Development 125, 3667-3680 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV5223

The β-catenin homolog BAR-1 and LET-60 Ras coordinately regulate the Hox gene *lin-39* during *Caenorhabditis elegans* vulval development

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Accepted 10 July; published on WWW 25 August 1998

SUMMARY

In *C. elegans*, the epithelial Pn.p cells adopt either a vulval precursor cell fate or fuse with the surrounding hypodermis (the F fate). Our results suggest that a Wnt signal transduced through a pathway involving the β-catenin homolog BAR-1 controls whether P3.p through P8.p adopt the vulval precursor cell fate. In *bar-1* mutants, P3.p through P8.p can adopt F fates instead of vulval precursor cell fates. The Wnt/*bar-1* signaling pathway acts by regulating the expression of the Hox gene *lin-39*, since *bar-1* is required for LIN-39 expression and forced *lin-39* expression rescues the *bar-1* mutant phenotype. LIN-39 activity is also regulated by the anchor cell signal/*let-23* receptor tyrosine kinase/*let-60* Ras signaling pathway. Our genetic and molecular experiments show that the vulval precursor cells can integrate the input from the BAR-1 and LET-60 Ras signaling pathways by coordinately regulating activity of the common target LIN-39 Hox.

Key words: β-catenin, Vulva, *Caenorhabditis elegans*, Wnt, Hox, BAR-1, LET-60 Ras

INTRODUCTION

Extracellular signals play a crucial role in controlling differentiation pathways during development. In many cases, several external signals act together to coordinately regulate the developmental fate of individual cells or tissues. Examples include mesoderm induction in *Xenopus* (Northrop et al., 1995; Watabe et al., 1995), limb formation in the chick (Johnson and Tabin, 1997), and imaginal disc and midgut development in *Drosophila* (Axelrod et al., 1996; Riese et al., 1997). Although we have learned much about how cells respond to individual signaling pathways, we are only beginning to understand how cells coordinate input from multiple pathways into a single developmental decision. In this paper, we show that the *C. elegans* vulval precursor cells integrate the input from two widely used signal transduction pathways, a Wnt signaling pathway and a receptor tyrosine kinase (RTK)/Ras pathway. These two signaling pathways coordinately control cell fate specification by regulating a common Hox target gene during vulval development in *C. elegans*.

C. elegans vulval formation begins in the first larval stage (L1) when six cells from the left side and six cells from the right side interdigitate in a semi-stochastic fashion, forming a single row of 12 cells along the ventral midline that are renamed P1 to P12 in their anterior to posterior order (Sulston and Horvitz, 1977). Each of the Pn cells (P1 through P12) divides to generate a Pn.a neuroblast and a Pn.p hypodermal (epithelial) cell. Five Pn.p cells (P1.p, P2.p, P9.p, P10.p, P11.p) adopt the F (fused) fate which is to fuse with the surrounding hypodermal syncytium in the L1 stage. An additional cell (P3.p) adopts the F fate in the late L2 stage in about 50% of wild-type animals (Sulston and White, 1980; Sternberg and Horvitz, 1986). One cell (P12.p) generates an anterior daughter that fuses with the syncytial hypodermis and a posterior daughter that undergoes programmed cell death. The remaining five or six cells (P4.p through P8.p and sometimes P3.p) become the vulval precursor cells (see Fig. 7A).

P4.p always adopts the vulval precursor cell fate, while P3.p can adopt either the F fate or the vulval precursor cell fate. The difference between the P3.p cell fate and the P4.p cell fate is not due to differences in cell lineage, as either cell can be derived from an ABpl descendant from the left (P3/4L) or an Abpr descendant from the right (P3/4R). Instead, the cell fate difference between P3.p and P4.p is likely to be due to extracellular signaling, since whichever cell ends up anterior following migration into the ventral midline (defined as P3) generates a posterior daughter that adopts the F fate in 50% of animals whereas whichever cell ends up posterior (defined as P4) generates a daughter that always adopts the vulval precursor cell fate (Sulston and Horvitz, 1977; Sulston and White, 1980). This observation indicates that either P3.p and/or P4.p respond to environmental cues that specify their position along the anterior to posterior axis.

After the vulval precursor cells have been generated, additional cell signaling interactions specify whether these cells adopt the 1°, 2° or 3° vulval cell fates in the late L2/early

L3 stage (reviewed in Eisenmann and Kim, 1994; Greenwald, 1997; Kornfeld, 1997). First, the anchor cell in the somatic gonad sends an inductive signal that causes P6.p to adopt the 1° vulval cell fate, which is to generate eight progeny cells that form the inner parts of the developing vulva. Specification of the 1° cell fate involves activation of an RTK/Ras/MAP kinase signaling pathway in P6.p. Key components of this pathway include the RTK LET-23, the Ras protein LET-60, the MAP kinase MPK-1/SUR-1, the Winged-Helix (WH) transcription factor LIN-31 and the Ets transcription factor LIN-1. P6.p then sends a lateral signal that causes the adjacent vulval precursor cells (P5.p and P7.p) to adopt the 2° vulval fate (Simske and Kim, 1995), which is to generate seven progeny cells that form the outer parts of the developing vulva. Specification of the 2° cell fate involves activation of a *lin-12 Notch* signaling pathway. Some experiments suggest that low levels of the anchor cell signal can also contribute to specification of the 2° cell fate (Katz et al., 1995). Vulval precursor cells that do not receive either the anchor cell signal or the lateral signal adopt the uninduced 3° cell fate, which is to generate two epithelial cells that fuse with the hypodermal syncytium.

The Hox gene *lin-39* is required two times during Pn.p cell fate specification, first for the specification of the vulval precursor cell versus F fate (during the L1 larval stage) and then again for the specification of the 1° versus 2° versus 3° cell fates (in the early L3 larval stage). Mutations that eliminate *lin-39 Hox* gene activity cause P3.p through P8.p to become F cells rather then vulval precursor cells (Clark et al., 1993; Wang et al., 1993). Mutations that partially reduce *lin-39 Hox* activity permit some of the Pn.p cells to become vulval precursor cells. These vulval precursor cells then exhibit defects in the specification of the 1°, 2° and 3° cell fates during the L3 stage. Specifically, vulval precursor cells adopt 3°, F and hybrid cell fates instead of 1° and 2° cell fates (Clandinin et al., 1997; Maloof and Kenyon, 1998). *lin-39 Hox* expression is regulated by the activity of the *let-60 ras* pathway, since loss-of-function mutations in *lin-3* (encodes a homolog of epidermal growth factor) or *let-23 RTK* result in diminished LIN-39 expression, and gain-of-function mutations in *let-60 ras* result in elevated expression (Maloof and Kenyon, 1998).

In this paper, we present data suggesting that the signaling process controlling the F versus vulval precursor cell fate is a Wnt signaling pathway involving a β-catenin-related protein. Wnt signaling pathways regulate pattern formation in many animals and a key protein in the Wnt signaling pathway is vertebrate β-catenin/*Drosophila* Armadillo (Arm) (reviewed in (Miller and Moon, 1996; Cadigan and Nusse, 1997; Moon et al., 1997)). In the absence of Wnt ligand, the activities of the tumor suppressor gene product APC and the serine/threonine kinase GSK3 (glycogen synthase kinase 3) lead to the degradation of β-catenin protein that is present in the cytoplasm. When Wnt proteins bind to their receptors (seven transmembrane receptors related to the *Drosophila frizzled* gene product), the activity of GSK3 is inhibited and cytoplasmic β-catenin is stabilized. This β-catenin interacts with transcription factors of the LEF-1/TCF family to form a protein complex that regulates expression of downstream target genes.

Results presented here lead to three main conclusions. First, the choice of vulval precursor cell fate versus F fate for P3.p through P8.p is mediated by the β-catenin/Armadillo-related protein BAR-1, most likely functioning as a component of a Wnt signaling pathway. Second, the *bar-1* signaling pathway functions to maintain the expression of the Hox gene *lin-39*. Finally, the *bar-1* signaling pathway and *let-23 RTK*/*let-60 ras* signaling pathway coordinately regulate *lin-39 Hox* activity in order to specify vulval precursor cell fates.

MATERIALS AND METHODS

Genetic methods and alleles

Methods for culturing, handling and genetic manipulation of *C. elegans* were as described (Brenner, 1974). The animals described as wild type were *C. elegans*, variety Bristol, strain N2. All experiments were performed at 20°C unless indicated. All strains were constructed by standard genetic methods (Brenner, 1974). The following genes and alleles were used in this work (unless otherwise indicated, the references for these alleles are Wood (1988) and Riddle et al. (1997).

LGI: *unc-29(e1072)*

LGII: *lin-31(n1053)* (Ferguson and Horvitz, 1985), *let-23(sy1)* (Aroian and Sternberg, 1991)

LGIII: *ncl-1(e1942)*

LGIV: *lin-1(n304)* (Beitel et al., 1995), *let-60(n1046)* (Ferguson et al., 1987), *dpy-20(e1282)*, *unc-30(e191)*

LGV: *him-5(e1490)*

LGX: *bar-1(ga80)* (D. M. E. and S. K. K., unpublished data), *uDf1*

The mutation designated *gaIs37* is an integrated array of plasmids that express the *D. melanogaster* Mek gene *Dsor-1* and an activated form of the *C. elegans* MAP kinase encoded by the *mpk-1* gene (Lackner and Kim, 1998). This array causes a temperature-sensitive multivulva phenotype.

Ablation of the cells Z1 and Z4, the precursors of the somatic gonad (including the anchor cell), in newly hatched *bar-1(ga80)* L1 larvae was performed as described (Miller et al., 1993).

Mosaic analysis of *bar-1* was performed essentially as described (Simske and Kim, 1995; Miller et al., 1996) using the strain *unc-29; ncl-1; unc-30; bar-1; gaEx117*[F35D3; C33C3; pSC11; pDE204].

Heat shocks were done essentially as described by Maloof and Kenyon (1998) on animals containing the integrated array *muIs23* [pCH17.1 (hs-*lin-39*); pMH86 (*dyp-20(+)*)] (Hunter and Kenyon, 1995). Briefly, a PTC-100 thermal cycler (MJ Research) was used to deliver an 11-minute, 31°C heat shock once ever 4 hours from hatching to early L3 (7 heat pulses total). After an additional 4 hours, a single 8-minute, 33°C heat shock was given.

The cell fates adopted by P3.p-P8.p were scored in living, early L4 hermaphrodites observed with Nomarski differential interference contrast optics using the criteria for designation of cell fate described in Sternberg and Horvitz (1986). Cells were assigned a fate of either 1°, 2°, 3°, I (Induced – cells adopted an abnormal, partial or hybrid lineage) or F (Fused). At 25°C, the majority of *unc-30; bar-1*; *gaIs37* animals burst at the vulva at the L4 molt and die, while these animals are wild type at 15°C. Therefore, to measure vulval induction in this strain, eggs from a *gaIs37* strain and Egl adults from a *bar-1*; *gaIs37* strain were moved from 15°C to 25°C and allowed to develop at that temperature. Early L4 stage animals from both strains were picked from 25°C and observed by Nomarski photomicroscopy for the extent of vulval induction and for the fate of P12.p. In this experiment, we scored those animals with inductions at P3.p, P4.p or P8.p as 'Muv' in Table 1 and those animals with no inductions at these cells as non-Muv, even though adult animals were not scored.

Molecular biology

Standard techniques for DNA manipulations were used (Ausubel et al., 1987). The plasmid pDE204 rescues the phenotypes of a *bar-1(ga80)* strain (100% wild type for Egl, Unc and P12.p phenotypes, *n*=200) and contains a 10.4 kb *Spe*I-*Bgl*II fragment of genomic DNA from cosmid F43E12 cloned into the vector pUC119 digested with

*Xba*I and *BamH*I. This construct contains unique *Bam*HI sites in the first and last exons of the *bar-1* open reading frame. The plasmid pDE248 was constructed by replacing the genomic DNA between these *Bam*HI sites with a similar fragment from a complete *bar-1* cDNA. The *bar-1::GFP* reporter plasmid pDE218 was constructed by ligating together: (1) a 6.3 kb *Pst*I-*Cla*I fragment containing *bar-1* upstream DNA (from the *Bgl*II site of pDE204 to the *Cla*I site in the open reading frame), (2) a 3.1 kb *Cla*I-*Bam*HI fragment containing *bar-1* genomic DNA encoding residues 1-761, and (3) the GFP reporter vector pPD95.75 digested with *Pst*I and *Bam*HI. The resulting plasmid rescues the *bar-1* mutant phenotypes. The plasmid pDE274 (*pbar-1::lin-39*) was made by introducing *Bam*HI sites at the start and stop codons of the *lin-39* cDNA by PCR amplification from plasmid 160.111 (Wang et al., 1993), and inserting this *Bam*HI fragment into pDE204 digested with *Bam*HI. The resulting fusion encodes the first four amino acids of BAR-1 fused to full-length LIN-39, with *bar-1* 5′ and 3′ genomic sequences.

Using partial sequence information for the *bar-1* rescuing cosmids generated by the *C. elegans* Genome Sequencing Consortium, we designed oligonucleotide primers and obtained *bar-1* cDNAs by the RACE technique. Using oligo(dT) and *bar-1* primers, we obtained partial cDNAs containing the 3′ end of the *bar-1* transcript. Using primers designed against the spliced leader sequence SL1 and *bar-1* primers, we obtained partial cDNAs containing the 5′ end of the *bar-1* transcript. We then designed nested primers for further PCR and isolated a complete *bar-1* cDNA (plasmid pDE219). All PCR products were subcloned into the vector pCRII (Invitrogen). The 2634 bp *bar-1* cDNA encodes a 22 bp SL1 spliced leader sequence, 12 bp of 5′ untranslated sequence, 17 exons, and a 3′ untranslated region of 149 bp, followed by a poly(A) tail. We have completely sequenced both strands of the *bar-1* cDNA and verified the splicing pattern indicated in Fig. 2. The complete bar-1 cDNA nucleotide sequence has been deposited in the GenBank sequence library under Accession number AF063646.

To identify the mutation in the *bar-1(ga80)* strain, we used the method of RNAse Cleavage Mismatch Detection (Ambion). Briefly, 6 sets of overlapping, nested primers were used to amplify the genomic region encompassing the BAR-1 open reading frame from N2 and *bar-1(ga80)* animals. The top and bottom strands of the resulting PCR products were then converted into RNA by in vitro transcription with SP6 and T7 polymerases. These separate RNA products were mixed together in the following manner, N2+N2, *ga80*+*ga80* and N2+*ga80*, and reannealed. These mixtures were treated with RNAse, loaded on an agarose gel and analyzed for differences in the pattern of bands seen in the hybrid mixture lane versus the parental mixture lanes. In this way, the *bar-1(ga80)* mutation was localized and the region containing the difference was amplified and sequenced from two independent *bar-1(ga80)* animals.

See http://cmgm.Stanford.EDU/~kimlab/ for a list of primers used.

Germline transformation

Germline transformation experiments with *unc-30(e191); bar-1(ga80)* animals were performed as described (Mello et al., 1991). Each cosmid and plasmid DNA was injected at 50-100 μg/ml. The plasmid pSC11(*unc-30(+)*) (Jin et al., 1994) was used as a cotransformation marker, and transformants were identified by their non-Unc phenotype. The arrays *gaIs45* and *gaIs46* contain plasmids pDE218 and pSC11 or plasmids pDE204 and pSC11 integrated into the genome at an unknown site. For each construct tested for *bar-1(ga80)* rescue or BAR-1 expression, two or more transformed lines were analyzed. For the mosaic analysis of *bar-1*, the *unc-29; ncl-1; unc-30; bar-1(ga80)* strain was transformed with a mixture of the following four DNAs: pSC11 (*unc-30(+)*), pDE204 (*bar-1(+)*), C33C3 (*ncl-1(+)*) and F35D3 (*unc-29(+)*).

Antibody production, staining and immunofluorescence

To create a TrpE-BAR-1 fusion protein, we subcloned the *Bam*HI fragment from pDE219 encoding BAR-1 amino acids 4-762 into the *Bam*HI site of the pATH10 vector (Koerner et al., 1991), creating pDE223. This protein was expressed in *E. coli*, purified by preparative SDS-PAGE and injected into rats and rabbits (Josman Labs). We also prepared a GST-BAR-1 fusion protein by subcloning a piece of pDE219 encoding BAR-1 amino acids 179-794 into the vector pGEX-1lambdaT (Pharmacia Biotech) to create pDE216. Antisera from immunized rats and rabbits was purified against this GST-BAR-1 fusion protein immobilized on nitrocellulose as described (Olmsted, 1981). One purified rat antisera (T1) stained worms containing the array *gaIs46*, but not wild-type worms, indicating that the antisera recognized BAR-1 only when overexpressed. The pattern of staining observed with T1 antisera is similar to that observed with BAR-1-GFP from *gaIs45*.

Partially synchronous populations of animals were obtained by first starving L1 animals and then harvesting them for staining at the L2 and L3 stages. For stains with monoclonal antibody MH27 (Francis and Waterston, 1991), LIN-31 antibodies (Tan et al., 1998) or BAR-1 (T1) antibodies, larvae were fixed and stained as described by Finney and Ruvkun (1990) with minor modifications, and observed by immunofluorescent microscopy. LIN-39 and MH27 co-staining was done as described (Maloof and Kenyon, 1998), except that methanol fixation was increased to 5 minutes, and fluorescein-conjugated goat anti-mouse antibodies were used to detect MH27.

RESULTS

bar-1(ga80) results in defects in Pn.p cell fate specification

The *bar-1(ga80)* mutation was isolated in a genetic screen to identify mutants showing partial defects in vulval induction that cause animals to have a protruding vulva phenotype (D. M. E. and S. K. K., unpublished data). *bar-1(ga80)* animals exhibit two defects in cell fate specification by P3.p through P8.p. First, the *bar-1(ga80)* mutation can cause P3.p through P8.p to adopt the F fate rather than the vulval precursor cell fate. In *bar-1* mutants, P3.p and P4.p almost always adopt the F fate (greater than 90% of cells), while P5.p, P6.p, P7.p and P8.p adopt the F fate less frequently (about 12-36% of cells) (Table 1; Fig. 1).

In wild-type animals, Pn.p cells adopt the F cell fate in the L1 stage (P1.p, P2.p, P9.p, P10.p and P11.p) (Sulston and Horvitz, 1977) or the late L2 stage (P3.p) (Sternberg and Horvitz, 1986) (Fig. 6C). To determine when Pn.p cells adopt the F fate in *bar-1(ga80)* mutants, we examined the time at which they fuse with the hypodermal syncytium, hyp7. The monoclonal antibody MH27 stains the cell junctions of all epithelial cells including the vulval precursor cells but does not stain F cells after they have fused with hyp7 (Francis and Waterston, 1991). During the late L1/early L2 stage, MH27 staining of P3.p through P8.p in *bar-1* mutants was identical to that of wild-type animals, indicated that at this time none of these cells had yet fused with the hypodermal syncytium to become F cells (Fig. 1D,E; at this time, P1.p, P2.p, P9.p, P10.p and P11.p have already adopted the F fate). However, during the late L2 period, MH27 staining in P3.p through P8.p in *bar-1(ga80)* mutant animals was often missing; specifically, MH27 staining was absent from P3.p and P4.p in greater than 90% of these cells in different animals, and was absent from P5.p, P6.p, P7.p or P8.p between 2% and 17% of the time in these cells in individual animals. (Fig. 1G; Table 3). Thus, P4.p through P8.p in *bar-1(ga80)* mutants adopt the F fate at a similar time (late

Table 1. Phenotypes of *bar-1* single and double mutants

	F fates:3° fates observed*								
Strain	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	N†	% Muv‡	N†
N2	44:56	0:100	0:0	0:0	0:0	0:100	80	0	300
bar-1	96:4	94:6	36:6	17:7	14:30	12:88	100	0	180
let-60	2:15	0:13	0:0	0:0	0:0	0:40	55	95	300
let-60; bar-1	94:6	90:2	4:0	0:2	11:6	10:77	52	22	200
gaIs37 at 25°	nd	nd	nd	nd	nd	nd		94	148
gaIs37; bar-1 at 25°	nd	nd	nd	nd	nd	nd		52	278
lin-1	2:5	0:0	0:0	0:0	0:0	0:0	40	100	200
lin-1; bar-1	48:13	35:11	2:0	0:0	4:0	10:8	52	95	274
lin-31	14:31	3:28	0:6	0:0	0:29	1:70	78	88	78
lin-31; bar-1	18:13	10:32	0:2	0:0	2:10	2:72	60	89	147

*Each column gives the percentage of the time each Pn.p cell was observed to adopt the F fate and 3° fate. The remaining percentage of the time, the Pn.p cell adopted an induced cell fate (1°, 2° or hybrid). Strains were observed at the L3 stage using Nomarski photomicroscopy. The following alleles were used: *bar-1(ga80), let-60(n1046), lin-1(n304)* and *lin-31(n1053)*. The integrated array *gaIs37*, which expresses *Drosophila* MEK and an activated form of *C. elegans* MAP kinase, is described in Materials and Methods. nd, not determined.

†Indicates the number of animals scored.

‡Indicates the penetrance of the Multivulva phenotype of the indicated strain as observed on plates using a dissecting microscope (except for *gaIs37* and *gaIs37; bar-1*; see Materials and Methods).

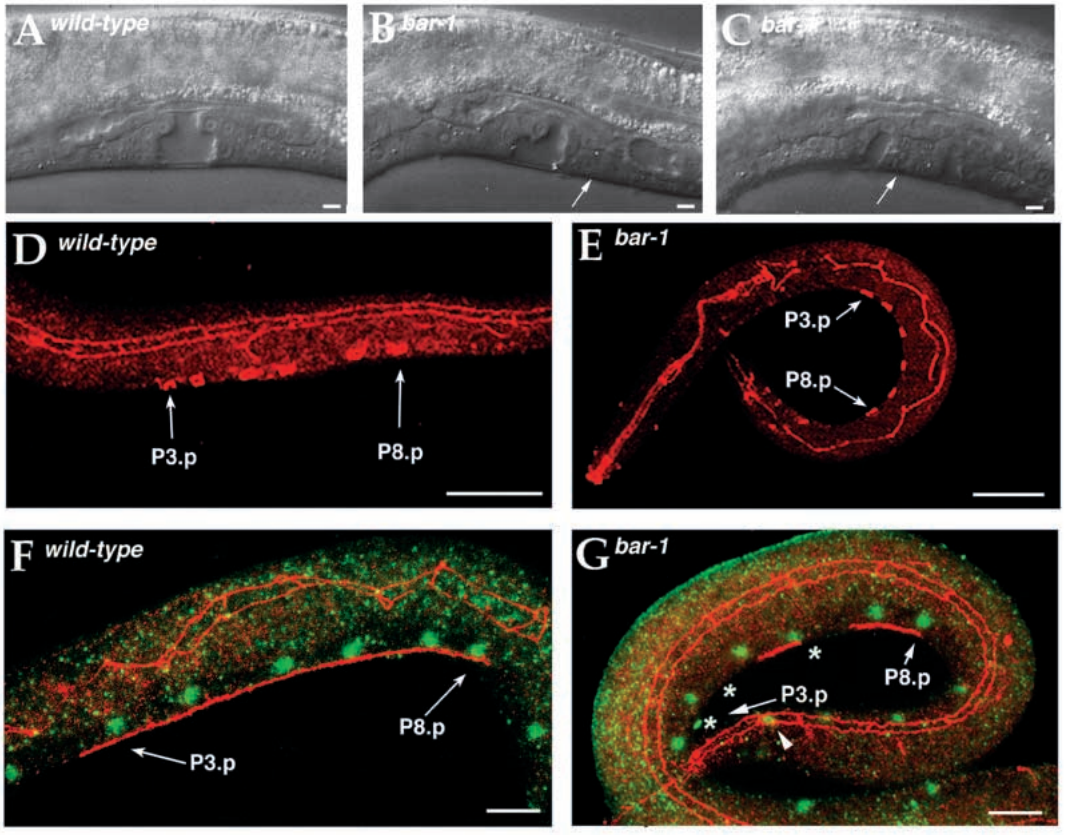


Fig. 1. Defects in the vulval equivalence group in *bar-1* mutants. (A-C). Photomicrographs taken using Nomarski differential interference contrast microscopy of (A) wild-type and (B,C) *bar-1(ga80)* L4 larvae. (A) P5.p-P7.p have finished dividing and their 22 progeny cells are undergoing morphogenesis to form the vulval opening. (B) In this *bar-1(ga80)* animal, P7.p adopted the 3° fate (arrow). (C) In this *bar-1(ga80)* animal, P6.p adopted the F fate (arrow). (D,E). Staining of a wild-type (D) and *bar-1(ga80)* mutant (E) L1 stage larva with the antibody MH27 are equivalent. Wild-type (F) and *bar-1(ga80)* mutant (G) late L2/early L3 stage larva (24-26 hours after hatching, before any vulval precursor cells have begun their first divisions) co-stained with the antibody MH27 (red) and antisera against the transcription factor LIN-31 (green), which is expressed in the cells P1.p-P11.p. (F) Six Pn.p cells (P3.p-P8.p) have not adopted the F fate, indicated by expression of the MH27 antigen. (G) In this *bar-1(ga80)* animal, P3.p, P4.p and P6.p have fused with the hypodermal syncytium, as indicated by their lack of MH27 staining. Note also P12.p expresses LIN-31 in this animal (arrowhead), indicating that P12.p (which does not normally express LIN-31; Tan et al., 1998) has adopted the fate of P11.p (which does). The animal in D is viewed from a slight ventrolateral angle, such that rings of MH27 staining (outlining the cell junctions) are seen. (E-G) Views are completely lateral, so MH27 staining appears as a line, rather than rings. Bars, 10 μm.

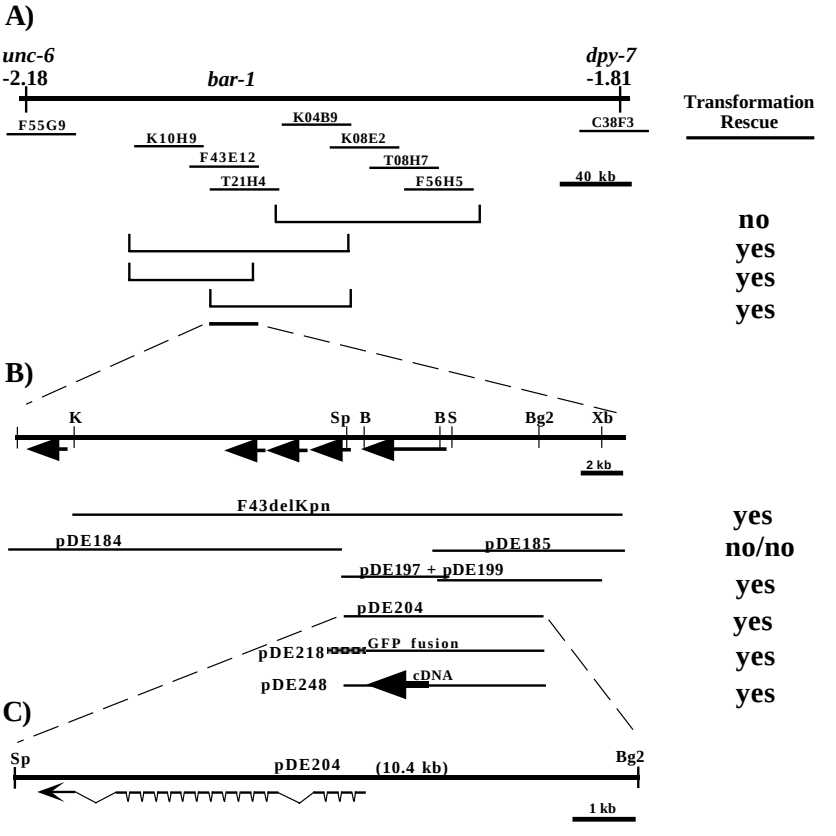
L2) to that of P3.p in the wild type, suggesting that *bar-1(+)* functions in a signaling pathway that distinguishes the P3.p cell fate from the P4.p through P8.p cell fates.

The second *bar-1(ga80)* phenotype is that vulval precursor cells are defective in the specification of the 1°, 2° and 3° cell fates during the L3 stage; specifically, P5.p, P6.p and P7.p sometimes adopt the 3° fate in *bar-1* mutants instead of the 2°, 1° and 2° cell fates as in wild-type animals, respectively (Table 1; D. M. E. and S. K. K., unpublished data). Activation of the anchor cell and lateral signaling pathways causes the vulval precursor cells to express the 1° and 2° cell fates. The *bar-1* vulval cell specification phenotype is similar to the partial reduction-of-function phenotype of genes in the anchor cell signaling pathway, such as *let-23 RTK*, *let-60 ras* and *mpk-1* (Greenwald, 1997; Kornfeld, 1997). This result suggests that *bar-1(+)* activity may be necessary for the vulval precursor cells to efficiently transduce the anchor cell signal, or to efficiently respond to activation of the anchor cell signaling pathway.

bar-1 encodes a β-catenin/Armadillo-related protein

To understand how *bar-1* functions at the molecular level, we positionally cloned the *bar-1* gene. *bar-1* was genetically mapped (D. M. E. and S. K. K., unpublished data), and a pool of four cosmids from the this region was found to rescue the *bar-1(ga80)* mutant phenotypes in germline transformation experiments (Fig. 2). Next, the rescuing activity was localized to a 10.4 kb DNA fragment (pDE204) which contains a single predicted gene (predicted by Acedb (Eeckman and Durbin, 1995)). The single gene corresponds to *bar-1*, since expression of a cDNA copy of this gene from its own promoter rescues the *bar-1* phenotypes in transformation experiments (Fig. 2), and a point mutation in *bar-1(ga80)* genomic DNA causes a Glu to Stop codon change at amino acid 97 of the predicted BAR-1 protein (Fig. 3).

We performed RT-PCR on wild-type polyA RNA and obtained a full-length *bar-1* cDNA clone (see Materials and Methods). The *bar-1* transcript encodes a predicted protein of 811 amino acids that is similar to members of the β-catenin/Armadillo/plakoglobin family of proteins (Figs 2D, 3). Members of this β-catenin family function in Wnt signal transduction pathways and in epithelial cell adhesion (Gumbiner, 1995; Miller and Moon, 1996). BAR-1 and other



D)

protein (species)	% identity/% similarity to BAR-1
β-catenin (Hs)	25% / 38%
Armadillo (Dm)	24% / 41%
plakoglobin (Hs)	23% / 43%
HMP-2 (Ce)	28% / 45%
WRM-1 (Ce)	16% / 33%
p120 (Mm)	17% / 35%

Fig. 2. *bar-1* encodes a β-catenin/Armadillo-related protein. (A) Genetic map (above the line) and physical map (below the line) are shown. Pools of cosmids that rescue the *bar-1(ga80)* phenotype in transformation rescue experiments are shown. (B) The region of overlap between cosmids F43E12 and T21H4 contains five predicted open reading frames, indicated with arrows. Plasmid pDE204 is sufficient to rescue the *bar-1(ga80)* mutant phenotype and contains only a single predicted open reading frame. Plasmid pDE218 contains the *bar-1* genomic promoter and open reading frame sequences with GFP (Green Fluorescent Protein; (Chalfie et al., 1994)) coding sequences inserted after BAR-1 residue 761. pDE218 expresses GFP in P3.p-P8.p (Fig. 5). pDE248 contains a *bar-1* cDNA sequence in place of *bar-1* genomic open reading frame sequences in plasmid pDE204. Restrictions enzyme sites are indicated as follows: *Kpn*I (K), *Spe*I (Sp), *Bam*HI (B), *Sal*I (S), *Bgl*II (Bg2) and *Xba*I (Xb). (C) Exon (thick lines) and intron (slanted lines) structure of the *bar-1* gene. *bar-1* cDNAs begin with an SL1 leader and end with a polyA tail (arrowhead). (D) Percent identical and similar amino acids in the predicted BAR-1 protein compared to other β-catenin protein sequences in the Genbank database (Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Mm, *Mus musculus*).

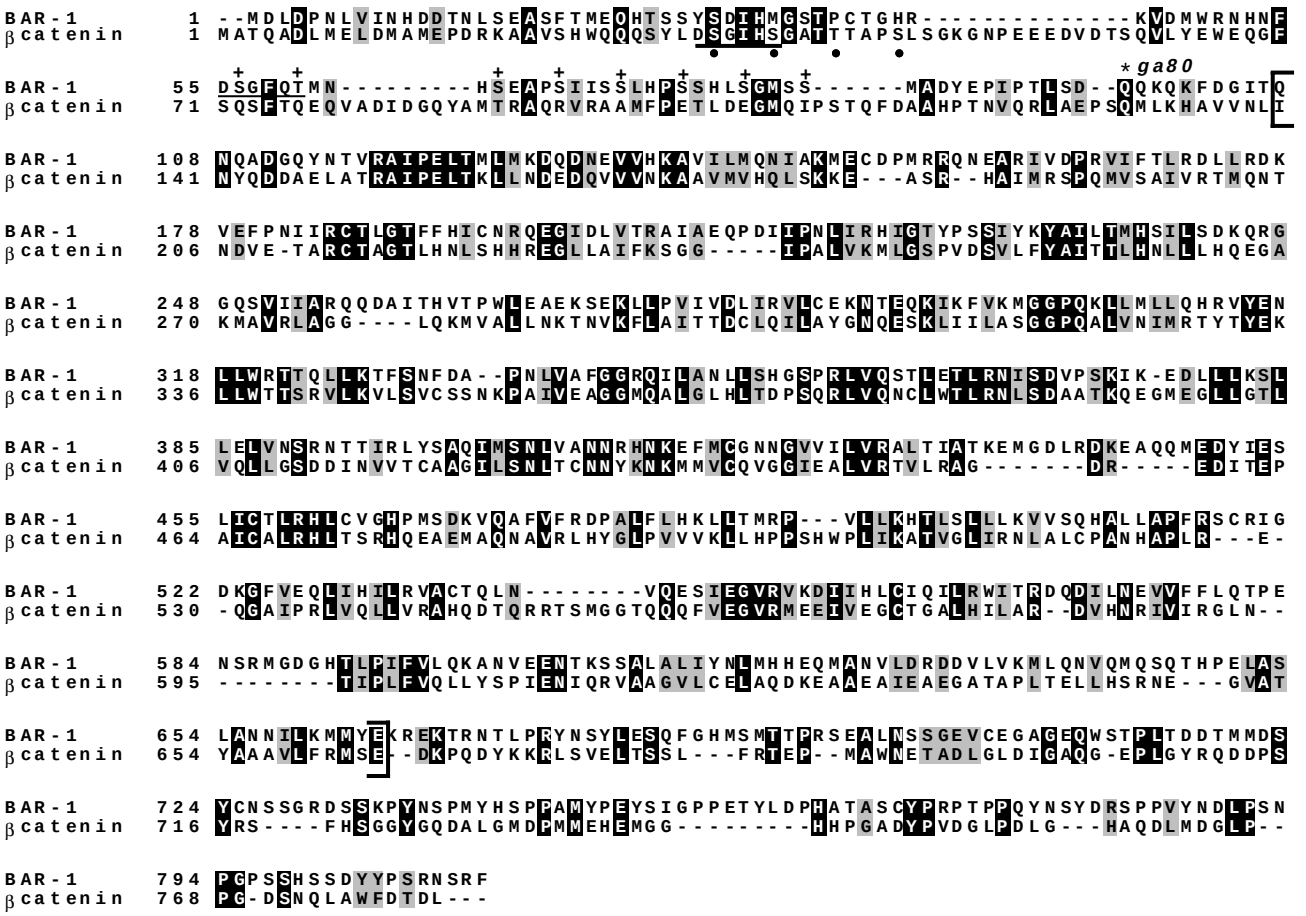


Fig. 3. Homology of BAR-1 and β-catenin. Alignment of the predicted BAR-1 and human β-catenin protein sequences made using the program Clustal (Higgins et al., 1996). Identical residues are boxed in black, similar residues in gray. The 'Arm repeat' region of β-catenin**,** as defined by X-ray crystallographic study (Huber et al., 1997), is indicated within brackets **[ ]**. A sequence in β-catenin and BAR-1 similar to one found in IkB and Cactus and shown to be required for ubiquitin-mediated degradation (Aberle et al., 1997; Orford et al., 1997) is indicated with an underline. Filled circles below the β-catenin sequence indicate consensus phosphorylation sites for the serine/threonine kinase GSK-3. Putative GSK3β phosphorylation sites are indicated above the BAR-1 sequence with '+' signs. An asterisk '*' indicates the position of the CAA to TAA mutation in *bar-1(ga80)*, leading to the insertion of a Stop codon at position 97 of the BAR-1 open reading frame.

members of this family have a common structure consisting of a large, central domain containing 12 Arm repeats flanked by shorter amino-terminal and carboxy-terminal domains (Peifer et al., 1994a; Miller and Moon, 1996; Huber et al., 1997). Because of this protein sequence similarity, we have named this locus *bar-1* for β-catenin/Armadillo-related gene.

Three results indicate that the *ga80* mutation eliminates *bar-1* gene activity. First, the *ga80* mutation is predicted to truncate 88% of the BAR-1 protein, including all of the Armadillo repeats. The truncated region in BAR-1 corresponds to a region in other β-catenins that is essential for interactions with TCF/LEF1, α-catenin and APC (Miller and Moon, 1996; Cadigan and Nusse, 1997). Second, mutations in the *Drosophila armadillo* gene that truncate this region of the protein are null (Peifer and Wieschaus, 1990). Third, the *bar-1(ga80)* homozygous phenotype is similar to the *bar-1(ga80)/uDf1* phenotype (*uDf1* deletes the *bar-1* locus) (data not shown). This result shows that the *ga80* mutation behaves similarly to a deletion of the *bar-1* locus, indicating that *ga80* may be a null allele.

Mosaic analysis of *bar-1* function

To determine which cells require *bar-1* gene function during normal development, we performed a genetic mosaic analysis. We generated mosaic animals in which some cells had *bar-1(+)* activity and other cells lacked it, and then scored these mosaics to see if they exhibited a *bar-1* mutant phenotype. To obtain mosaic animals, we constructed a strain containing loss-of-function mutations in the chromosomal copies of *bar-1* and the cell lineage marker *ncl-1* (Hedgecock and Herman, 1995), and also carrying an extrachromosomal DNA array containing the *bar-1(+)* and *ncl-1(+)* genes. In this strain, the wild-type *bar-1* and *ncl-1* gene activities are only derived from the extrachromosomal array. The extrachromosomal array can be stochastically lost at any cell division in the lineage, generating a founder cell that gives rise to a defined clone of mutant cells. We first scored the Ncl phenotype of individual cells to determine which cells had lost the extrachromosomal array, and then scored the mosaic animals for the *bar-1* phenotype.

Our results indicate that *bar-1* acts in a cell autonomous fashion in P4.p (Fig. 4). We analyzed 13 mosaic animals in

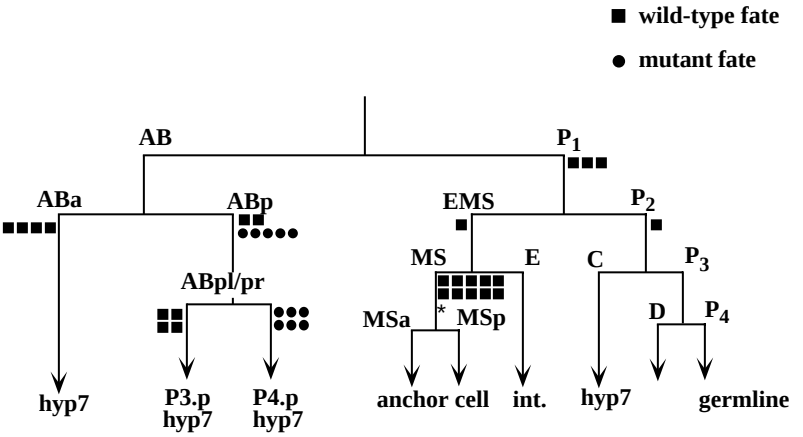


Fig. 4. Genetic mosaic analysis of *bar-1* function in P4.p. Animals from the strain *unc-29(e1072); ncl-1(e1942); unc-30(e191); bar-1(ga80); gaEx117*[F35D3; C33C3; pSC11; pDE204] were identified that had lost the *gaEx117* array in a specific point in the cell lineage based on their Unc and Ncl phenotypes, and then the cell fate adopted by P4.p in these mosaic animals was scored (see Materials and Methods). A portion of the *C. elegans* cell lineage (Sulston and Horvitz, 1977; Sulston et al., 1983) is shown. Vertical lines with arrows indicate many cell divisions. Relevant cell types produced from each lineage branch are indicated underneath. Six Pn.p cells are derived from the ABpl lineage and six from the ABpr lineage, indicated by Abpl/pr. When P3.p is derived from Abpl, P4.p must be derived from Abpr, and vice versa. The anchor cell is derived from either the MSa or MSp lineage. hyp7 and int indicate the hypodermal syncytium and the intestine, respectively. Each square or circle represents an individual animal that was determined to have lost the *gaEx117* array at that point in the lineage. A square indicates a wild-type fate adopted by P4.p. A circle indicates a mutant fate adopted by P4.p (F). An asterisk indicates an animal in which P4.p-P7.p adopted induced fates.

which P4.p lacked the extrachromosomal array and found that this cell adopted the F fate in 11 cases (85% in genetic mosaics compared to 91% in *bar-1(ga80)* control animals). We also analyzed 24 mosaic animals in which P4.p contained the array but in which other cells had lost the array, and found that P4.p did not adopt the F fate in any case. Specifically, we found that *bar-1* activity was not required in any cells derived from ABa, EMS or P1, nor was it required in P3.p for P4.p to develop normally (Fig. 4).

BAR-1 is expressed in the vulval precursor cells

To determine which cells express BAR-1 and where BAR-1 is localized within the cell, we performed immunocytochemistry experiments with BAR-1 antibodies and examined expression of a *bar-1::GFP* reporter gene. We prepared affinity-purified polyclonal antibodies to a recombinant BAR-1 fusion protein produced in *E. coli.* These antibodies did not exhibit BAR-1 staining in wild-type worms, but they did exhibit BAR-1 staining in worms that overexpress *bar-1(+)* from an integrated DNA array (see Material and Methods). We also constructed a *bar-1::GFP* reporter gene (Chalfie et al., 1994) and showed that it rescues all of the *bar-1* mutant phenotypes in transformation rescue experiments, indicating that it is expressed in the cells that require *bar-1* function (Fig. 2). We obtained similar expression patterns using either the BAR-1 antibodies in immunocytochemistry experiments or by direct observation of the functional BAR-1-GFP.

We found that BAR-1 is present in P3.p through P8.p in the late L1/early L2 stage and disappears in the mid-L3 stage before the first divisions of P3.p-P8.p (Fig. 5D-F and data not shown). We did not observe BAR-1 in P1.p, P2.p, P9.p, P10.p or P11.p at this time. P6.p occasionally showed higher BAR-1 expression than the other vulval precursor cells, and was often observed to be the last vulval precursor cell to lose BAR-1 staining (data not shown and Fig. 5H). These expression studies indicate that BAR-1 is expressed in the vulval precursor cells, and are consistent with the genetic mosaic results suggesting that BAR-1 acts cell-autonomously in P4.p, and with the observation of cell fate specification defects in *bar-1* mutants in the L2 and L3 stages.

In the vulval precursor cells, BAR-1 is predominantly cytoplasmic, with some protein present in the nucleus and some at cell junctions (Fig. 5D,H). Localization of BAR-1 in the nucleus is consistent with a function for BAR-1 in a Wnt signaling pathway, since β-catenin is known to translocate into the nucleus in response to Wnt signals. Localization of BAR-1 at the cell junctions is consistent with a function for BAR-1 in cell adhesion (see Miller and Moon, 1996). However, the subcellular localization of the transgenic BAR-1 observed in these experiments may not accurately reflect the subcellular localization of endogenous BAR-1, since transgenic BAR-1 is likely to be overexpressed from this integrated array.

In addition to the vulval precursor cells, BAR-1 is expressed in several other postembryonic cells, including the seam cells (Fig. 5G), cells in the somatic gonad (data not shown) and P12 (Mike Herman, personal communication).

bar-1 regulates *lin-39 Hox* expression in the vulval precursor cells

The *bar-1* phenotype is similar to the phenotype caused by partial reduction-of-function mutations in the Hox gene *lin-39* (Clandinin et al., 1997; Maloof and Kenyon, 1998). Reduction-of-function *lin-39 Hox* mutations result in an incompletely penetrant phenotype in which P3.p to P8.p sometimes adopt the F fate rather than the vulval precursor cell fate. Those cells that do become vulval precursor cells often express 3° cell fates instead of 1° or 2° cell fates as in wild type. The similarity of the *bar-1* null phenotype to the *lin-39 Hox* reduction-of-function phenotype suggests that these two genes might act in the same pathway. In addition, like *bar-1*, *lin-39* has been shown to function cell autonomously in the vulval precursor cells (Clark et al., 1993). One possibility is that *bar-1* might activate *lin-39 Hox* expression, such that vulval defects in *bar-1(ga80)* mutants might be caused by reduced expression of *lin-39 Hox*.

To address this, we examined LIN-39 protein levels in wild-type and *bar-1(ga80)* animals using anti-LIN-39 antibodies (Maloof and Kenyon, 1998) in immunocytochemistry experiments (Fig. 6). We found that *bar-1(ga80)* causes some vulval precursor cells to lose LIN-39 expression at the late L2/early L3 stage (Fig. 6I-L). In wild-type animals at this stage, P4.p through P8.p always exhibit LIN-39 staining and

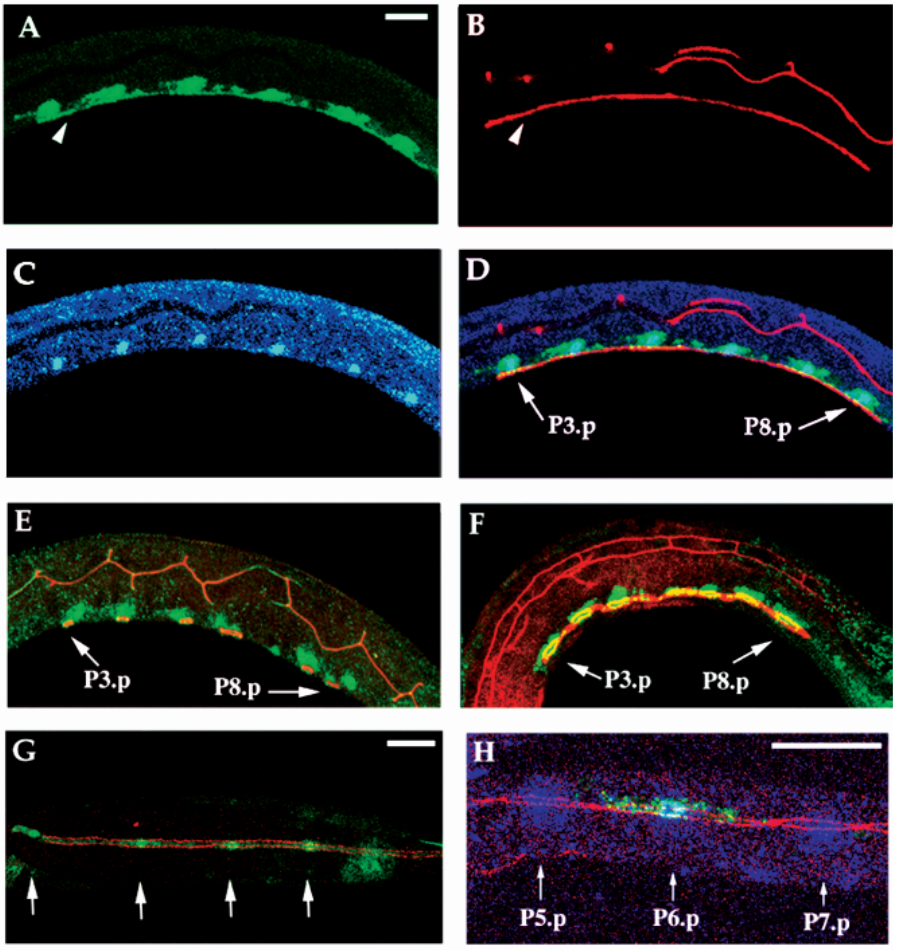


Fig. 5. Expression of BAR-1 and BAR-1-GFP in the vulval precursor cells. (A-D) Lateral views of the mid-body region of an L2/L3 stage animal containing the integrated array *gaIs46* triple-stained with antisera against BAR-1 (A, green), the antibody MH27 (B, red), and antisera against LIN-31 (C, blue). The merged image is shown in D. Note that BAR-1 protein localizes in the cytoplasm, nucleus and at cell junctions (arrowhead). (E-H) The mid-body region of transgenic animals containing the integrated array *gaIs45*, which encodes a BAR-1-GFP fusion protein, stained with MH27 antibody (red) (E-G) or MH27 (red) and anti-LIN-31 antisera (blue) (H). BAR-1-GFP is expressed in P3.p-P8.p in the late L1/L2 stage (E) (lateral view) and late L2/L3 stage (F) (ventrolateral view). BAR-1-GFP appears to concentrate in the nuclei of the seam cells of adult animals (G) (lateral view of the animal's posterior half is shown; arrows point to seam cell nuclei) and can sometimes be seen in the nuclei of vulval precursor cells (H). (H) Ventral view of the mid-body region of an L3 stage animal in which BAR-1-GFP has faded from all of the vulval precursor cells except P6.p. The BAR-1-GFP localization is similar to that of LIN-31 in this cell. Arrows point to nuclei of P5.p-P7.p. Bars, 10 μm.

P3.p shows LIN-39 staining in approximately 50% of animals (Fig. 6A-D) (Maloof and Kenyon, 1998). In *bar-1(ga80)* mutants, the vulval precursor cells express normal levels of LIN-39 in the L1 stage and early L2 stage (Fig. 6G,H). However, at the late L2 stage, P3.p and P4.p lose LIN-39 staining in greater than 90% of cases (n=54; Fig. 6I-L). P5.p, P6.p, P7.p and P8.p exhibit less penetrant defects in LIN-39 expression. Therefore, *bar-1* is required for proper expression of *lin-39 Hox* in P3.p through P8.p.

We next asked if *lin-39 Hox* is a functionally important target of the *bar-1* signaling pathway, such that defects in *lin-39 Hox* expression in *bar-1* mutant animals cause the F cell fate phenotype. If so, forced expression of *lin-39 Hox* should rescue the *bar-1* mutant phenotype. We expressed a *lin-39 Hox* cDNA driven from a heat-shock promoter (Hunter and Kenyon, 1995) in *bar-1(ga80)* mutants, and found that heat-shock-induced *lin-39 Hox* expression rescued the F fate defect of P3.p and P4.p (Table 2).

bar-1 and the *let-23 RTK* signaling pathway act together to maintain *lin-39 Hox* expression in the vulval precursor cells

The results described above show that *bar-1* is involved in maintaining *lin-39 Hox* expression in P3.p through P8.p. Previous results have shown that the *let-23 RTK*/*let-60 ras* signal transduction pathway regulates *lin-39 Hox* expression in P5.p through P7.p (Maloof and Kenyon, 1998). Thus, *lin-39 Hox* activity may be regulated by *bar-1* alone in P3.p and P4.p, but by both *bar-1* and the *let-23* signaling pathway in P5.p, P6.p and P7.p. This redundant regulation of *lin-39 Hox* in P5.p-P7.p might explain why the F cell fate phenotype of *bar-1* mutants is strong for P3.p and P4.p, but weak for P5.p through P7.p. (It in unclear why P8.p exhibits a weaker F cell fate phenotype in *bar-1* mutants. P8.p also expresses the Hox gene *mab-5* (Salser et al., 1993), and *mab-5* activity can affect cell

Table 2. Heat shock expression of *lin-39 Hox* can rescue the *bar-1* F fate phenotype

	% F fates observed*						
Strain	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	n†
N2	50	0	0	0	0	0	24
bar-1	100	100	60	0	7	0	15
bar-1; hs:lin-39 + heat shock	44	31	6	0	0	0	16

*Numbers indicate the percentage of the time that each vulval precursor cell was found to have fused with the hypodermal syncytium in each strain, as determined by loss of MH27 staining. The actual strains used were *dpy-20(e1282); bar-1(ga80)* and *dpy-20(e1282); bar-1(ga80); muIs23*. *muIs23* is an integrated array containing the plasmids pCH17.1, which contains the *lin-39 Hox* cDNA behind a heat shock promoter, and pMH86, which contains the wild type *dpy-20* gene (Hunter and Kenyon, 1995).

†Indicates the number of animals scored.

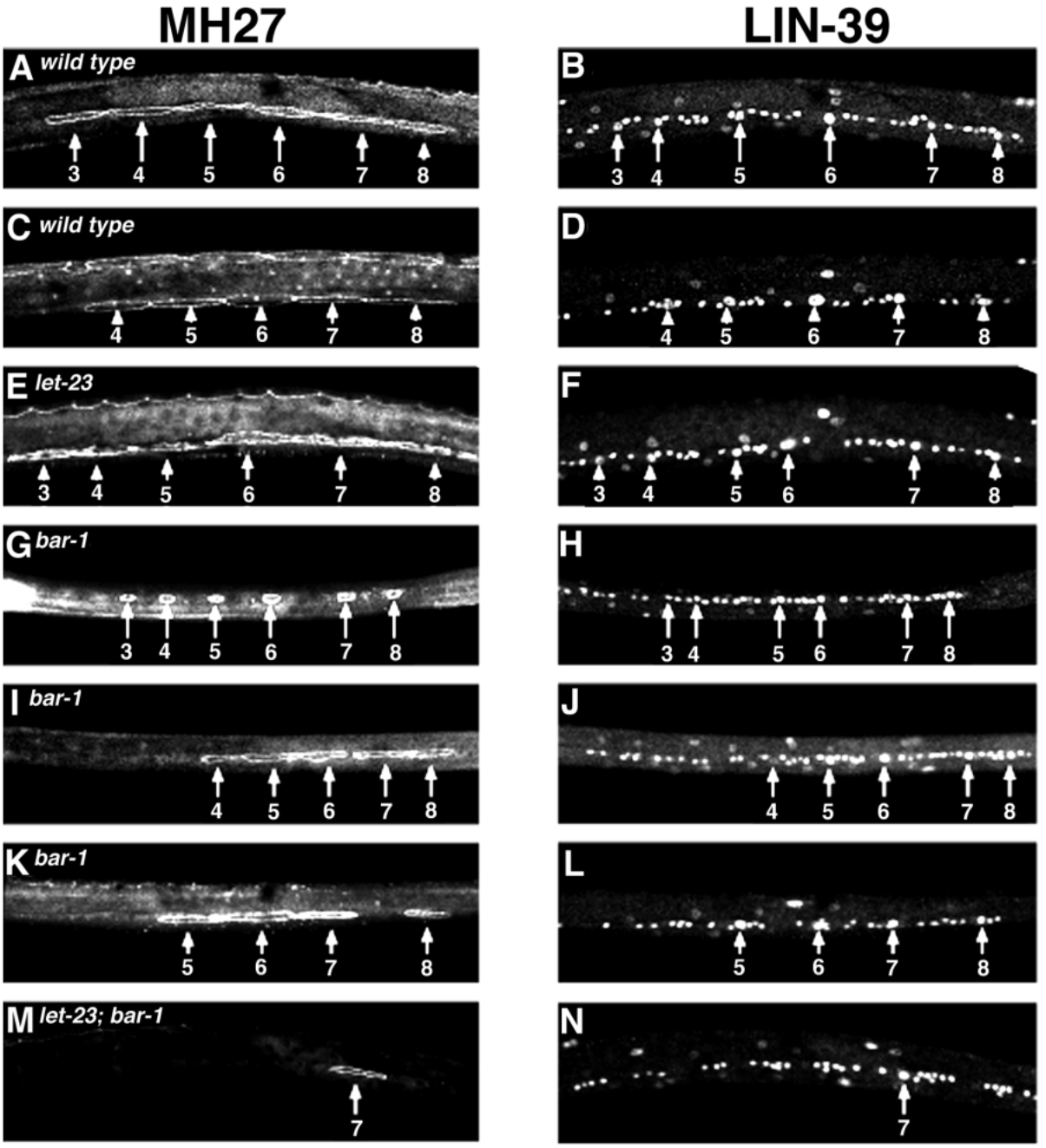


Fig. 6. LIN-39 expression requires *bar-1* activity. Wild-type (A-D), *let-23(sy1)* (E,F), *bar-1(ga80)* (G-L), and *let-23(sy1)*; *bar-1(ga80)* (M,N) animals were double stained with the MH27 antibody and antisera against the LIN-39 protein (Maloof and Kenyon, 1998). All animals are late L2/early L3 stage, except G,H which is an early L2 stage larva, and ventrolateral views are shown. The numbers 3-8 indicate the cell boundaries (left panels) or nuclei (right panels) of the cells P3.p-P8.p. The other nuclei exhibiting LIN-39 staining are mid-body ventral cord neurons. The larger nuclei staining with LIN-39 are the vulval precursor cell nuclei; only vulval precursor cell nuclei that exhibit LIN-39 staining are noted with an arrow. (A,B) A wild-type animal in which P3.p adopted the vulval precursor cell fate. (C,D) A wild-type animal in which P3.p adopted the F fate.

fate determination by the vulval precursor cells (Clandinin et al., 1997), so it is possible that *mab-5* may be expressed independently of BAR-1 activity, allowing P8.p to still adopt the vulval precursor cell fate in *bar-1* mutants.)

To demonstrate the overlapping functions of *bar-1* and the *let-23 RTK* signaling pathway, we examined *let-23(sy1)*; *bar-1(ga80)* double mutants. We scored whether the Pn.p cells adopted the vulval precursor cell fate or the F fate by directly observing these cells using Nomarski microscopy to see if they divide, by staining with the MH27 cell junction marker to show if they had fused with the hypodermal syncytium and by examining levels of LIN-39 expression.

Our results show that the F cell fate phenotype in P5.p, P6.p and P7.p is stronger in the *let-23*; *bar-1* double mutant than in

Table 3. Reduction of *let-23 RTK* activity enhances the *bar-1* mutant phenotype

Strain	% F fates observed*						
	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	*n*†
N2	50	0	0	0	0	0	24
bar-1	94	91	17	6	2	17	54
let-23	35	6	0	0	0	0	32
let-23; bar-1	95	89	36	36	53	44	95

*Data as in Table 2. Actual strains used were *bar-1(ga80), let-23(sy1)* and *let-23(sy1); bar-1(ga80).*

†Indicates the number of animals scored.

either the *bar-1* or *let-23 RTK* single mutants (Table 3). In wild-type or *let-23 RTK* mutants, P5.p through P7.p do not adopt the F cell fate and always stain with the MH27 marker. In *bar-1(ga80)* mutants, these cells adopt the F fate and lose MH27 staining infrequently. In *let-23(sy1); bar-1(ga80)* double mutants, these cells adopt the F fate and lose MH27 staining in 36-53% of cells. We also found that fewer animals showed LIN-39 staining in P5.p through P7.p in *let-23(sy1); bar-1(ga80)* animals than in *bar-1* or *let-23 RTK* single mutants (Fig. 6M,N). In a similar fashion, ablation of the gonad (the source of the inductive signal LIN-3 (Hill and Sternberg, 1992)) with a laser microbeam in *bar-1* mutants increases the F cell fate of P5.p, P6.p and P7.p (data not shown). These experiments show that *bar-1* and *let-23 RTK* have partially redundant functions in P5.p, P6.p and P7.p to regulate LIN-39 levels and maintain the vulval precursor cell fate.

bar-1 acts during the specification of 1°, 2° and 3° cell fates

Previous studies have shown that *lin-39 Hox* has two functions in vulval development. At an early time (the L1 stage), *lin-39(+)* is required for P3.p through P8.p to be vulval precursor cells rather than F cells (Clark et al., 1993; Wang et al., 1993). At a later time (the late L2/early L3 stage), *lin-39 Hox* is required for the vulval precursor cells to adopt the 1° and 2° cell fates correctly (Clandinin et al., 1997; Maloof and Kenyon, 1998). Since *bar-1* regulates *lin-39 Hox* expression, we examined whether *bar-1* might function in the specification of 1°, 2° and 3° cell fates in addition to specification of vulval precursor cell versus F fate.

First, as described above (Table 1), *bar-1(ga80)* causes defects in 1° and 2° cell fate specification that are similar to those caused by a reduction in *lin-39 Hox* activity, suggesting that *bar-1* may function in this later process (Clandinin et al., 1997; Maloof and Kenyon, 1998).

Second, we used genetic epistasis analysis to show that *bar-1* interacts with the anchor cell signaling pathway during the specification of the 1°, 2° or 3° fates. We determined whether the function of certain key genes in the anchor cell signaling pathway was dependent on *bar-1* activity by building double mutant strains containing *bar-1(ga80)* and a mutation in these genes that causes a multivulva (Muv) phenotype. We used four mutations or transgenic constructs that cause a Muv phenotype: a gain-of-function mutation in *let-60 ras* (Ferguson and Horvitz, 1985; Beitel et al., 1990; Han et al., 1990), an integrated array that expresses activated forms of both *Drosophila* MEK and worm MPK-1 (*gaIs37*) (Lackner and Kim, 1998), and loss-of-function mutations in *lin-1 Ets* (Horvitz and Sulston, 1980; Beitel et al., 1995) and *lin-31 WH* (Ferguson and Horvitz, 1985; Miller et al., 1993), which encode transcription factors believed to jointly act as repressors of genes required for vulval induction (Tan et al., 1998). The *bar-1(ga80)* mutation partially suppresses the Muv phenotype of *let-60(gf)* and *gaIs37*, but does not strongly suppress the Muv phenotypes of either *lin-1* or *lin-31* (Table 1).

These epistasis results indicate that *bar-1* is required during the specification of the 1°, 2° and 3° cell fates. If *bar-1* were to function only at an earlier time (to cause P3.p through P8.p to become vulval precursor cells rather than F cells) and not later, then the *bar-1(ga80)* mutation should suppress the Muv phenotype of all four mutations equally well. This is because the *bar-1* mutation would cause a Pn.p cell to be a vulval precursor cell or a F cell equally in all four Muv strains, and the Muv mutation would then cause the vulval precursor cells to adopt the 1° or 2° cell fate equally in all four strains. However, the observed epistasis results do not match this prediction, since *bar-1(ga80)* suppresses the Muv phenotype of *let-60 ras* and the *mek(gf)*/*mpk-1(gf)* transgenic array but does not strongly suppress the Muv phenotype of either *lin-1* or *lin-31*.

These results also show that *bar-1* activity is required for the Muv phenotype caused by either *let-60(gf)* or *mek(gf) mpk-1(gf)*, suggesting that *bar-1* acts downstream of *let-60 ras*, *mek* and *mpk-1*. However, *bar-1* activity is not required for the Muv phenotypes caused by either *lin-1(lf)* or *lin-31(lf)*, suggesting that *bar-1* acts upstream or in parallel to *lin-1 Ets* and *lin-31 WH*. One appealing possibility is that *bar-1* encodes a component of a Wnt signaling pathway acting in the vulval precursor cells, and that the *bar-1* and *let-23 RTK* pathways intersect at this point to coordinately regulating LIN-39 expression (see Discussion).

Third, we determined whether specification of the 1°, 2° and 3° cell fates by the *bar-1* signaling pathway might also be mediated by *lin-39 Hox*. In *bar-1(ga80)* mutants, low *lin-39 Hox* expression might cause vulval precursor cells to adopt 3° rather than 1° or 2° cell fates. If so, then forced expression of *lin-39 Hox* in P5.p through P7.p should rescue the *bar-1* vulval cell fate determination defect. We created a DNA construct in which a *lin-39 Hox* cDNA (Wang et al., 1993) is expressed from the *bar-1* promoter (*pbar-1::lin-39*) and used this construct to transform *bar-1(ga80)* mutants. We found that *pbar-1::lin-39* can partially rescue the defect in the specification of the 1° and 2° cell fates in *bar-1* mutants (Table 4). We found that the frequency at which P5.p through P7.p adopted incorrect cell fates was reduced in animals carrying *pbar-1::lin-39* compared to a *bar-1* control strain, leading to a decreased penetrance of the egg-laying defective (Egl) and protruding vulva (Pvl) phenotypes. These results indicate that the defect in the specification of the 1°, 2° and 3° cell fates in *bar-1* mutants is at least partly caused by loss of *lin-39 Hox* expression.

DISCUSSION

Our analysis suggests the following model of *bar-1* function in the Pn.p cells (Fig. 7). First, that a Wnt signal is required for P3.p to P8.p to adopt the vulval precursor cell fate rather than

Table 4. Forced expression of *lin-39 Hox* partially rescues the *bar-1* induced cell fate phenotype

	% non-wild-type fates observed*								
Strain	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	N†	% Egl‡	N†
bar-1	96	94	42	24	44	12	100	58	381
bar-1; pbar-1::lin-39	93	83	12	0	11	1	83	16	148

*L4 stage animals were observed by Nomarski microscopy and the percentage of non-wild-type cell fates adopted by P3.p - P8.p was determined. The actual strains used were *bar-1(ga80)* and *unc-30(e191); bar-1(ga80)* containing pDE274(*pbar-1::lin-39*) and pSC11(*unc-30(+)*) on an extrachromosomal array. For purposes of this table, the F fate is considered a non-wild-type fate when adopted by P3.p.

†Indicates the number of animals scored.

‡Indicates the percentage of animals for each strain having an egg-laying defective phenotype as observed on plates using a dissecting microscope.

the F cell fate (Fig. 7A). In wild-type animals, the Wnt signal would be strong near P4.p through P8.p, and cause these cells to adopt the vulval precursor cell fate rather than the F cell fate in all individuals. The Wnt signal would be weak near P3.p, and cause this cell to adopt the vulval precursor cell fate in only 50% of animals. *bar-1* encodes a protein similar to β-catenin and transduces this Wnt signal in the Pn.p cells. In *bar-1* mutants, all six Pn.p cells can adopt the F cell fate rather than the vulval precursor cell fate due to defects in Wnt signal transduction.

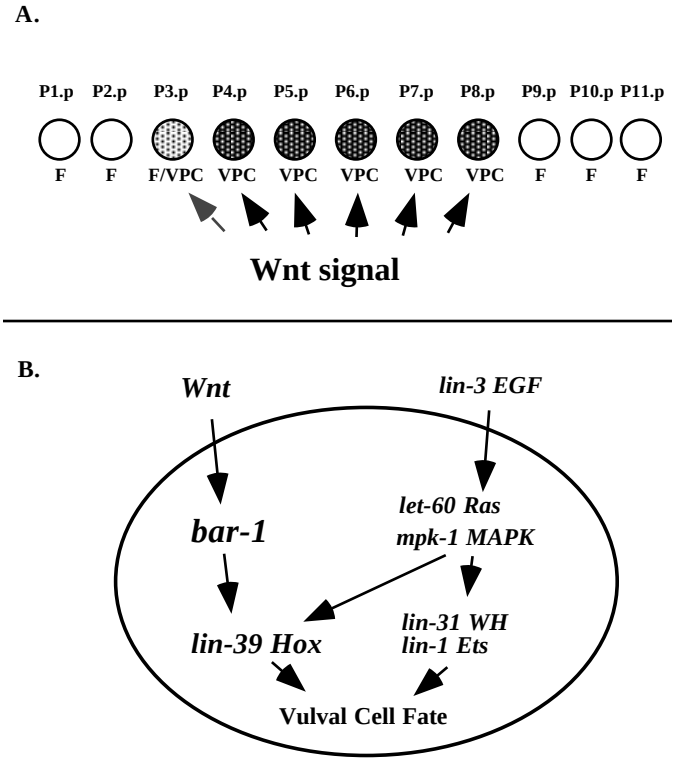


Fig. 7. Model for *bar-1* function in a Wnt pathway in the vulval precursor cells. (A) A Wnt/BAR-1 signaling pathway causes P4.p-P8.p to adopt the vulval precursor cell fate rather than the F fate. Weak levels of the Wnt signal at P3.p causes it to adopt the vulval precursor cell fate in approximately 50% of animals. (B) The Wnt/*bar-1* and the anchor cell signal/*let-60 ras* pathways coordinately regulate the Hox gene *lin-39*. *lin-39* is required in the L1/L2 for P3.p-P8.p to adopt and maintain their vulval precursor cell identity, and then again in the L2/L3 for these cells to express the 1° or 2° cell fates. In addition to LIN-39, specification of the 1°, 2° and 3° cell fates requires other transcription factors such as LIN-31 WH and LIN-1 Ets, which are believed to function by repressing genes necessary for induced vulval cell fates (Tan et al., 1998). The LET-60 Ras pathway could act directly by phosphorylating the LIN-39 protein (the LIN-39 protein sequence contains a MAP kinase consensus phosphorylation site (Clark et al., 1993; Wang et al., 1993), or could act through regulation of *lin-39* expression by downstream transcription factors such as LIN-1 (Clandinin et al., 1997; Maloof and Kenyon, 1998).

Second, that the BAR-1 signaling pathway acts by maintaining expression of the Hox gene *lin-39* in the Pn.p cells (Fig. 7B). *lin-39 Hox* acts two times during Pn.p cell fate specification, first to specify vulval precursor cell versus F cell fates (Clark et al., 1993; Wang et al., 1993) and later in the specification of 1°, 2° and 3° vulval cell fates (Clandinin et al., 1997; Maloof and Kenyon, 1998). BAR-1 is required for both types of Pn.p cell fate specification and for proper LIN-39 expression.

Third, *lin-39* is also regulated by the *let-23 RTK*/*let-60 ras*/*mpk-1* signaling pathway (Maloof and Kenyon, 1998), and the *bar-1* mutant phenotype is enhanced by reduced activation of this pathway. Thus, the *bar-1* signaling pathway and the *let-60 ras* signaling pathways interact to control the fates of the Pn.p cells by coordinately regulating *lin-39 Hox*.

BAR-1 is similar to vertebrate β-catenin and *Drosophila* Armadillo

BAR-1 has the same overall structure as β-catenin. It has a large, central domain composed of 12 Arm repeats flanked by less-conserved amino- and carboxy-terminal domains (Peifer and Wieschaus, 1990; Peifer et al., 1994a; Huber et al., 1997), eight consensus phosphorylation sites for the serine/threonine protein kinase GSK-3 in its amino-terminal domain (Peifer et al., 1994b) and a sequence (DSGFQT) similar to a region from β-catenin that leads to ubiquitin-mediated–degradation when phosphorylated (Aberle et al., 1997; Orford et al., 1997) (Fig. 3). Also, like β-catenin and Arm (Behrens et al., 1996; van de Wetering et al., 1997), the carboxy terminal domain of BAR-1 contains a transcriptional activation domain, since it can stimulate transcription in yeast when fused to the GAL-4 DNA-binding domain (D. M. E., unpublished observations). Finally, BAR-1 has the same pattern of subcellular localization as β-catenin and Arm (Gumbiner, 1995; Miller and Moon, 1996) (Fig. 5).

Similar to β-catenin and Arm, BAR-1 regulates the expression of a homeodomain gene. In *Xenopus*, β-catenin/TCF regulates the homeodomain-containing gene *siamois* during Spemann organizer formation and, in *Drosophila*, Arm/dTCF regulates the homeotic gene *Ubx* during development of the midgut (Brannon et al., 1997; Riese et al., 1997; van de Wetering et al., 1997). In worms, we have shown that *bar-1* regulates expression of the homeotic gene *lin-*

39 during Pn.p cell fate specification. For these reasons, we believe it is likely that BAR-1 is *a C. elegans* homolog of β-catenin and Armadillo.

A Wnt signaling pathway specifies the vulval precursor cell fate

Our results suggest that BAR-1 functions in a Wnt signaling pathway to cause Pn.p cells to adopt the vulval precursor cell fate rather than the F cell fate. The fates of P3.p and P4.p are determined by their position rather than their lineage, indicating that cell-cell signals set the fates of P3.p and P4.p (Sulston and Horvitz, 1977; Sternberg and Horvitz, 1986). The anterior cell adopts the F fate in 50% of animals and the posterior cell adopts the vulval precursor cell fate in 100% of animals, regardless of which cell is derived from ABpl and which is derived from ABpr. A *bar-1* loss-of-function mutation causes P3.p through P8.p to sometimes adopt the F fate rather than the vulval precursor cell fate, and *bar-1* is similar to β-catenin, a central component of Wnt signal transduction pathways. These results suggest that the extracellular signal acting on the Pn.p cells is a Wnt molecule, and that this signal is transduced by *bar-1*. The cellular source and identity of the putative Wnt signal acting on P3.p through P8.p are not known. It is unlikely that the Wnt signal originates from the gonad since ablation of the entire somatic gonad does not prevent Pn.p cells from adopting vulval precursor cell fates.

An alternative model is that BAR-1, like β-catenin, may function to mediate cell adhesion in the Pn.p cells and that defects in cell adhesion could cause the adoption of F cell fates in *bar-1* mutant animals. In addition, it is possible that BAR-1 may represent a *C. elegans* β-catenin-like protein functioning in a novel or distinct manner from vertebrate and *Drosophila* β-catenin family members. Although these possibilities are not mutually exclusive, we prefer the Wnt signaling model outlined above because (1) we have observed no obvious cell adhesion defects in other epithelial cells in *bar-1* mutants, (2) *bar-1* regulates *lin-39 Hox* expression, suggesting a function in a signaling pathway rather than as a cell adhesion molecule, and (3) *bar-1* mutants have defects in the specification of the P12 fate that resemble those caused by mutations in Wnt pathway components and other Hox genes (see below).

In addition to BAR-1, there are two other *C. elegans* proteins similar to β-catenin in structure and function, HMP-2 and WRM-1. HMP-2, which has 26% amino acid identity to β-catenin/Armadillo, may function predominantly in epithelial cell morphogenesis and migration, as it is localized to epithelial cell junctions along with α-catenin and cadherin homologs, and *hmp-2* mutants have defects in specific epithelial cells during embryogenesis (Costa et al., 1998). WRM-1, which has 15% overall amino acid identity to β-catenin, may have a role in Wnt signaling, since RNA-mediated inhibition of *wrm-1* function leads to a Wnt signaling defect similar to that caused by mutations in the Wnt gene *mom-2* and the *frizzled* gene *mom-5* (Rocheleau et al., 1997; Thorpe et al., 1997). Thus, the primary defect seen in the three β-catenin mutants are different, suggesting that these β-catenins may have separate functions. In this case, it is interesting that the diverse functions of β-catenin in other species may have been divided amongst three proteins in *C. elegans*: HMP-2 may function in cell morphogenesis/migration, WRM-1 may function in embryonic Wnt signaling and BAR-1 may function in postembryonic Wnt signaling. However, it is also possible that these three proteins may have partially overlapping functions; for example, one of these β-catenin-related genes might also be expressed in the Pn.p cells in the L3 and might partially compensate for the loss of *bar-1* activity in *bar-1* mutants. Thus, the *bar-1* null phenotype described here might correspond to a reduction of Wnt signaling in the Pn.p cells and not the complete absence of Wnt signaling.

Integration of the BAR-1 and LET-60 Ras signaling pathways by coordinate regulation of LIN-39 Hox expression

Our results show that *bar-1* maintains the expression of the Hox gene *lin-39* in P3.p through P8.p. First, the phenotype caused by *bar-1* null mutations is similar to the phenotype caused by *lin-39 Hox* partial reduction-of-function mutations, suggesting that these two genes act in the same genetic pathway. Second, *lin-39 Hox* expression is eliminated in *bar-1* mutants in P3.p through P8.p. Third, forced expression of *lin-39 Hox* in *bar-1* mutants can partially rescue the *bar-1* mutant phenotypes, indicating that *lin-39 Hox* is a functionally important target of the *bar-1* signaling pathway.

Previous results indicate that the *let-23 RTK* signaling pathway activates *lin-39 Hox* expression in P5.p through P7.p (Maloof and Kenyon, 1998). (*lin-39 Hox* expression in P5.p and P7.p may be regulated by the lateral signal/*lin-12 Notch* pathway instead of directly by the anchor cell signal/*let-23 RTK* pathway). Our results suggest that, in P3.p and P4.p, *lin-39 Hox* expression is maintained by the *bar-1* signaling pathway, but that, in P5.p, P6.p and P7.p, *lin-39 Hox* activity is activated by both the *bar-1* and the *let-23 RTK* signaling pathways. In wild-type animals or *let-23 RTK* mutants, *bar-1(+)* activity is sufficient to maintain *lin-39 Hox* expression so that P3.p through P8.p all adopt the vulval precursor cell fate rather than the F cell fate. In *bar-1* mutants, LIN-39 Hox expression is strongly reduced in P3.p and P4.p, so that these cells often adopt the F fate. However, in P5.p, P6.p and P7.p, LIN-39 expression is partially maintained by the *let-23 RTK* signaling pathway (and perhaps the lateral signaling pathway), and these cells sometimes adopt the F fate. In *let-23*; *bar-1* double mutants, *lin-39 Hox* expression is severely reduced in all of the Pn.p cells, and all of these cells frequently adopt the F cell fate. Thus, *lin-39 Hox* is a crucial downstream target that is coordinately regulated by both the *bar-1* and *let-23 RTK* signaling pathways to regulate vulval precursor cell versus F cell fate determination.

Does *bar-1* play an instructive or permissive role during the specification of the 1°, 2° and 3° cell fates? When the anchor cell signaling pathway is defective, LIN-39 levels are equivalent in all six vulval precursor cells and all of the cells express 3° cell fates (Maloof and Kenyon, 1998), suggesting that the *bar-1* pathway is permissive since it is not sufficient to instruct these cells to adopt the 1° cell fate in the absence of *let-23 RTK* signaling. It seems likely that the function of *bar-1* is to maintain *lin-39 Hox* expression in the vulval precursor cells, permitting these cells to retain the potential to adopt the 1°, 2° or 3° cell fates. The *let-23 RTK* and the *lin-12 Notch* signaling pathways then have instructive roles in specifying the 1°, 2° or 3° cell fates.

Interestingly, we and others have found that the same genetic

regulatory network specifies the fate of the cell P12. The fate of P12 is regulated by a Wnt signaling pathway involving *lin-44 Wnt* (Herman and Horvitz, 1994; Herman et al., 1995) and *bar-1* mutants have the same defect in P12 fate specification as seen in *lin-44* mutants (D. M. E. and S. K. K., unpublished data). *bar-1::GFP* is expressed in P12 (M. Herman, personal communication) and genetic mosaic analysis indicates *bar-1* acts cell autonomously in the determination of P12 fate (data not shown). The P12 fate is also regulated by *let-23 RTK* (Aroian and Sternberg, 1991), *lin-45 raf* (Han et al., 1993) and the Hox gene *egl-5* (Chisholm, 1991). Recently, it has been shown that the *let-23 RTK/let-60 ras* and *lin-44 Wnt* pathways coordinately regulate *egl-5 Hox* activity in P12 to control cell fate specification (Jiang and Sternberg, 1998). These results suggest that conserved Ras and Wnt signaling pathways may interact to regulate Hox targets in multiple cell fate decisions.

We thank Mike Costa, Mike Herman, Rueyling Lin, Paul Sternberg and Patrick Tan for communicating results prior to publication. We thank Andy Fire for the gift of the GFP reporter construct pPD95.75 and Yishi Jin for pSC11. We thank Albert Candia, Valerie Reinke and Patrick Tan for critical reading of the manuscript and all members of the Kim laboratory for advice and support. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources (NCRR). This research was supported by grants to S. K. K. from the Lucille P. Markey Charitable trust, the Searle Scholars Program/The Chicago Community Trust and the National Institutes of Health. D. M. E. was funded by an American Cancer Society (National) Postdoctoral Fellowship award. J. N. M. was supported in part by a HHMI pre-doctoral fellowship. C. K. was supported by NIH grant R01 GM37053. S. K. K. was a Lucille P. Markey Scholar and a Searle Scholar.

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