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The nematode *even-skipped* homolog *vab-7* regulates gonad and vulva position in *Pristionchus pacificus*

Benno Jungblut and Ralf J. Sommer*

Max-Planck Institut für Entwicklungsbiologie, Abt. Evolutionsbiologie, Spemannstrasse 37-39, 72076 Tübingen, Germany
*Author for correspondence (e-mail: ralf.sommer@tuebingen.mpg.de)

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SUMMARY

In free-living nematodes, developmental processes like the formation of the vulva, can be studied at a cellular level. Cell lineage and ablation studies have been carried out in various nematode species and multiple changes in vulval patterning have been identified. In *Pristionchus pacificus*, vulva formation differs from *Caenorhabditis elegans* with respect to several autonomous and conditional aspects of cell fate specification. To understand the molecular basis of these evolutionary changes, we have performed a genetic analysis of vulva formation in *P. pacificus*. Here, we describe two mutants where the vulva is shifted posteriorly, affecting which precursor cells will form vulval tissue in *P. pacificus*. Mutant animals show a concomitant posterior displacement of the gonadal anchor cell, indicating that the gonad and the vulva are affected in a similar way. We show that mutations in the *even-skipped* homolog of nematodes, *vab-7*, cause these posterior displacements. In addition, cell ablation studies in the *vab-7* mutant indicate that the altered position of the gonad not only changes the cell fate pattern but also the developmental competence of vulval precursor cells. Investigation of *Cel-vab-7* mutant animals showed a similar but weaker vulva defective phenotype to the one described for *Ppa-vab-7*.

Key words: *Pristionchus pacificus*, *Caenorhabditis elegans*, Gonad displacement, Vulva development, *vab-7/even-skipped*

INTRODUCTION

In free-living nematodes, developmental processes can be studied at a single cell level, providing a cellular basis for organ formation and morphogenesis during embryonic and post-embryonic development. Across species borders, blast cells are homologous to one another so that evolutionary changes in developmental processes can be addressed at a cellular level (Sommer, 1997; Félix, 1999). Cell lineage analysis of the development of the vulva, the copulatory organ and the egg-laying structure of nematode females and hermaphrodites, has been carried out in more than 50 species representing six nematode families. These comparative studies indicate that multiple alterations occur in the inductive interactions required for vulva formation and that the cell fate specification of homologous vulval precursor cells (VPCs) changes during nematode evolution (Sommer, 2000). In addition to cell lineage studies, genetic analysis of vulva development is now possible in two selected species, *Pristionchus pacificus* and *Caenorhabditis elegans*, of the families Diplogastridae and Rhabditidae, respectively (Sommer and Sternberg, 1996; Eizinger and Sommer, 1997). Comparison of loss-of-function mutations in the two homeotic genes *lin-39* and *mab-5* between the two species has indicated changes in the actual function of these genes during vulva development (Eizinger et al., 1999). Therefore, the comparative analysis of vulva development among nematodes can reveal how the cellular, genetic and molecular aspects of a developmental system evolve.

The vulva derives from the ventral epidermis, which consists of 12 precursor cells, denoted P(1-12).p, according to their relative anteroposterior position (Fig. 1A) (Sulston and Horvitz, 1977). P(1-12).p adopt region-specific fates and a subset of these cells divide during larval development to form the vulva. In *C. elegans*, P(3-8).p form a vulva equivalence group as all cells have the potential to adopt vulval fate (Fig. 1B) (Sternberg and Horvitz, 1986). P(5-7).p finally form the vulva with a 2°-1°-2° pattern. P(5,7).p have a 2° fate, generate seven progeny each and form the anterior and posterior part of the vulva, respectively. P6.p has a 1° fate, generates eight progeny and forms the central part of the vulva (Fig. 1B). P(3,4,8).p have a 3° fate, divide once and fuse with the hypodermal syncytium. Cells with a lower fate (i.e. 3° cells) can replace cells with a higher fate (i.e. 2° or 1° cells) after cell ablation, indicating a hierarchy of cell fates. Vulva formation requires a signal from the gonadal anchor cell (AC; Kimble, 1981). The AC resides above P6.p and establishes the physical connection between the gonad and the vulva by making specific contacts with some of the progeny of P6.p.

It is now understood that at least four different signaling systems are involved in vulva formation (Fig. 1C; for a review see Kornfeld, 1997). The inductive signal from the AC is a secreted epidermal growth factor-like molecule encoded by the gene *lin-3* (Fig. 1C) (Hill and Sternberg, 1992). This signal is

transmitted in P(5-7).p via an EGF/RAS/MAPK signaling pathway. In addition, cell-cell interactions among the VPCs are also involved in cell fate specification (Fig. 1C). P6.p, after receiving a sufficient amount of inductive signal, will signal its two neighbors via the *lin-12*/Notch pathway to adopt a 2° fate (Simske and Kim, 1995). The EGF/RAS/MAPK and the *lin-12*/*Notch* pathways act in a redundant fashion. Furthermore, two redundant negative signaling pathways are involved in vulva formation. Genes within these pathways antagonize EGF/RAS/MAPK signaling and prevent inappropriate vulva differentiation (Fig. 1C). Mutants defective in negative signaling display ectopic vulva differentiation by P(3,4,8).p, a phenotype that has been designated as 'multivulva'. The cloning of two such genes, *lin-35* and *lin-53*, has revealed that they encode proteins similar to retinoblastoma (Rb) and its binding protein RbAp48 (Lu and Horvitz, 1998). Finally, WNT-signaling has also been shown to play an important role during vulva formation in *C. elegans* (Eisenmann et al., 1998).

Cell fate specification during vulva formation differs among nematodes. Multiple differences in both autonomous and non-autonomous cell fate specification have been observed between *P. pacificus* and *C. elegans*, although both species form their vulvae from homologous cells, P(5-7).p, adopting a 2°-1°-2° pattern (Fig. 1B,D) (Eizinger and Sommer, 1997; Jungblut and Sommer, 2000). Specifically, ventral epidermal cells in the anterior and posterior body region fuse with the hypodermal syncytium *hyp7* in *C. elegans*, but die of programmed cell death in *P. pacificus* (Sommer and Sternberg, 1996). Within the vulva equivalence group, P8.p is the only cell in *P. pacificus* not participating in vulva formation. Systematic cell ablation studies have indicated that P8.p in *P. pacificus* represents a new cell type in the ventral epidermis. P8.p in *P. pacificus*, unlike its homologous cell in *C. elegans*, is incompetent to respond to gonadal signaling in the absence of other vulva precursor cells, but can respond to lateral signaling from a neighboring vulval precursor. P8.p provides an inhibitory signal that determines the developmental competence of P(5,7).p. For example, after the ablation of P(6,7).p, P5.p will adopt a 2° fate in the presence of P8.p. In contrast, after ablation of P(6-8).p, an isolated P5.p can adopt the 1° fate (Jungblut and Sommer, 2000). This lateral inhibition acts via the mesoblast M and is regulated by the homeotic gene *Ppa-mab-5* (Fig. 1E) (Jungblut and Sommer, 2000). In addition, P8.p provides a negative signal that inhibits gonad-independent vulva differentiation (Fig. 1E; Jungblut and Sommer, 2000). In *C. elegans*, no such properties of P8.p have been observed. Finally, vulva induction by the gonad also differs between *C. elegans* and *P. pacificus*. In *C. elegans*, only the AC has inductive properties, whereas multiple cells of the somatic gonad are involved in vulva induction in *P. pacificus* (Sigrist and Sommer, 1999).

These cellular differences in vulva formation, allow us to use genetic analysis in *P. pacificus* to identify the molecular players associated with vulval evolution. Here, we describe the role of the *even-skipped* homolog *vab-7* in the regulation of gonad and vulval position in *P. pacificus*. In *Ppa-vab-7* mutants, the gonad and the AC are posteriorly displaced, thereby affecting the subset of VPCs forming vulval tissue. Cell ablation studies in this mutant background indicate that the altered position of the gonad has an influence on the developmental competence of P8.p.

MATERIALS AND METHODS

Strains and cultures

All experiments were carried out using the laboratory strain *P. pacificus* PS312, which is a derivative of a wild isolate from Pasadena, CA (Sommer et al., 1996). Worms were grown on *E. coli* OP50 as described elsewhere (Sommer et al., 1996). For larval stages, the nomenclature J1-J3 is used according to Félix et al. (Félix et al., 1999).

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 4 hour s at 20°C. The suspension was washed in M9 five times and the worms were spotted onto the surface of NG plates (Wood, 1988). After 1 hour, excess liquid had been absorbed and individual motile L4 hermaphrodites were picked individually to plates. In case of the TMP/UV mutagenesis, worms were incubated for 20 minutes with 33µg/ml TMP and then UV irradiated for 50 seconds with an intensity of 500 $\mu W/cm^2$. In the F_2 generation, egg-laying defective mutants were isolated and their progeny were reanalyzed for vulval defects using Nomarski microscopy. Mutant hermaphrodites were backcrossed (Nomarski screen) using wild-type males. Both mutants were backcrossed several times. Complementation tests were carried out using the morphological marker *Ppa-dpy-1*, which was previously shown to be linked to the homeotic genes *Ppa-lin-39* and *Ppa-mab-5* (Eizinger and Sommer, 1997; Jungblut and Sommer, 1998).

General DNA analysis

General DNA manipulation and analysis were carried out using standard protocols. The genomic library screened to obtain *Ppa-vab-7* clones has been described elsewhere (Sommer et al., 1996). Subclones were made using the pBluescript vector (Stratagene Cloning Systems, La Jolla, CA).

PCR experiments and mutant sequencing

Ppa-vab-7 was cloned by PCR using the degenerate primers 5′ CGRCGRTAYMGRACNGCNTT-3′and 5′-ACTGCATTCWSNMG-NGARCA-3′in the N-terminal and 5′-NCKYTGNCKYTTRTCYTT-CAT-3′and 5′-CATGCKACKRTTYTGRAACA-3′in the C-terminal region. To clone the 5′and 3′region of *Ppa-vab-7*, we used RACE (rapid amplification of cDNA ends) experiments (Frohmann, 1994; Ranasinghe and Hobbs, 1998). The TMP/UV allele was analyzed by genomic Southern blot analysis. *Eco*RI-digested genomic DNA was hybridized with a *Ppa-vab-7* fragment and a10kb *Ppa-vab-7* hybridization signal of wild-type DNA was reduced in size to approximately 1.5 kb in DNA of *tu113* mutant animals. This 1.5 kb fragment was cloned and sequenced by inverse PCR (Ochman et al., 1988). The EMS allele was analyzed by PCR. DNA was isolated from two independent batches of animals, amplified by three independent PCR reactions, cloned into pBluescript and sequenced. The GenBank Accession Number for the sequence reported in this paper is AF286591.

Immunofluorescence

Immunofluorescence studies were performed by collecting newly-hatched larvae over a five hour time interval and subsequent ageing of these larvae to the particular time point of interest (20°C). Larvae were fixed and stained as described elsewhere (Finney and Ruvkun, 1990). Cy3-conjugated goat anti-mouse antibodies (Dianova) were used to detect MH27.

Cell ablation experiments

Cell ablation experiments were carried out using standard techniques described for *C. elegans* in Epstein and Shakes (Epstein and Shakes,

1995) and using a 'Laser Science' dye laser of the type described by Avery and Horvitz (Avery and Horvitz, 1987). Animals were picked into M9 buffer placed on a pad of 5% agar in water containing 10 mM sodium azide as anesthetic. All ablation experiments were carried out within 1 hour of hatching (20°C).

Cell lineage characters and cell fate terminology

The different cell fates of the VPCs are distinguished using the terminology 1°, 2°, 3° and 4° for cell fates, and T (transversal), L (longitudinal), N (non-dividing) and O (oblique) for cell division patterns (Sommer and Sternberg, 1995; Sommer and Sternberg, 1996). During normal development, P6.p has the 1° fate and generates six progeny with the cell division pattern TNNT. The two 'N' cells (P6.pap and P6.ppa), which do not divide (in contrast to *C. elegans*), attach to the AC. P(5,7).p have a 2° fate and generate seven progeny each, with a cell division pattern LLLN (for P5.p). After ablation of other VPCs, an isolated 1° and an isolated 2° cell can be distinguished from one another by several cell lineage characteristics. In the intermediate four-cell stage (after two rounds of cell divisions of a VPC) of a 1° cell, the AC moves between the two central cells P6.pap and P6.ppa. In the final six-cell stage, the cells are located symmetrically around the AC. In the four-cell stage of a 2° cell, the AC does not move between the central Pn.pxx cells. When the invagination is formed, the distribution of the seven progeny is asymmetric and variable with respect to the AC. VPCs that remain epidermal in the absence of vulva induction were designated as 3°. The fate of P8.p was designated as 4° based on the finding that this cell loses its competence to form vulval tissue during early larval development (Jungblut and Sommer, 2000).

RESULTS

A homeotic mutation resulting in vulva formation by P(6-8).p instead of P(5-7).p

To understand the genetic and molecular processes underlying evolutionary changes in vulval patterning, we used a genetic approach in *P. pacificus*. Specifically, we looked for mutations that alter VPC specification and result in an abnormal vulval pattern. Mutants were isolated based on their egg-laying defective phenotype. Cell lineage analysis using Nomarski microscopy was used to determine the precise vulval patterning defects of individual mutants. From several ethyl methanesulfonate (EMS) and trimethylpsoralen (TMP)-UV mutagenesis screens, we identified two mutants with a vulva 'shift' phenotype, namely *tu113* and *tu125* (Table 1A). In approximately 50% of mutant animals, P7.p instead of P6.p had a 1° fate and P(6-8).p formed the vulva. In all animals with a shifted vulva, the AC was posteriorly displaced and resided above P7.p instead of P6.p.

Complementation tests showed that these mutants were allelic. When homozygous *Ppa-dpy-1 tu113* hermaphrodites were crossed to heterozygous *tu125* males, half of the progeny had a mutant phenotype. Furthermore, genetic analysis indicated that both mutants were linked to the recessive visible mutation *Ppa-dpy-1*, a gene that has previously been shown to be linked to the homeotic genes *Ppa-lin-39* and *Ppa-mab-5* (Eizinger and Sommer, 1997; Jungblut and Sommer, 1998). *P.*

Table 1. Vulva differentiation in *P. pacificus* and *C. elegans* mutant strains and in ablated animals

A	Differentiation	P5.p	P6.p	P7.p	P8.p	Number of animals
	Ppa-vab-7(tu113)	2°	1°	2°	4°	13/28
		3°	2°	1°	2°	9/28
		3°	2°	1°	4°	4/28
		2°	2°	1°	2°	1/28
		3°	2°	2°	4°	1/28
	Ppa-vab-7(tu125)	2°	1°	2°	4°	15/34
		3°	2°	1°	2°	12/34
		3°	2°	1°	4°	4/34
		2°	2°	1°	2°	2/34
		2°	2°	1°	4°	1/34
B	P7.p Ablation	P5.p	P6.p	P7.p	P8.p	Number of animals
	Ppa-vab-7(tu113)	2°	1°	-	4°	4/8
		2°	1°	-	2°	3/8
		3°	1°	-	2°	1/8
	Wild-type	2°	1°	-	2°	5/10*
		2°	1°	-	4°	5/10*
C	P(5,6).p Ablation	P5.p	P6.p	P7.p	P8.p	Number of animals
	Ppa-vab-7(tu113)	-	-	1°	2°	6/8
		-	-	1°	4°	2/8
	Wild-type	-	-	2°	4°	17/20*
		-	-	3°	4°	2/20*
		-	-	1°	4°	1/20*
D	P(5-7).p Ablation	P5.p	P6.p	P7.p	P8.p	Number of animals
	Ppa-vab-7(tu113)	-	-	-	2°	5/22
		-	-	-	1°	2/22
		-	-	-	4°	15/22
	Wild-type	-	-	-	4°	22/22*

E	Differentiation	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	Number of animals
	Cel-vab-7(e1562)	3°	3°	2°	1°	2°	3°	46/50
		3°	3°	3°	2°	1°	2°	4/50

Cells were ablated within one hour after hatching. Cell fates are defined in Materials and Methods.
*Data from Jungblut and Sommer, 2000.

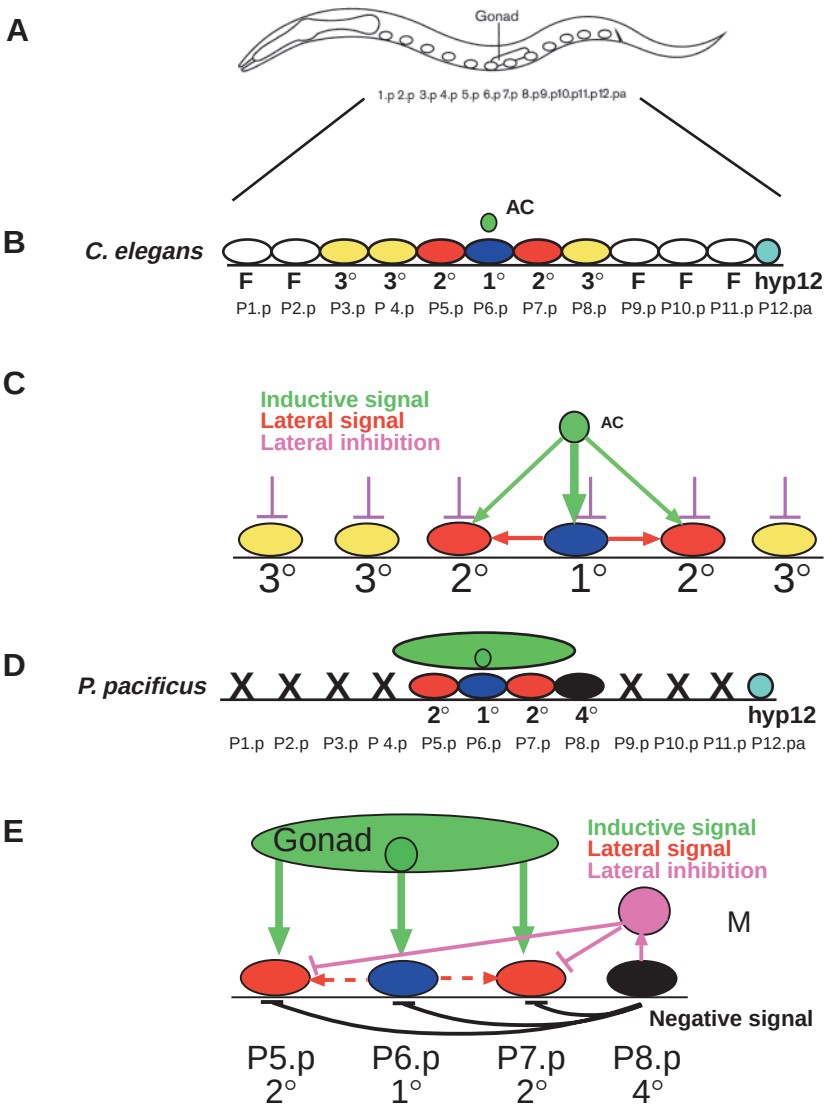

Fig. 1. The position and the fate of ventral epidermal cells in *Caenorhabditis elegans* and *Pristionchus pacificus*. (A) The 12 Pn.p cells (with 'n' denoting cell number) are equally distributed between pharynx and rectum. (B) Cell fate specification in *Caenorhabditis elegans*. P(1,2,9-11).p fuse with the hypodermal syncytium *hyp7* (white ovals, F). P(3-8).p form the vulva equivalence group and adopt one of three alternative cell fates. P(3,4,8).p remain epidermal and have a 3° fate (yellow ovals). P(5,7).p have a 2° fate and generate seven progeny, which form part of the vulva (red ovals). P6.p has the 1° fate and generates eight progeny, which form the central part of the vulva (blue oval). The AC (green circle) is born dorsally to P6.p, induces vulva formation and makes contact with the progeny of P6.p. *hyp12* is a special cell and forms part of the rectum. (C) Signaling interactions during vulva formation in *C. elegans*. An inductive EGF-like signal originates from the AC (green arrows). P6.p signals its neighbors to adopt a 2° fate via 'lateral signaling' (red arrows). Negative signaling (pink bars) prevents inappropriate vulva differentiation. (D) Cell fate specification in *Pristionchus pacificus*. P(5-7).p form the vulva with a 2°-1°-2° pattern. P8.p does not form part of the vulva and has a special fate designated as 4° (black oval). See text for details. (E) Model for cell-cell interactions during vulva development in *P. pacificus*. P8.p provides a lateral inhibition to P(5,7).p, mediated by the mesoblast M (pink bars). Lateral inhibition influences the 1° vs. 2° cell fate decision of P(5,7).p. P8.p also provides a negative signal (black bars), which influences the vulva vs. non-vulval cell fate decision. Lateral signaling occurs between P6.p and P8.p (not indicated) and perhaps also between P6.p and P(5,7).p (broken red arrow).

pacificus has six chromosomes, as does *C. elegans* (Sommer et al., 1996). *tu113* and *tu125* animals were uncoordinated and displayed a bobbed tail phenotype, reminiscent of *C. elegans vab-7* mutants (Fig. 2). *Cel-vab-7* is the homolog of the *Drosophila* pair-rule gene *even-skipped* (Ahringer, 1996; Ahringer, 1997). Given the similarity of the bobbed tail phenotype between *Cel-vab-7* and *P. pacificus tu113/tu125* and the linkage of both mutants to the Hox cluster, a feature also known from *Cel-vab-7*, we hypothesized that the phenotype might be caused by mutations in *Ppa-vab-7*.

Mutations in the *even-skipped* homolog *vab-7* cause the vulva shift phenotype of *tu113* and *tu125*

We cloned the *vab-7* homolog from *P. pacificus* by polymerase chain reaction (PCR) using degenerate primers within the homeodomain. The resulting fragment was used to screen a genomic library and to perform 5′ and 3′ RACE experiments. The *Ppa-vab-7* gene is similar in size to the *Cel-vab-7* gene, but contains three additional introns (Fig. 3A,D). Sequence similarity is high in the homeobox region (97% similarity and 90% identity), but is restricted to small patches in the N and C-terminal parts of the protein (32% similarity and 19% identity) (Fig. 3B). Such a conservation pattern was also observed for other genes, such as the homeotic genes *lin-39* and *mab-5* (Eizinger and Sommer, 1997; Jungblut and Sommer, 1998).

We determined the genomic sequence of *Ppa-vab-7* in mutant animals and identified mutations in *tu113* and *tu125*. *tu113* contained a large deletion in the *Ppa-vab-7* locus. Inverse PCR experiments indicated an approximately 9kb deletion, removing 5′ regulatory regions and the first three exons (Fig. 3C,D). *tu125* had a nonsense mutation in the first exon (Fig. 3D). Taken together, these results indicate that molecular lesions in the *even-skipped* homolog *Ppa-vab-7* cause the vulva phenotype of *tu113* and *tu125*. Given the similar phenotype of these alleles and the nature of the mutations, these alleles most likely represent strong loss-of-function alleles.

Ppa-vab-7 mutations result in mispositioning of the gonad primordium

We asked whether the mispositioning of the epidermal cells or

Fig. 2. Nomarski photomicrographs of juvenile hermaphrodites, all of which are in the L3 stage (*C. elegans*) and J2 stage (*P. pacificus*), respectively. (A) *Pristionchus* wild-type with an elongated tail. (B) *Ppa-vab-7* and (C) *Cel-vab-7*, both with a bobbed tail. Scale bar: 50 μm.

of the gonad primordium were responsible for the alterations in vulval patterning. At hatching, the gonad primordium consists of four cells, Z1-Z4. Z(2,3) constitute the germ line, whereas Z(1,4) are the precursor of the somatic gonad. The AC, which is the source of the inductive signal for vulva formation in *C. elegans*, is one of the great grand-daughters of either Z1 or Z4.

Examination of the gonad primordium in early larval stages revealed that the gonad was displaced relative to the ventral epidermis in *Ppa-vab-7* animals. In wild-type animals, the gonad primordium resides between the two neuroblasts P6.a and P7.a. More specifically, the posterior-most cell of the gonad primordium, Z4, resides above P7.a (Fig. 4A,G). As a consequence of this spatial arrangements, the AC is born in its final position, dorsally above P6.p (Fig. 4C). In *Ppa-vab-7* animals, the neuroblasts P(1-12).a are equally spaced in the ventral cord, as in wild-type animals. However, the gonad primordium is posteriorly displaced and Z4 resides close to or above P8.a in *Ppa-vab-7* mutants (Fig. 4B,G). As a result, the AC is born between P6.p and P7.p and can connect to either cell during subsequent development (Fig. 4D). Thus, the vulva shift in *Ppa-vab-7* animals is caused by the posterior displacement of the gonad primordium relative to the ventral epidermis.

Mispositioning of the gonad can influence the fate of all Pn.p cells in *P. pacificus*

As the AC can be connected to either P6.p or P7.p in *Ppa-vab-7* mutants, patterning is variable, leading to a vulva formed by P(5-7).p or P(6-8).p, respectively. In principle, this could result from a random migration of the AC towards the anterior or posterior VPC or from a stochastic bias of the VPCs to connect to the AC. Previous work has shown that P6.p and P7.p differ in their response to lateral inhibition in wild-type animals (Jungblut and Sommer, 2000). Specifically, P8.p can prevent P7.p, but not P6.p, from adopting a 1° fate after the ablation of other VPCs. It remains unclear, however, if P6.p is intrinsically different from P7.p or if the closer contact of P6.p to the AC might be responsible for the different response to lateral inhibition.

To determine if P6.p and P7.p have identical or different developmental potentials, we ablated P6.p or P7.p, respectively, in *Ppa-vab-7* mutants. After ablation of P7.p in *Ppa-vab-7* mutants, P6.p had a 1° fate in all eight ablated animals (Table 1B). Similarly, after ablation of P(5,6).p, P7.p had a 1° fate in all ablated animals (Table 1C). In 75% of these animals, P8.p had a 2° fate and formed part of the partial vulva (Table 1C). These results suggest that in *vab-7* mutants, P6.p and P7.p have identical developmental potentials and that the AC is in contact with P6.p or P7.p in a random fashion in unablated animals.

Gonad displacement in *Ppa-vab-7* mutants also influences the specification of P8.p. Previous studies indicate that P8.p differs from other VPCs (Jungblut and Sommer, 2000). In wild-type animals, P8.p fuses with the hypodermis before the birth of the AC and is unable to adopt a vulval fate after ablation of the other VPCs (Fig. 4E, Table 1D). In addition, P8.p is involved in lateral inhibition and provides a negative signal that prevents gonad-independent differentiation of P(5-7).p (Jungblut and Sommer, 2000).

One important difference between P8.p and P(5-7).p, which may account for the fate difference of P8.p, is that in wild-type animals P8.p has no direct contact with the gonad. Since *Ppa-vab-7* regulates gonad position, we asked whether the closer position of P8.p to the gonad in mutant animals had an influence on P8.p specification. We used the adherens junction-specific antibody MH27 to monitor cell fusion of Pn.p cells. MH27 antibody labels unfused cells, whereas fused cells are unlabeled. Indeed, MH27 antibody staining revealed that P8.p remained unfused in approximately 70% of *Ppa-vab-7* mutant animals, indicating its competence to adopt vulval fate in response to gonadal signaling (Fig. 4F). These observations have been confirmed by cell ablation experiments, testing for the developmental competence of P8.p in *Ppa-vab-7* versus wild-type animals. After ablation of P(5-7).p, P8.p adopted a 2° fate in 23% and a 1° fate in 9% of *Ppa-vab-7* mutants (Table 1D). In contrast, in wild-type animals, P8.p had an epidermal fate in 100% of P(5-7).p ablated animals (Jungblut and Sommer, 2000). These results suggest that P8.p has a greater competence to adopt vulval fate in *Ppa-vab-7* than in wild-type animals.

Cel-vab-7 also regulates gonad and vulva position

Given the posterior displacement of the gonad in *Ppa-vab-7* mutants, we sought to determine whether the position of gonad and vulva was similarly affected in *Cel-vab-7*. *Cel-vab-7* mutants exhibit a strong posterior patterning defect in the body muscles and epidermis (Ahringer, 1996). *Cel-vab-7* is expressed in both, muscles and epidermal cells in the posterior body region and is regulated by the caudal homolog *pal-1* (Ahringer, 1997). However, gonad and vulva development have as yet not been analyzed in *Cel-vab-7* mutants.

We re-analyzed the *Cel-vab-7* allele *e1562* for defects in gonadal and vulval patterning. *Cel-vab-7(e1562)* contains a stop codon early in the homeodomain of *Cel-vab-7* and is therefore a presumptive null allele (Ahringer, 1996; Ahringer, 1997). Cell lineage observation of early larval stages of *Cel-vab-7(e1562)* mutants indicated that both, the position of the

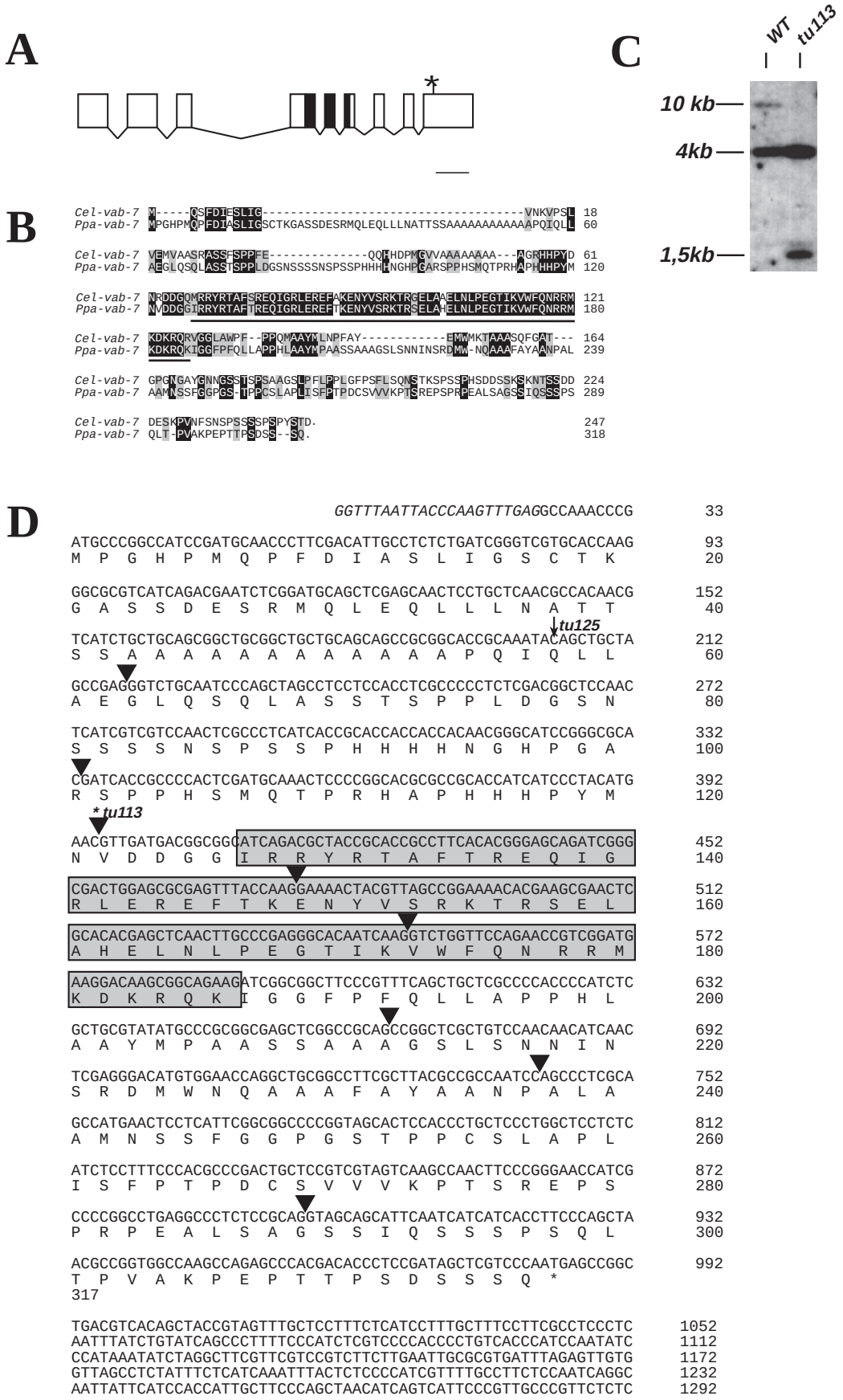

Fig. 3. *Ppa-vab-7* genomic sequence and protein comparison. (A) *Ppa-vab-7* genomic structure. The homeodomain is indicated in black. The asterisk indicates the stop codon. (B) Amino acid sequence comparison of *Ppa*-VAB-7 and *Cel*-VAB-7 proteins using the clustal method. Filled residues indicate identical and shaded residues indicate similar amino acids. The homeobox is underlined. (C) Genomic Southern blot. Wild-type and *tu113* mutant DNA were digested with *Eco*RI and hybridized with a fragment containing the homeodomain of *Ppa-vab-7*. An approximately 10 kb hybridization signal of wild-type DNA is reduced to 1.5 kb in the *tu113* mutant. (D) *Ppa-vab-7* cDNA sequence as obtained from 5′and 3′RACE experiments. SL-1 leader sequence is indicated in italics. Conceptual translation starts with the first in-frame ATG codon after the SL-1 splice acceptor site. Introns are indicated by inverted black triangles. Because of the limited sequence similarity outside of the homeodomain, most introns cannot be aligned between the two species. The proximal breakpoint of the deletion in *tu113* is in the third intron (asterisk). *tu125* contains a cytosine-to-thymine transition (arrow), resulting in a stop codon.

gonad and the distribution of ventral epidermal cells were altered, relative to wild-type animals. The position of the gonad primordium relative to the ventral epidermis was variable and in some animals the AC was born between P6.p and P7.p, as in *Ppa-vab-7* mutants. As a result, 8% of *Cel-vab-7* animals had a vulva formed by P(6-8).p (Table 1E).

In addition to the gonad primordium, the Pn mother cells (with n denoting any of the 12 precursor cells) were no longer homogeneously distributed in the ventral region in *Cel-vab-7* animals. In general, the Pn cells are in a lateral position at hatching. Several hours later, they migrate into the ventral region and divide to give rise to a neuroblast, the Pn.a cell and an ectoblast, the Pn.p cell. In *Cel-vab-7* mutants, the posterior Pn and Pn.p cells were misplaced and their spacing relative to one another varied from animal to animal. For example, P7.p was in close proximity to P8.p in 50% of *Cel-vab-7* animals. The abnormal spacing of Pn.p cells might be a secondary consequence of the posterior patterning defects, which affect both the preanal and postanal region. In contrast, *Ppa-vab-7* mutant animals had defects only in the postanal region, so that the distribution of the Pn.a and Pn.p cells was unaltered from wild-type animals.

Thus, *Cel-vab-7* and *Ppa-vab-7* mutants show similar and different developmental defects. In both species, *vab-7* mutations can affect vulval patterning in a similar way, resulting in a vulva formed by P(6-8).p rather than P(5-7).p. Nonetheless, *Cel-vab-7* has a more dramatic effect on body organization, changing not only the position of the gonad but also the positions of postembryonic blast cells, like the Pn cells, relative to one another.

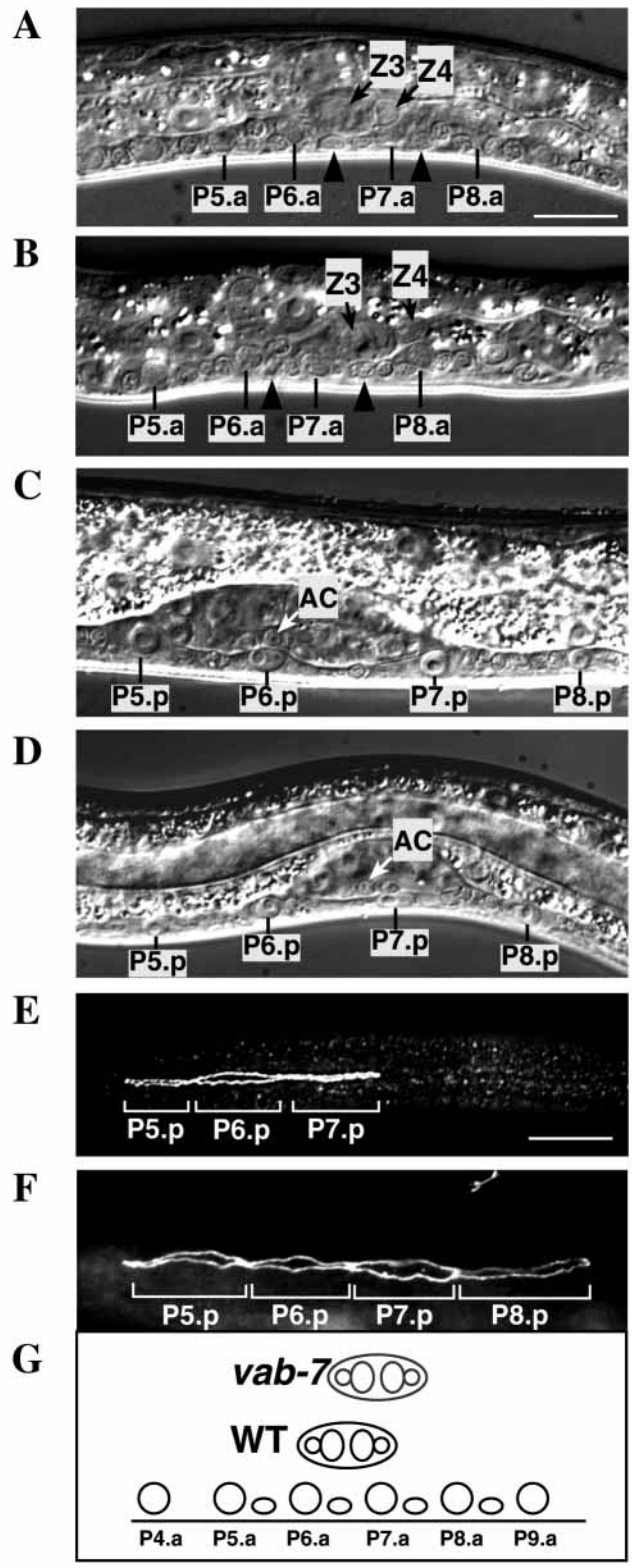

Fig. 4. Nomarski (A-D) and immunofluorescence (E,F) photomicrographs of *Pristionchus* wild-type (A,C,E) and *vab-7* mutants (B,D,F). (A) *Pristionchus* wild-type animal, 0-1 hour after hatching. Z4 of the gonad primordium is located in the same region as the neuroblast P7.a. (B) *Ppa-vab-7* mutant animal, 0-1 hour after hatching. The gonad primordium is posteriorly displaced and Z4 is located above P8.a. (C) *Pristionchus* wild-type animal, 25 hours after hatching. The AC is located above P6.p. (D) *Ppa-vab-7* mutant animal 25 hours after hatching. The AC is located above P7.p. (E) MH27 staining of a *P. pacificus* wild-type animal, 20-25 hours after hatching. P8.p is unlabeled indicating cell fusion. (F) *Ppa-vab-7* mutant animal, 20-25 hours after hatching. P8.p is labeled and remained unfused. (G) Gonad position in wild-type and *Ppa-vab-7* mutant animals at hatching. Scale bar: 10 μm.

DISCUSSION

We have isolated and characterized mutations in the *even-skipped* homolog *vab-7* in *P. pacificus*. *Ppa-vab-7* plays a role in the regulation of the position of the gonad relative to the ventral epidermis. In mutant animals, the gonad is posteriorly displaced, thereby affecting which VPCs will form vulval tissue. *Cel-vab-7* mutants show similar defects in gonad position but also affect the positioning of other blast cells, like the Pn.p cells themselves.

Previous work on *even-skipped* homologs in multiple metazoan organisms has suggested a role for this gene in posterior patterning. This observation is mostly based on the posterior expression pattern of *even-skipped*-like genes in many organisms and the conserved linkage to the posterior end of the Hox cluster in vertebrates (Dollé et al., 1994). Linkage of *vab-7* to the Hox cluster is also observed in *P. pacificus* and *C. elegans*. Detailed analysis of *Ppa-vab-7* and *Cel-vab-7* mutant animals allows us to draw two major conclusions.

A new gene regulating gonad position

Since there was no systematic screen for gonad displacement

mutants in *C. elegans*, *vab-7* represents one of the few genes shown to regulate gonad position in nematodes. Besides *vab-7*, mutations in the *dig-1* gene in *C. elegans* causes a displacement of the gonad (Thomas et al., 1990). In *dig-1* mutants, the gonad is usually shifted towards the anterior and in approximately 13% of the animals, it is also dorsally displaced. Therefore, gonad displacement of *dig-1* mutants affects vulva development in the opposite way to *vab-7* mutants: P(4-6).p instead of P(5-7).p can form vulval tissue in *dig-1* mutants (Thomas et al., 1990).

Another *C. elegans* mutant affecting AC and vulval positioning is the *Cel-mab-5* gene. Clandinin et al. observed that in certain *loss-of-function* mutations of *Cel-mab-5*, the vulva can be formed by P(6-8).p and that the AC is posteriorly displaced in such animals (Clandinin et al., 1997). Similar effects have also been observed in *Ppa-mab-5* mutants (Jungblut and Sommer, 1998). However, the frequency with which gonad and vulva position is affected is much higher in *Ppa-vab-7* mutants, than in *Cel-mab-5* or *Ppa-mab-5* mutants, e.g. only 5% of *Ppa-mab-5* mutant animals in strong alleles had a shifted vulva, whereas approximately 50% of both *Ppa-vab-7* alleles had a posteriorly displaced AC. When we generated the *Ppa-vab-7 Ppa-mab-5* double mutant, no strong enhancement of the vulva shift phenotype was seen. In seven of 19 animals (37%), P6.p had a 1° fate, whereas in 11 of 19 animals (58%) P7.p had a 1° fate. In contrast, in *tu113* and *tu125* mutant animals, P7.p had a 1° fate in 50% and 56% of the animals, respectively. These results suggest that there is no strong synergy between *Ppa-vab-7* and *Ppa-mab-5* with regard to gonad displacement. One could speculate therefore that both genes act in a linear pathway.

P6.p and P7.p have identical developmental potentials

Recent ablation studies in *P. pacificus* had indicated that P5.p and P7.p have identical developmental potentials (Jungblut and Sommer, 2000). For example, both cells are equally influenced by a lateral inhibitory signal from the mesoblast cell M (Fig. 1D). At the same time, additional experiments indicated that P6.p was inert to lateral inhibition (Jungblut and Sommer, 2000). It remains unknown however, if the different response of P6.p is due to an intrinsic difference of P6.p to P(5,7).p or due to the closer contact of P6.p to the gonadal AC. In the latter scenario, AC signaling might override the inhibitory effect of the M cell. It should be noted, however, that in contrast to *C. elegans*, the AC is not the only signaling source in *P. pacificus*, because vulva induction by the somatic gonad starts long before the AC is born (Sigrist and Sommer, 1999).

To distinguish if P6.p and P(5,7).p have identical or different developmental potentials, the AC displacement in *Ppa-vab-7* mutant animals can be taken as a test system. Indeed, ablation experiments suggest that P6.p and P7.p have identical developmental potentials as in the absence of one cell, the other cell always adopts the 1° cell fate (Table 1B,C). These results suggest that the different response to lateral inhibition as seen in wild-type animals results from a closer contact of P6.p to the AC and that P(5-7).p have similar developmental potentials. However, it is also possible that in wild-type animals, the difference between P6.p and P7.p relies on a cell autonomous program requiring VAB-7. A third possibility would be that mutations in *Ppa-vab-7* influence the fate of P8.p in a way that interactions with neighboring Pn.p cells are secondarily altered. To rule out these alternative hypotheses, we would have to perform mosaic analysis, a technique that is not yet available in *P. pacificus*.

Ppa-vab-7 and gonad position influence the fate of P8.p

Detailed analysis of the *Ppa-vab-7* mutant phenotype indicates that besides P(5-7).p, P8.p can also be affected. P8.p is the only surviving Pn.p in the central body region of *P. pacificus* that is normally not involved in vulva formation. In wild-type animals, P8.p fuses with *hyp7* before the birth of the AC and is unable to adopt a vulval fate in the absence of neighboring VPCs (Jungblut and Sommer, 2000). These original observations do not distinguish if P8.p adopts its wild-type fate in response to signaling with neighboring cells or as a result of a cell-autonomous program. Experiments described here and elsewhere suggest that the fate of P8.p is specified by a complex network of autonomous and non-autonomous specification events.

In *Ppa-vab-7* mutants, P8.p remained unfused and had a 2° cell fate whenever the AC was posteriorly displaced and made a connection to P7.p (Table 1A). After ablation of other VPCs, P8.p had a 1° or 2° fate (Table 1D). Thus, closer positioning of P8.p to the gonad had an influence on P8.p specification, suggesting that P8.p is responsive to external cues. However, these experiments cannot completely rule out the possibility that *Ppa-vab-7* acts cell-autonomously in P8.p, which can only be tested by mosaic analysis.

Another indication of interactions of P8.p with neighboring cells or tissues comes from the analysis of *Ppa-mab-5* mutants. In *Ppa-mab-5* mutants, P8.p differentiates ectopically and forms a posterior vulva-like structure (Jungblut and Sommer, 1998). The multivulva phenotype of *Ppa-mab-5* mutants is gonad independent, as P(7,8).p form vulva-like structures after the ablation of Z(1,4). Surprisingly, the ectopic differentiation depends on signaling from the mesoblast M or its derivatives (Jungblut and Sommer, 2000). When the M cell was ablated in *Ppa-mab-5* mutants, the proliferation of P8.p was substantially reduced from 80% to around 20%, indicating that P8.p responds to signaling from the mis-specified M lineage (Jungblut and Sommer, 2000). Together, the *Ppa-vab-7* and the *Ppa-mab-5* mutant phenotypes reveal that P8.p is able to change its competence and fate upon interactions with neighboring tissues. We speculate that gonadal signaling in *Ppa-vab-7* mutants and ectopic signaling from the M cell lineage in *Ppa-mab-5* mutants can override a probable intrinsic 'cell fusion' program in P8.p. Therefore, P8.p is most likely specified by the interaction of cell autonomous and non-autonomous programs.

However, the mechanism by which *vab-7* regulates gonad position in *C. elegans* and *P. pacificus* remains elusive. The expression of *vab-7* has been intensively studied in *C. elegans* (Ahringer, 1996; Ahringer, 1997). Nonetheless, no expression has been observed in the gonad or other midbody cells. Expression might either be undetectable in the gonad or, alternatively, the well-described expression in the posterior epidermis might have a long range effect on gonad position by for example, providing a repulsive cue. In the absence of the *vab-7* gene product, the gonad might be more attracted by the

posterior body region, resulting in the observed displacement of the gonad relative to the ventral epidermis.

Conclusions

In summary, VPC fate specification in *P. pacificus* requires multiple complex interactions among various cell types. The analysis of specific mutants, like *Ppa-mab-5* or *Ppa-vab-7*, has become a very useful tool for the analysis of cell fate specification. Mutant analysis allows to elucidate the importance of some of these interactions because certain cells are misplaced (like the AC in the case *Ppa-vab-7*) or mis-specified (like the M cell in *Ppa-mab-5*). Therefore, a more comprehensive understanding of vulva development in *P. pacificus* and with it, of the evolution of vulva development among nematodes, requires a more detailed genetic analysis of this process.

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