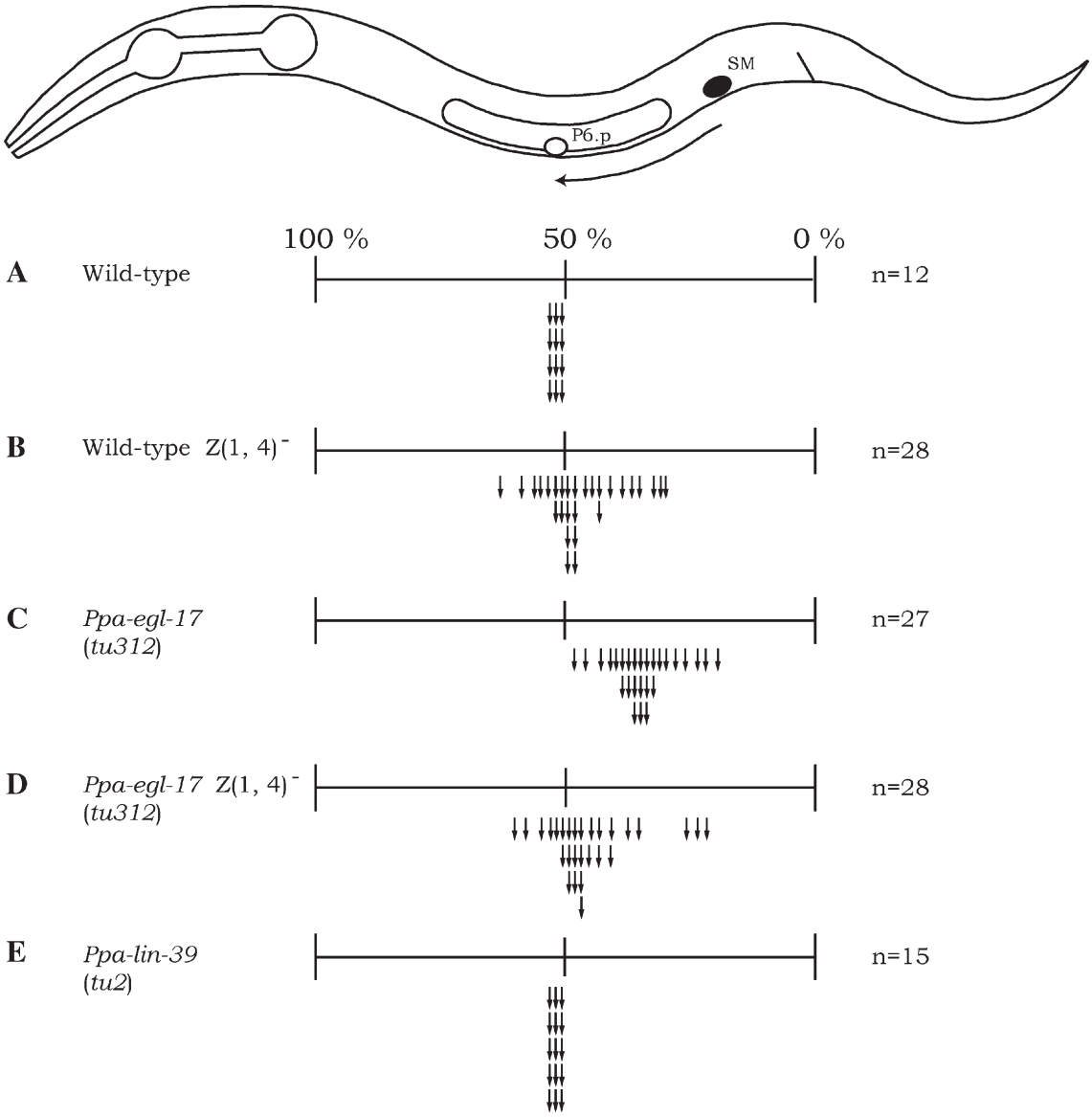

Fig. 6. Effects of gonad ablation on SM positioning in *P. pacificus* wild-type and mutant animals. Gonad ablation was performed at hatching by eliminating Z(1,4), the precursor cells of the somatic gonad. Each arrow represents the position of a single SM cell. (A) *P. pacificus* un-ablated wild-type animals. The SM cells are positioned above P6.p. (B) In gonad ablated wild-type animals, SM cells migrated anteriorly but stopped migration at different positions, mostly in the region between P6.p and P7.p. (C) In un-ablated *Ppa-egl-17(tu312)* mutant animals, SM cells remain in the posterior region where they were born. (D) In gonad-ablated *Ppa-egl-17(tu312)* mutant animals, cells migrate to the anterior region as in gonad-ablated wild-type animals. (E) In *Ppa-lin-39(tu2)* mutant animals, VPCs die of programmed cell death. SM migration is normal in these mutants.

6E). We conclude that VPCs in *P. pacificus* are not necessary for SM cell migration.

Discussion

We are interested in the evolution of the nematode egg-laying system and use comparative studies between *P. pacificus* and *C. elegans* to identify the genetic and molecular alterations that result in evolutionary novelty. The work on vulva formation revealed substantial differences in the genetic control in *P. pacificus* when compared to *C. elegans* (Sommer, 2005; Zheng et al., 2005). Surprisingly, the genetic and molecular analysis of the specification of the postembryonic mesoderm as described in this study revealed an unexpected level of similarity and conservation. We show the first results of an unbiased genetic analysis for M and SM cell-defective mutants that result in egg-

Fig. 5. Molecular cloning of *pmd-3/Ppa-egl-17*. (A) Map position of *Ppa-egl-17* on chromosome I. (B) Genomic structure of *Ppa-egl-17*. The gene spans a region of 2.5 kb and contains 8 introns. (C) Conceptual translation of *Ppa-egl-17*. Introns are shown as black triangles, the position of the mutation in *tu312* is indicated as an arrow. (D) Amino acid sequence comparison of EGL-17 between *P. pacificus* (*Ppa*) and *C. elegans* (*Cel*). Identical amino acids are shown in black, similar amino acids in grey. The internal homologous region that is common to all FGF proteins is marked in grey.

laying defective animals. We identified SM cell fate specification and SM migration mutants and could show that these phenotypes result from mutations in *Ppa-sem-4* and *Ppa-egl-17*, respectively. These results indicate a surprising level of conservation in SM specification and migration between *P. pacificus* and *C. elegans*. Even more striking is the finding that *Ppa-sem-4* and *Cel-sem-4* share a dual role in SM cell fate specification and vulval patterning. In addition, the complex logic of SM cell migration with a gonad-dependent attraction, a gonad-independent attraction, and a gonad-dependent repulsion is also conserved between both nematodes.

The *sem-4* gene is one of only a few genes in *C. elegans* that have a role in the development of both, the vulva and the sex muscles. Besides its prominent phenotype in the M lineage, *Cel-sem-4* mutants exhibit also a vulva phenotype. Loss of *Cel-sem-4* function results in the abnormal specification of the 2° cell lineages of P5.p and P7.p (Grant et al., 2000). Interestingly, mutations in *Cel-sem-4* have a stronger phenotype in P7.p than in P5.p. In *Cel-sem-4(ku200)*, P7.p remains epidermal in 34% of the animals, whereas P5.p has a 3° fate in only 15% of the animals (Table 1). This is strikingly similar to the *Ppa-sem-4* mutant phenotype described in this study. Thus, the two independent functions of a transcriptional regulator of vulva and sex muscle development are conserved over a period of more than 200 million years. It should be noted, however, that in general, the vulva phenotype of *Cel-sem-4(ku200)* shows a higher penetrance than the one of the *Ppa-sem-4* alleles.

Ppa-egl-17/FGF represents a component of the second major signaling system to be genetically characterized in *P. pacificus*. Previous studies had identified the Wnt receptor *Ppa-lin-17*/Frizzled, which shows a strong diversification of Wnt signaling function during the evolution of nematode vulva development (Zheng et al., 2005). Our analysis of the postembryonic mesoderm did not indicate a similar diversification of FGF signaling function. No obvious differences were observed comparing the mutant phenotype of *Ppa-egl-17* to *Cel-egl-17* mutants. One factor that might strongly influence the conservation and diversification of gene function, in particular for genes encoding signaling pathway components, is the copy number in the genome of interest. The *C. elegans* genome contains multiple genes for most Wnt pathway components; for example, there are five Wnt ligands, four Frizzled-type receptors and three Dishevelled-type adaptors. In contrast, only one FGF receptor and two FGF-type ligands are known in the *C. elegans* genome (Ruvkun and Hobert, 1998; www.wormbase.org). Although the *P. pacificus* genome project is not yet completed, preliminary analysis suggests that the different numbers of Wnt pathway components versus FGF pathway components is largely conserved between *C. elegans* and *P. pacificus*. One might speculate that the large number of Wnt pathway genes allows a high degree of subfunctionalization and redundancy and thereby provides the “freedom” for evolutionary change. Similarly, signaling pathways with components being encoded by single genes might lack subfunctionalization and redundancy and, therefore, could be evolutionarily constrained.

The conservation in SM cell fate specification and migration between *P. pacificus* and *C. elegans* is in strong contrast to our studies on vulva formation. In vulva development, many differences are observed between both organisms. For example, vulva induction relies on a signal from the single AC in *C. elegans* but requires input from multiple cells of the somatic gonad in *P. pacificus* (Kimble, 1981; Sigrist and Sommer, 1999). In addition, a negative signal from the VPCs counteracts vulva induction in *P. pacificus* and requires a Wnt signaling pathway (Zheng et al., 2005). The Hox gene *lin-39* plays a crucial role in *C. elegans* vulva induction and is a direct target of EGF and Wnt signaling (Eisenmann et al., 1998; Maloof and Kenyon, 1998). In contrast, *Ppa-lin-39* is dispensable for vulva induction (Sommer et al., 1998). Thus, the cell–cell interactions and the underlying molecular principles of vulva formation changed during nematode evolution.

The vulva is part of the reproductive system, which has a direct role in fitness. One explanation for the major differences in the genetic and molecular regulation of vulva formation between nematode species has been the fitness value of the egg-laying system. Given this line of argumentation, similar results would have been expected for the sex muscles, which form the second major part of the egg-laying system. Our study clearly indicates that the genetic control of SM fate specification and migration is conserved between *P. pacificus* and *C. elegans*. Thus, the genetic control of different parts of the egg-laying system is uncoupled and can change independently during nematode evolution. Therefore, our data might provide the first observation arguing against fitness as the major driving force of vulval patterning changes.

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