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Report

Wnt Signaling Induces Vulva Development in the Nematode *Pristionchus pacificus*

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Summary

The *Caenorhabditis elegans* vulva is induced by a member of the epidermal growth factor (EGF) family that is expressed in the gonadal anchor cell, representing a prime example of signaling processes in animal development [1]. Comparative studies indicated that vulva induction has changed rapidly during evolution [2]. However, nothing was known about the molecular mechanisms underlying these differences. By analyzing deletion mutants in five Wnt pathway genes, we show that Wnt signaling induces vulva formation in *Pristionchus pacificus*. A *Ppa-bar-1*/β-catenin deletion is completely vulvaless. Several Wnt ligands and receptors act redundantly in vulva induction, and *Ppa-egl-20*/Wnt; *Ppa-mom-2*/Wnt; *Ppa-lin-18*/Ryk triple mutants are strongly vulvaless. Wnt ligands are differentially expressed in the somatic gonad, the anchor cell, and the posterior body region, respectively. In contrast, previous studies indicated that *Ppa-lin-17*, one of the Frizzled-type receptors, has a negative role in vulva formation [3, 4]. We found that mutations in *Ppa-bar-1* and *Ppa-egl-20* suppress the phenotype of *Ppa-lin-17*. Thus, an unexpected complexity of Wnt signaling is involved in vulva induction and vulva repression in *P. pacificus*. This study provides the first molecular identification of the inductive vulva signal in a nematode other than *Caenorhabditis*.

Results and Discussion

In the satellite model organism *Pristionchus pacificus*, vulva induction involves multiple cells of the somatic gonad, whereas the single anchor cell (AC) induces the vulva in *Caenorhabditis elegans* [5]. Previous studies revealed evidence for a repressor function of Wnt signaling in *P. pacificus* vulva formation [4] (Figure 1). Mutations in *Ppa-lin-17*/Fz result in gonad-independent vulva differentiation and P7.p-lineage polarization defects. Morpholino (MO) knockdown experiments of several Wnt-pathway components in the *Ppa-lin-17* mutant background suggested that a Wnt pathway transmits this negative signaling function.

Given that MO treatment results in knockdown rather than knockout phenotypes, we generated deletion mutants in Wnt pathway genes. With the advanced draft of the *P. pacificus* genome (www.pristionchus.org), we identified five ligands, four Frizzled-like receptors, a *lin-18*/Ryk-like receptor, and two β-catenin-like genes. We defined orthology relationships for all the *P. pacificus* and *C. elegans* Wnt ligands and Frizzled-type proteins (Table S1 available online). The two β-catenin-like proteins are most similar to *Cel*-BAR-1 and *Cel*-HMP-2 (Table S2). We used trimethylpseuralen/ultra violet light (TMP/UV) mutagenesis in large-scale deletion library screens [6] to identify deletions in *P. pacificus* Wnt-pathway genes. We chose *Ppa-bar-1*, the "related to tyrosine kinase" (RYK)-like receptor gene *Ppa-lin-18*, and the Wnt ligands as mutagenesis targets and screened 4,300,000 gametes by TMP/UV in 18 fragments of seven target genes. We identified deletions in *Ppa-bar-1(tu362), Ppa-egl-20(tu364, tu382)*, *Ppa-mom-2(tu363)*, *Ppa-cwn-2(tu373)*, and *Ppa-lin-18(tu359)* (Figure S1). Most mutations result in deletions that eliminate large parts of the coding region and result in frame-shift mutations eliminating important functional domains of the proteins. Therefore, the alleles represent strong reduction-of-function, most likely loss-of-function, mutations.

Ppa-bar-1(tu362) is maternal-effect embryonic lethal, and homozygous mutant progeny of *Ppa-bar-1*/+ mothers are egg-laying defective (Figure 2A). Most of their progeny arrest during embryogenesis with only few larvae escaping (Figure 2B). In contrast, *Cel-bar-1* has no described maternal function [7]. To study the role of *Ppa-bar-1* in ventral epidermal cell-fate specification, we investigated the self-progeny of *Ppa-bar-1*/+ heterozygous animals. *Ppa-bar-1(tu362)* does not influence the apoptosis of ventral epidermal cells (Figure 2). Thus, *Ppa*-BAR-1 is dispensable for the generation of the vulva equivalence group (VEG), and *Ppa*-BAR-1 is not required for the regulation of the Hox gene *lin-39*, an observation that is again different from *C. elegans*. To determine whether *Ppa-bar-1* is involved in vulva induction, we studied J3 stage mutant progeny from heterozygous carriers. *Ppa-bar-1(tu362)* mutant animals are completely Vul (Figures 2C and 2D; Table 1). The 3° cell-fate phenotype of P(5–7).p is identical to the P(5–7).p phenotype of wild-type animals, in which the somatic gonad has been ablated at hatching [5]. Thus, *Ppa*-BAR-1 is strictly required for vulva formation, suggesting that Wnt signaling in *P. pacificus* is a key regulator of this process.

Cel-bar-1 has a dual role in vulva formation. Early in development, it regulates the competence of the VPCs, and later in development, it is involved in vulva induction, and *Cel-bar-1(ga80)* mutant animals have a partial Generation vulvaless (Gev) and Induction vulvaless (Vul) phenotype [7]. Because uninduced cells do not divide in *P. pacificus*, competence and induction defective phenotypes cannot be distinguished by cell lineage. To determine whether the Vul phenotype of *Ppa-bar-1* mutants is due to the missing competence of the VPCs to respond to the inductive signal or the missing response to the inductive signal itself, we generated double mutants with the Multivulva (Muv) gene *Ppa-groucho* [8]. *Ppa-groucho* is epistatic over *Ppa-bar-1* with respect to P(3,4).p cell death: P(3,4).p survive in all *Ppa-bar-1(tu362); Ppa-groucho(tu102)* double mutants, further indicating that *Ppa*-BAR-1 is not involved in the cell-death decision of ventral epidermal cells (Table 1). In contrast, P(3–7).p can adopt a vulval fate or a 3° fate in *Ppa-bar-1(tu362); Ppa-groucho(tu102)* double mutants (Table 1). These results indicate that *Ppa*-BAR-1 does not control VPC competence but rather is involved in the transmission of the inductive signal.

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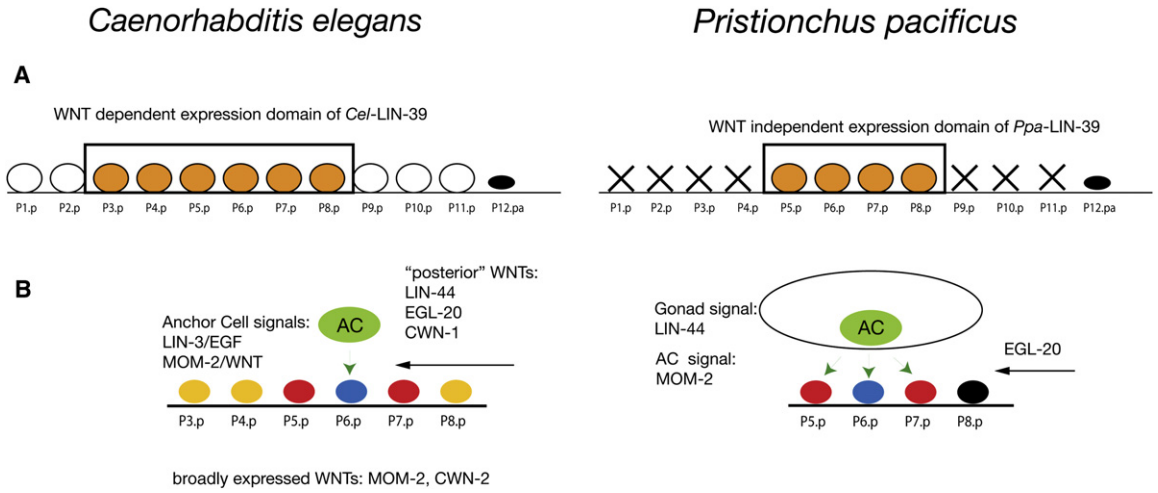

Figure 1. Cell-Fate Specification in the Ventral Epidermis in *C. elegans* and *P. pacificus* and the Role of Wnt Signaling

(A) *C. elegans* P(1,2,9–11).p fuse with the hypodermal syncytium *hyp7* (white circles). The Hox gene *Cel-lin-39* specifies P(3–8).p as the vulva equivalence group. LIN-39 activity requires Wnt signaling, and in *Cel-bar-1* mutants, some VPCs fuse with *hyp7* or remain uninduced (3° fate), resulting in a partial vulvaless phenotype [7, 13]. *P. pacificus* P(1–4,9–11).p die by programmed cell death (black cross), whereas P(5–8).p survive in response to *Ppa*-LIN-39 activity. Work in this study indicates that Wnt signaling plays no major role in the regulation of apoptosis and VPC competence.

(B) *C. elegans* P(3–8).p can adopt three alternative fates. In wild-type animals, P(3,4,8).p remain epidermal (3° fate, yellow ovals). P(5,7).p form part the anterior and posterior part of the vulva (2° fate, red ovals). P6.p forms the central part of the vulva (1° fate, blue oval) [1]. Cell-ablation studies revealed that the single anchor cell (AC, green circle) induces vulva formation (green arrow). The *C. elegans* vulva is induced by a member of the EGF family encoded by the gene *lin-3* that is specifically expressed in the AC [1]. Wnt signaling is also involved in *C. elegans* vulva induction. Expression-pattern analysis indicated that the five Wnt ligands are expressed broadly (*mom-2*, *cwn-2*) and in the posterior body region (*egl-20*, *cwn-1*, *lin-44*) [9, 13]. All five Wnt ligands have been shown to play a role in *C. elegans* vulva formation [9, 13]. In *P. pacificus*, P(5–7).p form vulval tissue with a 2°-1°-2° fate pattern similar to that in *C. elegans*. P8.p does not divide but influences the fate of P(5,7).p (4° fate, black oval). Cell-ablation studies revealed that vulva induction involves multiple cells of the somatic gonad (green arrows). Work in this study indicates that Wnt signaling plays a key role in vulva induction and that the three Wnt ligands *lin-44*, *mom-2*, and *egl-20* are differentially expressed in the somatic gonad, the AC, and the posterior body region, respectively.

To study whether a Wnt signaling pathway acts upstream of *Ppa*-BAR-1, we analyzed deletion mutants in the three Wnt ligands, in *Ppa-egl-20(tu364)*, in *Ppa-mom-2(tu363)*, and in *Ppa-cwn-2(tu373)*, and in the receptor mutant *Ppa-lin-18*/Ryk (Table 1; Figure S2). Animals carrying a deletion in any of these genes form a normal vulva, with only minor defects in a minority of animals (Table 1). For example, 5% of *Ppa-lin-18(tu359)* mutants are underinduced, and 2% show P7.p lineage polarization defects (Table 1), whereas 40% of *Cel-lin-18* mutants have such a phenotype [9]. Thus, single-deletion mutants of three Wnt ligands and *Ppa-lin-18* have no strong vulva induction defects.

To test whether these genes have redundant functions during vulva induction, we generated double- and triple-mutant animals (Table 1). The possible double- and triple-mutant combinations indicate that *Ppa-egl-20*, *Ppa-mom-2*, and *Ppa-lin-18* act redundantly in vulva induction: 56% of the VPCs had a 3° cell fate, and 39% of the animals were completely Vul in *Ppa-mom-2(tu363); Ppa-lin-18(tu359)* double mutants. In *Ppa-mom-2(tu363); Ppa-lin-18(tu359); Ppa-egl-20(tu364)* triple mutants, 83% of the VPCs remained uninduced, and 60% of the animals were Vul. Although *Cel-mom-2* and *Cel-lin-18* have been argued to form a ligand-receptor pair and show no synergistic effects [9], our results on *Ppa-mom-2* and *Ppa-lin-18* indicate synergism and show another difference between the two species. *Ppa*-CWN-2 is not involved in vulva formation, and *Ppa-cwn-2(tu373); Ppa-lin-18(tu359); Ppa-egl-20(tu364)* triple mutants form a normal vulva. These results further indicate that Wnt signaling is not involved in the regulation of apoptosis and VPC competence but provide three major conclusions for *P. pacificus* vulva induction. First, redundant signaling processes involving at least two Wnt ligands, *Ppa*-EGL-20 and *Ppa*-MOM-2, are necessary for vulva induction. Second, the Ryk-like receptor *Ppa*-LIN-18 has a redundant role in vulva induction but only a minor function in P7.p lineage polarization. Third, the *Ppa-bar-1* deletion represents the only known single-gene mutation with a completely penetrant Vul phenotype, suggesting that *Ppa*-EGL-20, *Ppa*-MOM-2, and *Ppa*-LIN-18 might act through *Ppa*-BAR-1.

To study the expression pattern of *Ppa*-BAR-1, we generated a polyclonal antibody against the *Ppa*-BAR-1 protein. We found *Ppa*-BAR-1 to be expressed in a punctuated pattern in most cells of the embryo and the hermaphrodite. This pattern is reminiscent of the localization of Armadillo homologs in adherens junctions (Figure 2) [10]. To determine the cellular source of the *P. pacificus* Wnt ligands, we used in situ hybridization experiments [11]. *Ppa-mom-2* is specifically expressed in the AC in the late J2 and J3 larval stages before the onset of VPC divisions but in no other cells of the developing gonad (Figure 3A). *Ppa-egl-20* is dynamically expressed in the posterior part of the animal. *Ppa-egl-20* expression starts in the J2 stage in the pre-anal blast cells U and Y and is in later stages also seen in the post-anal region (Figures 3B and 3C). Thus, *Ppa-egl-20* expression is very similar to that of *Cel-egl-20* [12].

Next, we analyzed the expression patterns of *Ppa-cwn-1*, *Ppa-cwn-2*, and *Ppa-lin-44*. Although there was no detectable expression of *Ppa-cwn-1* and *Ppa-cwn-2* (data not shown), *Ppa-lin-44* shows the most dynamic expression pattern of all Wnt ligands. *Ppa-lin-44* expression starts several hours after hatching in several cells of the somatic gonad. In the late J2 and J3 stage, *Ppa-lin-44* is continuously expressed in the central cells of the somatic gonad (Figure 3D). Together, *Ppa-mom-2*, *Ppa-egl-20*, and *Ppa-lin-44* show a differential and

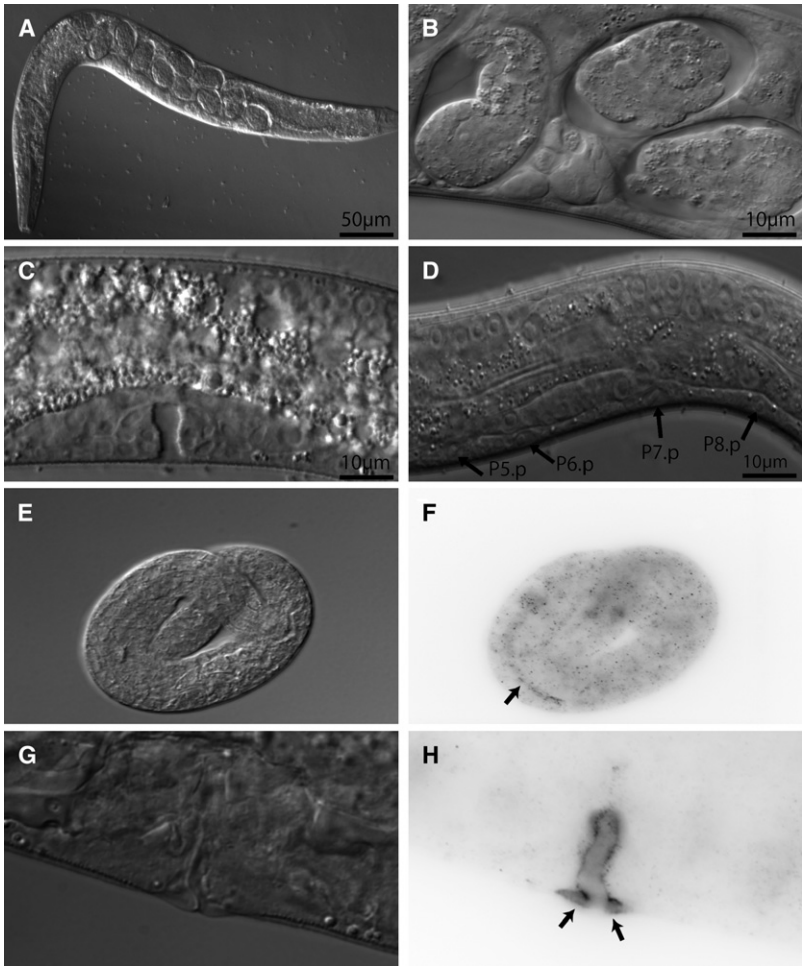

Figure 2. Mutant Phenotype of *Ppa-bar-1* and *Ppa*-BAR-1 Expression

(A–D) Nomarski microscopy of wild-type and *Ppa-bar-1-(tu362)* mutant animals. *Ppa-bar-1* homozygous mutant animals are maternally rescued and have an egg-laying defective phenotype (A). Higher magnification of the embryos inside of the animal in (A) is shown in (B). *Ppa-bar-1* mutants that are not maternally rescued show strong defects in early blastomere specification. A wild-type J4 stage animal with a normal vulva is shown (C). *Ppa-bar-1* homozygous mutants from heterozygous mothers are vulvaless, and P(5–7).p remain epidermal (arrows) (D). (E–H) Whole-mount immunostaining of wild-type *P. pacificus* worms with anti-*Ppa*-BAR-1 antibodies (F and H) and corresponding Nomarski photomicrographs (E and G). Fluorescein isothiocyanate (FITC) fluorescence signals were inverted for better visualization. A "3-fold" stage embryo is shown (E). *Ppa*-BAR-1 is widely expressed and specifically stains the embryonic gut (arrowheads) (F). The vulva of a young adult is shown (G). *Ppa*-BAR-1 localizes close to the lumen of the vulval invagination in all vulval cells, but especially strong to the apical domain of the outermost 2° cells (black arrowheads) (H).

dynamic expression during *P. pacificus* postembryogenesis. These expression patterns are reminiscent of the expression patterns of the orthologous genes in *C. elegans* [13]. In contrast, *Ppa-lin-3*, the only known TGF-alpha member in the *P. pacificus* genome, is not expressed in the somatic gonad at the time of vulva induction (Figures S3A and S3B). Also, *Ppa-lin-3* is not expressed in *Ppa-groucho* animals that are Muv and is therefore unlikely to play a major role in vulva induction (Figure S3C).

Ppa-lin-17/Fz is a negative regulator of vulva formation, and *Ppa-lin-17(tu108); Ppa-ced-3(tu104)* double mutants are Muv (Table 1). Several Wnt pathway genes, including *Ppa-bar-1* and *Ppa-egl-20*, were shown by MO studies to act together with *Ppa-lin-17* [4]. Because ectopic vulva formation in gonad-ablated *Ppa-lin-17* mutants must be independent of *Ppa-mom-2* and *Ppa-lin-44*, which are only expressed in the gonad, we studied the genetic interaction between *Ppa-lin-17* and *Ppa-egl-20.* We found that *Ppa-egl-20* completely suppressed the phenotype of *Ppa-lin-17*. First, *Ppa-lin-17(tu108); Ppa-egl-20(tu364)* double mutants form a wild-type vulva (Table 1). Second, after gonad ablation, all VPCs had a 3° cell fate in all 17 analyzed double-mutant animals, indicating that a mutation in *Ppa-egl-20* can suppress the gonad-independent differentiation of VPCs of *Ppa-lin-17* mutants (Table 1). *Ppa-bar-1* also suppressed *Ppa-lin-17*, and double mutants are Vul, whereas *Ppa-lin-17; Ppa-mom-2* double mutants undergo vulva differentiation (Table 1). These results suggest that several Wnt-pathway genes are involved in both an inductive and a repressive function of Wnt signaling.

This study provides the first genetic evidence for the molecular mechanism of vulva induction in a nematode outside of *Caenorhabditis*. In *P. pacificus*, cell ablation studies revealed a continuous inductive signal that involves multiple cells of the somatic gonad and lasts for more than 10 hr [5]. Ablation of the somatic gonad at different time points in larval development results in a successive increase of induced vulval fates. Only if the somatic gonad is ablated at hatching, do all VPCs have a 3° fate. *Ppa-bar-1* and Wnt triple mutants phenocopy the result of gonad-ablation experiments. Similarly, the differential and dynamic expression patterns, in particular the one of *Ppa-lin-44*, are consistent with the continuous induction of the *P. pacificus* vulva, suggesting a key role of Wnt signaling in *P. pacificus* vulva induction.

A complex network of Wnt signaling pathways is involved in vulva induction and vulva repression in *P. pacificus*. This conclusion is based on the combination of two reverse-genetic approaches, deletion-based knockout and MO-based knockdown. The original finding that *Ppa-bar-1*, *Ppa-egl-20*, and other Wnt-pathway genes act together with *Ppa-lin-17* were made in MO-treated *Ppa-lin-17* animals, indicating that the mutant background provides a sensitized system in which a weak reduction of gene function can be studied. Similar findings have been made in *C. elegans* for the application of RNA interference (RNAi) during vulva development [14]. Our data suggest that *bar-1* might act in the inductive and the repressive pathway, a finding that could be related to the reduced number of β-catenin-like genes in the *P. pacificus* genome.

Some aspects of the exact molecular mechanism of Wnt signaling in *P. pacificus* vulva formation, such as the suppression of *Ppa-lin-17* by *Ppa-egl-20* mutants, await further analysis and the isolation of mutants in additional Wnt genes. Although this study supports the importance of Wnt signaling, we have so far not obtained any evidence for EGF/RAS signaling in *P. pacificus* vulva induction (H. X. and R. J. S., unpublished data) (Figure 1). We speculate, therefore, that the role of EGF

Table 1. Multiple Wnts and *Ppa-lin-18* Are Involved in Vulva Induction

Strain	n	% Wild-Type (3)	% Underinduced (<3)	% Vulvaless (0)	% P7.p Defects (3)	% Overinduced (>3)	% Others (3)	Average Number Induced VPCs ± Standard Deviation
Wild-type *PS312*	100	100	0	0	0	0	0	3.0 ± 0.0
Ppa-bar-1(tu362)	118	0	1	99	0	0	0	0.0 ± 0.0
Ppa-groucho(tu102)	91	13	0	0	0	87	0	4.1 ± 0.6
Ppa-bar-1(tu362); Ppa-groucho(tu102)	50	0	64	2	0	10	24	2.1 ± 1.0
Ppa-egl-20(tu382)	50	100	0	0	0	0	0	3.0 ± 0.0
Ppa-egl-20(tu364)	48	100	0	0	0	0	0	3.0 ± 0.0
Ppa-mom-2(tu363)	56	83	11[a]	0	6	0	0	2.9 ± 0.3
Ppa-cwn-2(tu373)	50	100	0	0	0	0	0	3.0 ± 0.0
Ppa-lin-18(tu359)	43	93	5	0	2	0	0	3.0 ± 0.2
Ppa-mom-2(tu363); Ppa-lin-18(tu359)	62	2	35	39	19	0	5	1.3 ± 1.2
Ppa-mom-2(tu363); Ppa-lin-18(tu359); Ppa-egl-20(tu364)	52	0	40	60	0	0	0	0.5 ± 0.7
Ppa-mom-2(tu363); Ppa-egl-20(tu364)	103	81	19	0	0	0	0	2.8 ± 0.4
Ppa-cwn-2(tu373); Ppa-egl-20(tu364); Ppa-lin-18(tu359)	52	100	0	0	0	0	0	3.0 ± 0.0
Ppa-cwn-2(tu373); Ppa-egl-20(tu364)	50	100	0	0	0	0	0	3.0 ± 0.0
Ppa-egl-20(tu364); Ppa-lin-18(tu359)	41	98	2	0	0	0	0	3.0 ± 0.2
Ppa-cwn-2(tu373); Ppa-lin-18(tu359)	86	99	1	0	0	0	0	3.0 ± 0.1
Ppa-lin-17(tu33)	50	74	0	0	26	0	0	3.0 ± 0.0
Ppa-lin-17(tu108)	86	50	0	0	50	0	0	3.0 ± 0.0
Ppa-lin-17(tu108); Ppa-ced-3(tu104)	44	23	0	0	50	50	0	3.8 ± 0.8
Ppa-lin-17(tu108); Ppa-egl-20(tu364)	50	100	0	0	0	0	0	3.0 ± 0.0
Ppa-lin-17(tu108); Ppa-egl-20(tu382)	52	100	0	0	0	0	0	3.0 ± 0.0
Ppa-lin-17(tu33); Ppa-egl-20(tu364)	65	100	0	0	0	0	0	3.0 ± 0.0
Ppa-lin-17(tu33); Ppa-egl-20(tu382)	49	100	0	0	0	0	0	3.0 ± 0.0
Ppa-lin-17(tu108); Ppa-egl-20(tu364) Gonad $^-$	17	0	0	100	0	0	0	0.0 ± 0.0
Ppa-lin-17(tu108); Ppa-bar-1(tu362)	103	0	0	100	0	0	0	0.0 ± 0.0
Ppa-lin-17(tu108); Ppa-mom-2(tu363)	45	2	0	0	67	0	31	3.0 ± 0.0
Ppa-lin-17(tu108); Ppa-cwn-2(tu373)	51	47	6	0	47	0	0	2.9 ± 0.2
Ppa-cwn-2(tu373); Ppa-egl-20(tu364); Ppa-lin-17(tu108)	45	100	0	0	0	0	0	3.0 ± 0.0

Animals were scored as "Others" if three VPCs were induced but did not form a wild-type vulva. The number of induced VPCs is shown in parentheses.
[a] In *Ppa-mom-2*, P(5,7).p do not always undergo three rounds of cell division, resulting in a special case of underinduction.

signaling in *C. elegans* vulva induction represents a derived character that might have coevolved with the restriction of the inductive signal to the single anchor cell.

Supplemental Data

Experimental Procedures, four figures, and two tables are available at http://www.current-biology.com/cgi/content/full/18/2/142/DC1/.

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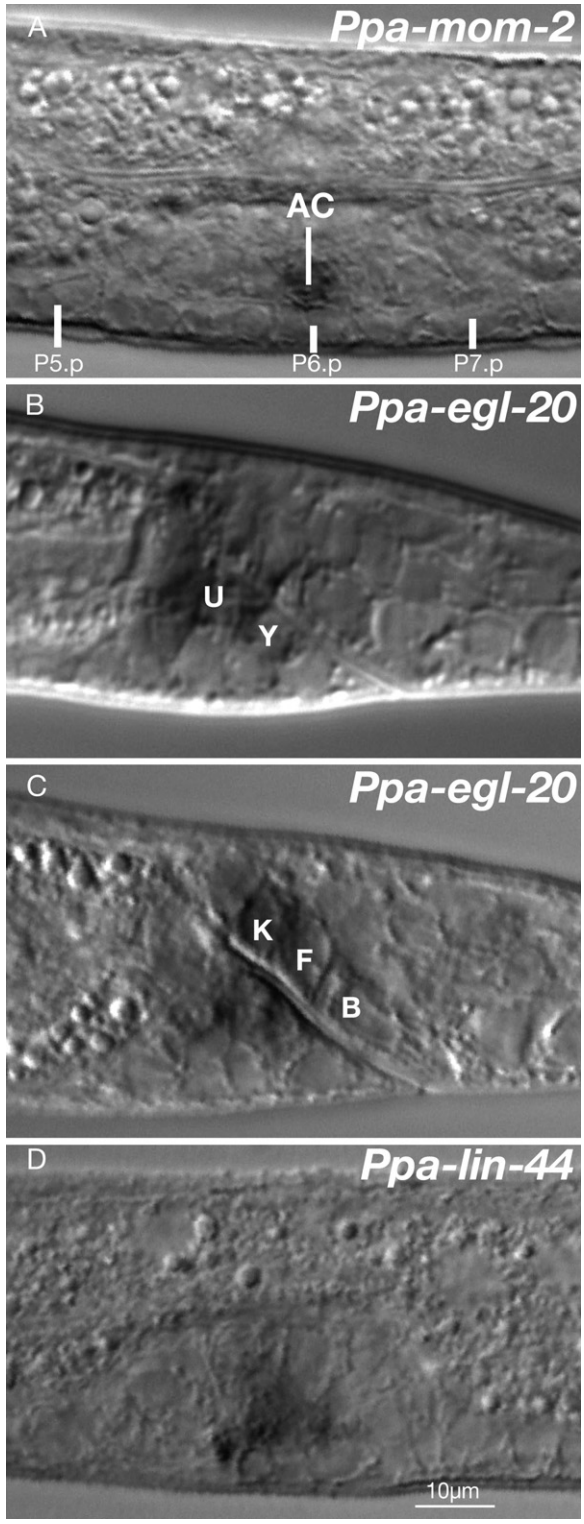

Figure 3. Expression-Pattern Analysis of the Wnt Ligands *Ppa-mom-2*, *Ppa-egl-20*, and *Ppa-lin-44* by In Situ Hybridization

(A) Early J3 stage animal, mid body region. *Ppa-mom-2* is specifically expressed in the AC.

(B) Late J2 stage, posterior body region. *Ppa-egl-20* is dynamically expressed in juvenile stages. *Ppa-egl-20* is expressed in the pre-anal region in the blast cells U and Y.

(C) Early J3 stage, posterior body region. *Ppa-egl-20* expression is also observed in the post-anal cells B and F and in the progeny of K.

(D) Mid J2 stage, mid body region. *Ppa-lin-44* is already expressed in the mid J2 stage and is the first Wnt gene to be expressed in the somatic gonad.

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