

Activation of Wnt signaling bypasses the requirement for RTK/Ras signaling during *C. elegans* vulval induction

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During *Caenorhabditis elegans* vulval development, activation of receptor tyrosine kinase/Ras and Notch signaling pathways causes three vulval precursor cells (VPCs) to adopt induced cell fates. A Wnt signaling pathway also acts in cell fate specification by the VPCs, via regulation of the Hox gene *lin-39*. We show here that either mutation of *pry-1* or expression of an activated BAR-1 β -catenin protein causes an Overinduced phenotype, in which greater than three VPCs adopt induced cell fates. This indicates that *pry-1*, which encodes a *C. elegans* axin homolog, acts as a negative regulator of Wnt signaling in the VPCs. Loss of activity of the APC homolog *apr-1* increases the penetrance of this Overinduced phenotype, suggesting that APR-1 may play a negative role in Wnt signaling in this process in *C. elegans* similar to APC proteins in other systems. The Overinduced phenotype is suppressed by reduction of function of the genes *pop-1* TCF and *lin-39* Hox. Surprisingly, the Overinduced phenotype caused by hyperactivated Wnt signaling is not dependent on signaling through the Ras pathway. These data suggest that hyperactivation of Wnt signaling is sufficient to cause VPCs to adopt induced fates and that a canonical Wnt pathway may play an important role during *C. elegans* vulval induction.

[Key Words: *C. elegans*; vulva; Ras; Wnt; axin; APC]

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Extracellular signaling pathways are used during the development of all metazoans to control the growth, differentiation, and death of cells. One such pathway is the Wnt signaling pathway, which is used during the development of many animals, from the cnidarian *Hydra* to humans (Cadigan and Nusse 1997; Wodarz and Nusse 1998; Hobmayer et al. 2000). In its most basic form, the Wnt signaling pathway becomes active when a Wnt family ligand binds to a transmembrane receptor of the Frizzled family. Ligand binding leads to the inhibition of a complex of proteins containing the tumor suppressor gene product APC, the scaffold protein Axin, and the serine/threonine kinase GSK3 β . In the absence of a Wnt signal, this complex allows GSK3 β to phosphorylate the amino terminus of the β -catenin protein, which targets β -catenin for ubiquitination and degradation by the proteasome (Maniatis 1999). Inhibition of this protein complex leads to the stabilization of β -catenin, which translocates into the nucleus and interacts with transcription factors of the TCF/LEF family to activate transcription of Wnt-inducible target genes (Eastman and Grosschedl 1999).

An excellent genetic model system for the study of conserved signaling pathways is the induction of the hermaphrodite vulva in the nematode *C. elegans* (for review, see Greenwald 1997; Kornfeld 1997). During vulval formation, six ventral hypodermal blast cells, P3.p–P8.p, known as the vulval precursor cells (VPCs), adopt different cell fates on the basis of activation of conserved signaling pathways. The anchor cell in the overlying somatic gonad sends an inductive signal that activates a receptor tyrosine kinase (RTK)/Ras pathway in the nearest VPC, P6.p, causing this cell to adopt the 1 $^\circ$ vulval fate. P6.p then signals to its neighboring cells, P5.p and P7.p, activating a Notch signaling pathway and allowing these cells to adopt the 2 $^\circ$ vulval fate. The remaining three VPCs, P3.p, P4.p, and P8.p, do not receive sufficient levels of either signal and adopt the 3 $^\circ$ nonvulval fate. In roughly half of wild-type animals, P3.p adopts the Fused (F) fate, which is to fuse with the syncytial hypodermis without dividing (Sulston and White 1980; Sternberg and Horvitz 1986). Cells adopting 1 $^\circ$ and 2 $^\circ$ fates (P5.p–P7.p) divide to generate 22 cells that form the adult vulva, whereas cells adopting the 3 $^\circ$ cell fate divide, and their progeny fuse with the syncytial hypodermis.

In addition to the requirement for RTK/Ras and Notch signaling pathways, previous work suggests that a Wnt signaling pathway also functions during vulval induc-

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tion. This is based on the analysis of strains mutant for the genes *bar-1*, which encodes a β -catenin-related protein (Eisenmann et al. 1998), *apr-1*, which encodes an APC-related protein (Rocheleau et al. 1997; Hoier et al. 2000), and *mig-14* (*mom-3*), which has not been cloned, but which appears to function in many Wnt-mediated processes (Harris et al. 1996; Thorpe et al. 1997; Eisenmann and Kim 2000). In these mutants, P4.p–P8.p can adopt the F fate instead of the normal 1°, 2°, and 3° fates, and P5.p–P7.p can adopt the 3° fate instead of the 1° and 2° fates, resulting in too few VPCs adopting induced fates. *bar-1* and *apr-1* have been shown to be required for maintenance of the Hox gene *lin-39* in VPCs, and cells that lose *lin-39* expression adopt the F fate (Eisenmann et al. 1998; Hoier et al. 2000). *lin-39* functions twice in vulval development, first in the L1 stage during generation of the VPCs (Clark et al. 1993; Wang et al. 1993), and later in the L3 stage during adoption of induced cell fates by the VPCs, when LIN-39 protein levels increase in response to activation of the RTK/Ras pathway (Clandinin et al. 1997; Maloof and Kenyon 1998). These results suggest that a Wnt pathway utilizing MIG-14, BAR-1, and APR-1 is active in the VPCs and that one target of this pathway is *lin-39*.

To further understand the role of Wnt signaling during vulval development, we examined the consequences of overactivation of the Wnt pathway. We find that hyperactivation of the Wnt pathway via a *pry-1* loss-of-function mutation or expression of an activated BAR-1 protein leads to an Overinduced phenotype in which extra VPCs adopt induced cell fates. This indicates that *pry-1*, which encodes a *C. elegans* axin homolog (Korswagen et al. 2002), negatively regulates Wnt signaling in the VPCs and that overactivation of the Wnt pathway causes cells to adopt vulval fates that would not normally do so. The *pry-1* (Axin) Overinduced phenotype is dependent on the activities of *bar-1* (β -catenin), *pop-1* (TCF), and the target gene *lin-39* (Hox). Reduction of *apr-1* (APC) activity enhances the *pry-1* Overinduced phenotype, suggesting that *apr-1* may negatively regulate Wnt signaling during vulval induction. Finally, we show that the Overinduced phenotype caused by Wnt pathway hyperactivation is not dependent on signaling through the Ras pathway. These results suggest that a canonical Wnt pathway may play an important role in specifying induced vulval cell fates during *C. elegans* vulval induction, as activation of this pathway is sufficient for vulval induction in the absence of Ras signaling.

Results

Loss of function of pry-1 (Axin) causes excessive vulval induction

To further characterize the function of Wnt signaling in vulval development, we took two approaches to overactivate the Wnt pathway. First, we examined *pry-1* mutants for any defects in vulval development. *pry-1* was first identified as a negative regulator of a Wnt signaling pathway acting during the migration of the progeny of the QL and QR neuroblasts. In this process, *pry-1* acts

downstream of *egl-20* (Wnt) and *lin-17* (Frizzled), but upstream of *bar-1* (β -catenin; Maloof et al. 1999). We found that >70% of *pry-1(mu38)* animals have an Overinduced vulval phenotype, in which more than three VPCs adopt induced cell fates. In *pry-1* mutants, extra vulval invaginations are seen in addition to the normal invagination formed by the progeny of P5.p–P7.p, indicating that P3.p, P4.p, and P8.p often adopt induced cell fates (Fig. 1). Consistent with this, lineage analysis of *pry-1(mu38)* animals at 15° shows that P3.p, P4.p, and P8.p can all divide ectopically, and that these VPCs can go through three rounds of divisions like P5.p–P7.p (data not shown). The *pry-1* Overinduced phenotype is cold sensitive, being more penetrant at 15°C than 20°C or 25°C (Table 1).

Epistasis analysis was performed with *pry-1(mu38)* and the mutations *bar-1(ga80)* and *mig-14(ga62)*, which cause too few VPCs to adopt induced fates. The *ga80* mutation introduces an early stop codon in BAR-1, and is predicted to cause a null mutant phenotype (Eisenmann et al. 1998). *mig-14(ga62)* is a viable, reduction-of-function mutation, which causes defects in multiple Wnt-mediated processes in embryogenesis and post-embryonic life (Eisenmann and Kim 2000). The Overinduced phenotype of *pry-1(mu38)* is still manifest in a double mutant with *mig-14(ga62)*, but is strongly reduced in a double mutant with *bar-1(ga80)* (Table 1). We also determined whether the *pry-1(mu38)* Overinduced phenotype requires the TCF homolog POP-1. For this analysis, the viable allele *pop-1(hu9)* was used. *hu9* encodes a protein with a missense mutation in the amino-terminal β -catenin-binding domain, and *pop-1(hu9)* animals have defects in Q progeny migration like those in *bar-1* mutants (Korswagen et al. 2002). Although *pop-1(hu9)* alone causes only a very weak effect on vulval development, the Overinduced phenotype of *pry-1(mu38)* was completely suppressed in the *pry-1(mu38) pop-1(hu9)* double-mutant strain (Table 1). These results indicate that *pry-1* acts downstream of *mig-14* and upstream of *bar-1* and *pop-1* during VPC fate specification, consistent with its site of action during Q neuroblast specification (Maloof et al. 1999). *pry-1* has been shown recently to encode a *C. elegans* Axin-like protein (Korswagen et al. 2002). Loss-of-function mutations in Axin lead to defects in vertebrate axis specification that are consistent with overactivation of the Wnt pathway (Zeng et al. 1997). Because loss-of-function mutations in genes like *mig-14*, *apr-1*, and *bar-1* lead to an Underinduced phenotype in which too few VPCs adopt induced cell fates, these results suggest that PRY-1 acts as a negative regulator of Wnt signaling in the VPCs, and that overactivation of the Wnt pathway causes VPCs to adopt induced cell fates.

Stabilization of the BAR-1 protein causes excessive vulval induction

To activate the Wnt pathway in a more direct manner, we deleted sequences in the *bar-1* gene encoding the amino-terminal region of BAR-1. This region of β -catenin proteins contains sites for phosphorylation by GSK3 β ,

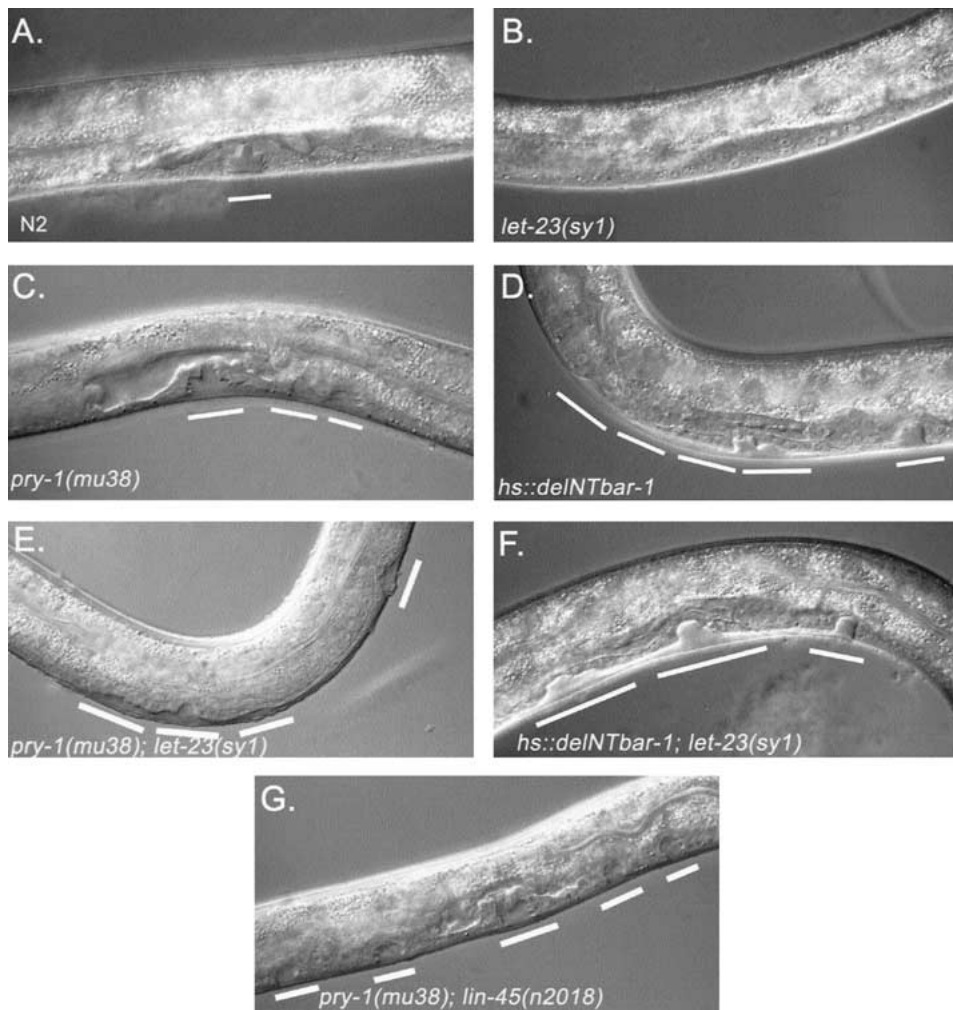


Figure 1. Activation of the Wnt pathway causes ectopic vulval induction in a Ras pathway-independent manner. Mid-body views of single, characteristic L4 stage larvae showing the extent of vulval induction. Anterior is left and dorsal is up. White bars indicate vulval cell invaginations, indicative of VPCs adopting induced cell fates. (A) Wild-type; only P5.p, P6.p, and P7.p adopt induced fates, and a single invagination is formed. (B) *let-23(sy1)*; Ras signaling is compromised in the VPCs and no vulval invagination is seen. (C) *pry-1(mu38)*; extra invaginations are seen, caused by cells other than P5.p–P7.p adopting induced fates. (D) *huIs1* subjected to two heat shocks starting in the L2; multiple invaginations are seen. (E) *pry-1(mu38); let-23(sy1)*, (F) *let-23(sy1); huIs1*, two heat shocks (G) *pry-1(mu38); lin-45(n2018cs)* at 15°C. In E–G, multiple vulval invaginations are still seen in the presence of mutations that compromise Ras signaling.

and mutation or deletion of this region creates a protein that is no longer degraded in the cytoplasm and that interacts with TCF/LEF family members to activate Wnt-regulated target genes (Polakis 2000). Because BAR-1 contains GSK3 β consensus phosphorylation sites in its amino terminus (Eisenmann et al. 1998), is known to interact with the TCF homolog POP-1 (Korswagen et al. 2000; Natarajan et al. 2001) and contains redundant transcription activation domains in its amino- and carboxy-terminal domains (Natarajan et al. 2001), it seemed likely that deletion of the BAR-1 amino terminus might result in a stabilized protein capable of constitutively activating Wnt target genes.

The delNTBAR-1 protein was first expressed from the *lin-31* and the *bar-1* promoters, which both express in the VPCs (Eisenmann et al. 1998; Tan et al. 1998); how-

ever, no effect on wild-type vulval development was observed (data not shown). Therefore, we expressed delNTBAR-1 from the *C. elegans* heat-shock promoter (*hs::delNTbar-1*) to produce higher levels of the truncated protein. When *hs::delNTbar-1* was introduced into wild-type animals on either an extrachromosomal (data not shown) or integrated array (*huIs1*) and subjected to heat shocks during the L2 and L3 stages, the majority of animals displayed an Overinduced phenotype (Table 1). Invaginations due to ectopic division of P3.p, P4.p, and P8.p were often seen (Fig. 1). Overexpression of native BAR-1 protein from the heat-shock promoter did not cause any defects in VPC fate specification (data not shown). This result verifies that activation of the Wnt pathway can cause VPCs to adopt induced fates that would not normally do so.

Table 1. Hyperactivation of Wnt signaling causes excess vulval induction

Strain	n	% Over induced	% wild type	% Under induced	% Vul	% Other	n	Average no. induced VPCs
N2 25°/20°/15°	200	0	100	0	0	0	50	3.0 ± 0.0
<i>pry-1(mu38)</i> 25°	376	37	46	4	0	13	56	3.2 ± 0.7
<i>pry-1(mu38)</i> 20°	309	49	36	10	0	5	55	3.4 ± 0.6
<i>pry-1(mu38)</i> 15°	124	76	13	0	1	10	112	4.0 ± 0.8
N2 + HS	200	0	100	0	0	0	50	3.0 ± 0.0
<i>huIs1</i> - HS	111	0	97	0	0	3	67	3.0 ± 0.0
<i>huIs1</i> + HS	416	79	13	2	0	6	104	4.8 ± 0.7
<i>bar-1(ga80); huIs1</i> - HS	111	0	47	42	8	3	111	1.9 ± 0.9
<i>bar-1(ga80); huIs1</i> + HS	138	60	1	20	0	20	52	4.4 ± 0.7
<i>mig-14(ga62)</i> 25°	103	0	39	57	2	2	110	2.3 ± 0.7
<i>pry-1(mu38); mig-14(ga62)</i> 25°	100	62	35	1	0	2	50	3.6 ± 0.8
<i>bar-1(ga80)</i> 25°	106	0	44	46	6	4	54	2.3 ± 0.9
<i>pry-1(mu38); bar-1(ga80)</i> 25°	105	4	46	50	0	0	50	2.7 ± 0.6
<i>pop-1(hu9)</i> 25°	103	0	71	1	0	28	103	3.0 ± 0.1
<i>pop-1(hu9) pry-1(mu38)</i> 25°	121	0	90	3	0	7	121	3.0 ± 0.2

Strains were grown at the indicated temperatures and scored for vulval induction as described in Materials and Methods. *pry-1(mu38)* double-mutant strains were grown at 25°C due to low viability at lower temperatures. *huIs1* is the integrated array containing the *hs::delNTbar-1* construct. (Vul) vulvaless; (other) animals with a malformed vulval induction that could not be unambiguously assigned to another category. (HS) Heat shock. Average number of induced VPCs is shown with standard deviation.

Expression of activated BAR-1 during the late L2 is sufficient for ectopic vulval induction

To determine the time during development when activation of the Wnt pathway can induce ectopic vulval fates, we subjected animals containing *hs::delNTbar-1* to a single heat shock at various times. A single heat shock at the time of the L2/L3 molt was very effective in causing an Overinduced phenotype, but heat shocks in the L1 or early L2 stages were not (Fig. 2). This result shows that VPCs are sensitive to overactivation of the Wnt pathway in the late L2 and early L3 stage, which is the time when inductive signaling leading to activation of the RTK/Ras pathway occurs (Greenwald 1997; Kornfeld 1997) and when up-regulation of LIN-39 levels is seen (Maloof and Kenyon 1998).

We also determined whether a single pulse of activated BAR-1 could rescue the *bar-1* mutant phenotype. In *bar-1* mutants, VPCs are born normally in the L1 and adopt Fused fates in the late L2 stage (Eisenmann et al. 1998). We again found that a single heat shock at the late L2 stage (22 h after feeding) caused 76% of *bar-1(ga80); hs::delNTbar-1* animals to display an Overinduced phenotype (Fig. 2), confirming that activation of the Wnt pathway in the late L2/early L3 can lead to adoption of excess vulval fates by the VPCs.

apr-1 (APC) may have a negative regulatory function during vulval induction

apr-1 encodes a *C. elegans* APC-related protein (Rocheleau et al. 1997). In vertebrates, APC acts as a negative regulator of β -catenin and Wnt signaling (Moon and Miller 1997; Polakis 2000), whereas in *C. elegans*, *apr-1* has been shown to function in a positive manner. During

Wnt signaling in embryogenesis, reduction of *apr-1* function by RNA interference causes a phenotype like that caused by loss of activity of *wrm-1* (β -catenin) or *mom-2* (Wnt) (Rocheleau et al. 1997). In vulval development, expression of an antisense *apr-1* cDNA in the VPCs causes a loss of *lin-39* expression and adoption of F fates, as in *bar-1* mutants (Hoier et al. 2000). Because reduction of *apr-1* activity has been reported to cause an Underinduced phenotype, we determined whether the Overin-

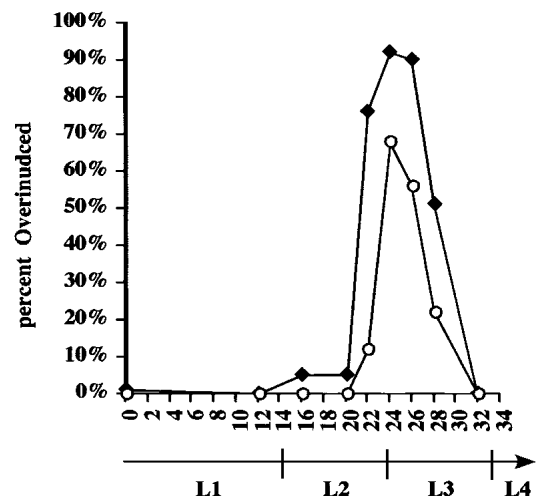


Figure 2. A single pulse of activated BAR-1 in the late L2 is sufficient to cause an Overinduced phenotype. Partially synchronized L1 larvae of the genotype *huIs1* (○) or *bar-1(ga80); huIs1* (◆) were grown at 20°C and given a single heat shock at the indicated time after placing them on plates with *E. coli*. The corresponding larval stages are indicated. The percentage of animals displaying an Overinduced phenotype is plotted for each time point.

duced phenotype caused by loss of *pry-1* or expression of activated BAR-1 was dependent on *apr-1* function.

To examine the role of *apr-1*, we used the technique of feeding RNAi, in which animals are grown on plates on which their food source is *Escherichia coli* expressing dsRNA for the gene of interest (Timmons et al. 2001). When wild-type L3 hermaphrodites were fed on bacteria expressing *apr-1* dsRNA, 95% of their progeny died as embryos, consistent with the embryonic lethal phenotype of an *apr-1* null mutant (Hoier et al. 2000). However, only a few surviving progeny had defects in vulval induction (Table 2). When *pry-1(mu38)* L3 animals were fed on *apr-1* dsRNA bacteria, their progeny still displayed an Overinduced phenotype, but the percentage showing the phenotype increased from 34% when fed on vector alone to 77% when fed on *apr-1* dsRNA at 25°C (Table 2).

To bypass the *apr-1(RNAi)* embryonic lethality and only reduce *apr-1* function during larval life, we fed N2 and *pry-1(mu38)* strains on *apr-1* dsRNA starting at the L1 stage and examined the vulval phenotype of these same animals later in the L4 stage. As a control, a strain expressing an APR-1::GFP fusion protein from the *apr-1* promoter (*zhIs2*; Hoier et al. 2000) was examined. APR-1 protein is expressed in the VPCs in the L3 stage (Hoier et al. 2000), and APR-1::GFP is expressed strongly in the descendants of the VPCs in the L4 stage (data not shown). When fed on bacteria containing feeding vector, 117/129 animals showed strong APR-1::GFP expression in the vulval cells in the L4 stage, whereas 1/136 animals

fed on bacteria expressing *apr-1* dsRNA showed strong vulval expression of APR-1::GFP, indicating that this method effectively reduced APR-1 expression in the vulval cells.

When this protocol was used to compromise *apr-1* function in wild-type animals, we found it caused only a weak effect on vulval development (Table 2). However, when *pry-1(mu38)* animals were treated in a similar manner, the percentage with an Overinduced phenotype increased from 44% when fed on vector alone to 84% when fed on *apr-1* dsRNA, and the average number of induced VPCs per animal increased from 3.4 to 4.2 ($P < 0.001$; two-tailed t-test). When a *hs::delNTbar-1* strain was fed on *apr-1* dsRNA starting in the L1 stage and subjected to heat shocks in the L2 and L3 stages, the penetrance of the Overinduced phenotype increased from 63% when fed on vector alone to 81% when fed on *apr-1* dsRNA, and the average number of induced VPCs increased from 4.3 to 4.7 ($P < 0.001$; two-tailed t-test) (Table 2). This enhancement of the *hs::delNTbar-1* phenotype was dependent on the presence of wild-type BAR-1 protein in this strain, as the enhancement of the Overinduced phenotype did not occur in a *bar-1(ga80)* mutant background (Table 2). Together, these results suggest that the Overinduced phenotype caused by loss of Axin or by stabilization of β -catenin is not dependent on APC function and further, that APR-1 in *C. elegans* may negatively regulate Wnt signaling during vulval development, consistent with APC function in other organisms.

Table 2. *apr-1* negatively regulates Wnt signaling in the VPCs

Strain	RNAi	<i>n</i>	% Over induced	% wild type	% Under induced	% Vul	% Other	<i>n</i>	Average no. induced VPCs
wild type P0	FV	180	0	97	2	0	1		nd
wild type P0	<i>apr-1</i>	115	0	87	5	3	5		nd
<i>pry-1(mu38)</i> P0	FV	185	34	47	3	0	15		nd
<i>pry-1(mu38)</i> P0	<i>apr-1</i>	133	77	14	0	0	9		nd
wild type	FV	564	0	100	0	0	0	332	3.0 ± 0.0
wild type	<i>apr-1</i>	553	0	96	1	0	3	343	3.0 ± 0.0
<i>pry-1(mu38)</i>	FV	622	44	41	5	0	10	146	3.4 ± 0.8
<i>pry-1(mu38)</i>	<i>apr-1</i>	636	84	10	1	0	5	152	4.2 ± 0.8
wild type + HS	FV	789	0	97	1	0	2	208	3.0 ± 0.0
wild type + HS	<i>apr-1</i>	788	2	89	3	0	6	211	3.0 ± 0.0
<i>huIsl</i> – HS	FV	376	0	99	0	0	1	179	3.0 ± 0.0
<i>huIsl</i> – HS	<i>apr-1</i>	381	0	99	0	0	1	180	3.0 ± 0.0
<i>huIsl</i> + HS	FV	273	63	5	0	0	32	180	4.3 ± 0.9
<i>huIsl</i> + HS	<i>apr-1</i>	303	81	3	0	0	16	169	4.7 ± 0.9
<i>huIsl</i> + HS*	FV	83	16	71	0	0	13	51	3.1 ± 0.5
<i>huIsl</i> + HS*	<i>apr-1</i>	98	49	41	0	0	10	62	3.4 ± 0.6
<i>bar-1(ga80); huIsl</i> – HS	FV	119	0	49	46	5	0	119	2.3 ± 0.8
<i>bar-1(ga80); huIsl</i> – HS	<i>apr-1</i>	143	0	46	52	1	1	143	2.4 ± 0.6
<i>bar-1(ga80); huIsl</i> + HS	FV	122	72	0	13	0	15	122	3.7 ± 0.8
<i>bar-1(ga80); huIsl</i> + HS	<i>arp-1</i>	117	57	1	14	0	28	117	3.4 ± 1.0

The RNAi column indicates whether animals were fed *E. coli* expressing double-stranded RNA for *apr-1* or for the control feeding vector (FV). P0 indicates that parent animals were fed on the given *E. coli* and allowed to produce progeny that were scored. For all other strains, L1 stage animals were moved onto plates with *E. coli* expressing the dsRNA, and these same animals were scored in the L4 stage. HS* indicates animals in which the timing of the heat-shock protocol was altered, resulting in a decreased penetrance of the phenotype. (nd) Not determined. Other terms are as defined in Table 1.

The Overinduced phenotype caused by activation of the Wnt pathway is dependent on lin-39

A known Wnt pathway target in the VPCs is the Hox gene *lin-39* (Eisenmann et al. 1998), so we determined whether the Overinduced phenotype caused by Wnt pathway activation depends on *lin-39* activity. Two approaches were taken to remove *lin-39* activity during the L2/L3 stages when the Wnt pathway is likely to act, but not to affect *lin-39* function in the early L1 stage when it is required for the generation of the VPCs (Maloof and Kenyon 1998). First, we used a weak temperature-sensitive allele of *lin-39*, *n709ts*, and grew animals at the restrictive temperature of 25°C (Clark et al. 1993), which has been shown to result in some VPCs adopting Fused fates (Clandinin et al. 1997). At both 15°C and 25°C the introduction of the *lin-39(n709)* mutation into the *pry-1(mu38)* background caused the penetrance of the Overinduced phenotype to decrease, and the average number of induced VPCs to drop below three (Table 3). Also, the average number of induced VPCs dropped from 4.8 in a *hs::delNTbar-1* strain to 3.4 in a *lin-39(n709ts); hs::delNTbar-1* strain (Table 3). As a control, it was shown that the Overinduced phenotype caused by an activating mutation in the *ras* gene, *let-60(n1046)* (Beitel et al. 1990; Han et al. 1990) is also suppressed by *lin-39(n709ts)*. Together, these results indicate that *lin-39* activity is required for the Overinduced phenotype caused by hyperactivation of the Wnt pathway.

As the *lin-39(n709ts)* mutation affects both the generation of the VPCs and cell-fate specification by the

VPCs (J.E. Gleason and D.M. Eisenmann, unpubl.), dsRNA-mediated interference was used to reduce *lin-39* activity only after the VPCs were born. A total of 87% of wild-type animals fed *lin-39* dsRNA only during larval life had an Underinduced or Vulvaless phenotype when scored at the L4 stage (Table 3). Examination of the VPCs in these larvae showed that 98% of animals had all VPCs unfused at the L1 molt ($n = 71$), 92% had all VPCs unfused in the mid L2 stage ($n = 39$), but only 31% had all VPCs unfused in the late L2 stage ($n = 141$), indicating that only the later function of *lin-39* was substantially compromised. With *pry-1(mu38)* animals, the percentage of Underinduced/Vulvaless animals increased from 10% when fed on vector alone to 35% when fed on *lin-39*, and the average number of induced VPCs dropped from 3.5 to 2.6. When *hs::delNTbar-1* animals were fed *lin-39* dsRNA by this method, the percentage of Underinduced/Vulvaless animals increased from 0% to 32%, and the average number of induced VPCs dropped from 4.4 to 2.9. Therefore, the results from these two methods of compromising *lin-39* activity indicate that the Overinduced phenotype caused by hyperactivating the Wnt pathway is dependent on the activity of *lin-39*, a Wnt pathway target in the VPCs.

The Overinduced phenotype caused by hyperactivation of the Wnt pathway does not require activation of the Ras signaling pathway

Previous work has shown that activation of the RTK/Ras pathway is necessary for VPCs to adopt induced cell

Table 3. *The Overinduced phenotype is dependent on lin-39 activity*

Strain	RNAi	n	% Over induced	% wild type	% Under induced	% Vul	% Other	n	Average no. induced VPCs
N2 25°	–	200	0	100	0	0	0	50	3.0 ± 0.0
<i>lin-39(n709ts)</i> 15°	–	112	0	54	39	0	7	112	2.5 ± 0.6
<i>lin-39(n709ts)</i> 20°	–	110	0	47	50	0	3	110	2.3 ± 0.8
<i>lin-39(n709ts)</i> 25°	–	114	0	45	52	0	4	114	2.4 ± 0.7
<i>pry-1(mu38)</i> 15°	–	124	76	13	1	0	10	112	4.0 ± 0.8
<i>pry-1(mu38); lin-39(n709ts)</i> 15°	–	121	17	1	50	4	28	121	2.5 ± 0.9
<i>pry-1(mu38)</i> 25°	–	105	54	32	2	0	12	56	3.2 ± 0.7
<i>pry-1(mu38); lin-39(n709ts)</i> 25°	–	115	23	3	71	3	0	51	2.3 ± 1.1
<i>huIsl</i> – HS	–	111	0	98	0	0	2	67	3.0 ± 0.0
<i>lin-39(n709ts); huIsl</i> – HS	–	85	0	39	59	1	1	85	2.3 ± 0.6
<i>huIsl</i> + HS	–	416	79	13	2	0	6	104	4.8 ± 0.7
<i>lin-39(n709ts); huIsl</i> + HS	–	128	64	6	18	0	12	56	3.4 ± 0.9
<i>let-60(n1046)</i> 25°	–	100	88	2	0	0	10	100	4.7 ± 0.9
<i>lin-39(n709ts); let-60(n1046)</i> 25°	–	115	23	3	71	3	0	108	2.6 ± 0.6
N2	FV	275	0	98	2	0	1	119	3.0 ± 0.0
N2	<i>lin-39</i>	309	0	10	64	23	6	79	1.4 ± 0.8
<i>pry-1(mu38)</i>	FV	242	49	36	10	0	5	79	3.5 ± 0.7
<i>pry-1(mu38)</i>	<i>lin-39</i>	286	37	7	34	1	21	79	2.6 ± 0.9
<i>huIsl</i> + HS	FV	300	83	3	0	0	14	57	4.4 ± 0.7
<i>huIsl</i> + HS	<i>lin-39</i>	230	42	1	30	2	25	57	2.9 ± 0.9
<i>let-60(n1046)</i>	FV	55	100	0	0	0	0	78	4.7 ± 0.8
<i>let-60(n1046)</i>	<i>lin-39</i>	86	20	0	45	27	8	88	1.6 ± 1.0

The RNAi column indicates whether animals were grown on *E. coli* expressing double-stranded RNA for *lin-39* or for the control feeding vector (FV). For RNAi, L1 stage animals were moved onto plates with *E. coli* expressing the dsRNA and these same animals were scored in the L4 stage. Other terms are as defined in Table 1.

fates. To determine whether the Overinduced phenotype caused by hyperactivation of the Wnt pathway is dependent on activity of the Ras pathway, double-mutant strains were built carrying *pry-1(mu38)* or *hs::delNTbar-1* and one of several mutations that reduce signaling through the Ras pathway. In all cases, the Overinduced phenotype was still present in strains in which the Wnt pathway was activated and the Ras pathway was compromised. For example, the *let-23(sy1)* mutation drastically reduces Ras signaling in the VPCs (Aroian and Sternberg 1991), and 95% of *let-23(sy1)* animals have a Vulvaless phenotype. However in a *pry-1(mu38); let-23(sy1)* double-mutant strain, 87% of animals display an Overinduced vulval phenotype, with 4.4 VPCs induced on average at 25°C (Table 4; Fig. 1). Similarly, 67% of animals carrying *lin-45(n2018cs)*, a reduction-of-function mutation in the *C. elegans raf* gene (Han et al. 1993), were Underinduced or Vulvaless, whereas 51% of *pry-1(mu38); lin-45(n2018cs)* were Overinduced (Table 4; Fig. 1). Finally, *let-60(n1531)* encodes a dominant-negative mutation in *let-60 ras* (Beitel et al. 1990; Han et al. 1990). A total of 56% of *let-60(n1531)/+* animals show a Vulvaless phenotype at 25°C, whereas 38% of *pry-1(mu38); let-60(n1531dn)* animals show an Overinduced phenotype. We also tested activated BAR-1 with the *let-23(sy1)* mutation. As with *pry-1*, whereas 100% of *let-23(sy1)* animals subject to heat shocks were Underinduced or Vulvaless, 56% of heat-shocked *let-23(sy1); hs::delNTbar-1* animals were Overinduced (Table 4; Fig. 1). Together, these results indicate that reducing the activity of the Ras pathway at the level of the receptor tyrosine kinase, Ras, or Raf does not abolish the Overinduced phenotype caused by activation of the Wnt pathway.

Discussion

Previous analysis of *bar-1* (β -catenin), *apc-1* (APC), and *mig-14* mutants suggested that a Wnt signaling pathway acts in the VPCs to regulate *lin-39* expression and influ-

ence cell fate specification (Eisenmann et al. 1998; Eisenmann and Kim 2000; Hoier et al. 2000). Due to the limited knowledge of the components of this pathway and its function, it has been unclear whether this putative pathway is similar to the canonical Wnt signaling pathway. Here, we extend our knowledge of the Wnt pathway acting in vulval development by showing that an Axin homolog, PRY-1 (Korswagen et al. 2002), acts in the VPCs, and that loss of *pry-1* activity causes VPCs in addition to P5.p–P7.p to adopt induced cell fates. The *pry-1* Overinduced phenotype is dependent on *bar-1* and *pop-1* activity, implying a site of action for *pry-1* (Axin) upstream of *bar-1* (β -catenin). The *pry-1* gene is expressed in the VPCs (Korswagen et al. 2002), suggesting that PRY-1 may function in the same cells as BAR-1. Because the Overinduced phenotype caused by loss of *pry-1* activity is opposite to the phenotype caused by loss of *bar-1* activity, this suggests that *pry-1* functions as a negative regulator of Wnt signaling in the VPCs, consistent with our knowledge of Axin function in other species.

We also show that overexpression of a BAR-1 protein containing an amino-terminal deletion causes an Overinduced phenotype like that seen in *pry-1* mutants. Mutation or deletion of this region in other β -catenins results in a stabilized protein that interacts with TCF proteins to hyperactivate Wnt pathway target genes (Polakis 2000). It has been shown that BAR-1 has transcription activation domains in its amino- and carboxy-terminal regions, that BAR-1 physically interacts with both POP-1 TCF and PRY-1 Axin, and that BAR-1 and POP-1 can activate transcription in tissue culture cells (Korswagen et al. 2000, 2002; Natarajan et al. 2001). Together, these results suggest that BAR-1 functions as a canonical β -catenin protein in *C. elegans*, that BAR-1 activity is negatively regulated by PRY-1 Axin, and that the Overinduced phenotype of *pry-1* mutants may be due to the hyperactivation of downstream target genes by a BAR-1–POP-1 complex.

Another member of the complex of proteins that nega-

Table 4. The Overinduced phenotype of *pry-1(mu38)* and activated BAR-1 does not require Ras signaling

Strain	n	% Over induced	% wild type	% Under induced	% Vul	% Other	n	Average no. induced VPCs
N2 25°	200	0	100	0	0	0	50	3.0 ± 0.0
<i>pry-1(mu38)</i> 25°	105	54	32	2	0	12	56	3.2 ± 0.7
<i>pry-1(mu38); let-23(sy1)</i> 25°	103	87	0	4	6	3	100	4.4 ± 0.8
<i>let-60(n1531dn)/+</i> ^a	200	0	44	0	56	0		nd
<i>pry-1(mu38); let-60(n1531dn)</i> 25°	114	38	46	9	2	5	50	3.4 ± 0.6
<i>pry-1(mu38)</i> 15°	124	76	13	0	1	10	112	4.0 ± 0.8
<i>pry-1(mu38); let-60(n1531dn)</i> 15°	60	70	12	8	0	10	60	3.8 ± 0.9
<i>lin-45(n2018)</i> 15°	50	0	53	29	38	0	55	1.6 ± 0.9
<i>pry-1(mu38); lin-45(n2018)</i> 15°	114	51	41	4	0	4	39	3.1 ± 0.7
N2 + HS	103	0	98	1	0	1	50	3.0 ± 0.0
<i>let-23(sy1)</i> + HS	208	0	0	32	68	0	50	0.5 ± 0.8
<i>huIsl</i> + HS	416	79	13	2	0	6	104	4.8 ± 0.7
<i>let-23(sy1); huIsl</i> – HS	178	0	3	16	81	0	50	0.5 ± 0.9
<i>let-23(sy1); huIsl</i> + HS	352	56	1	18	23	2	50	4.1 ± 1.6

^aData are from Beitel et al. (1990). [nd] Not determined. Other terms are as defined in Table 1.

tively regulates β -catenin stability is the APC gene product. In other systems, disruption of APC function results in overactivation of Wnt signaling (Moon and Miller 1997; Polakis 2000); however, previous work suggested that the *C. elegans* APC homolog APR-1 may play a positive role in Wnt signaling in the vulva. Specifically, postembryonic production of an antisense *apr-1* mRNA from the *lin-31* promoter causes loss of *lin-39* expression and adoption of Fused fates by the VPCs (Hoier et al. 2000). Here, we provide evidence that *apr-1* may have a negative regulatory function in Wnt signaling, like APC in other systems. When zygotic *apr-1* function was reduced by RNAi in animals in which the Wnt pathway was activated, the penetrance and expressivity of the Overinduced phenotype was consistently increased. It is not clear why our dsRNA feeding protocol resulted in a different effect on vulval development than the expression of antisense *apr-1* from the *lin-31* promoter. One possibility is that the enhancement of the Overinduced phenotype may not be due to reduction of *apr-1* function in the VPCs directly, because in our experiments, the entire animal was presumably affected by the ingested *apr-1* dsRNA. In this case, *apr-1* may function in another cell or cells, which then negatively regulate Wnt signaling in the VPCs. However, the loss of APR-1::GFP expression in the VPC descendants following *apr-1* RNAi suggests that *apr-1* activity was reduced in the VPCs. Alternatively, the effects caused by the *lin-31* driven, antisense *apr-1* construct used previously may not be due to a direct effect on *apr-1* activity. In fact, it has been noted that high-copy arrays containing the *lin-31* pro-

motor can sometimes cause a Fused fate phenotype (Kishore and Sundaram 2002; L. Miller, pers. comm.), suggesting that high levels of the *lin-31* promoter may titrate out some factor necessary for VPC fate specification. If this is the case, the observed loss of LIN-39 expression and adoption of Fused fates observed previously may be due to the use of *lin-31* promoter sequences. Finally, although loss of *apr-1* function enhanced the phenotype caused by Wnt pathway hyperactivation, *apr-1* RNAi caused little effect on vulval development in a wild-type background. It may be that the negative role for *apr-1* is only apparent in a sensitized background in which the Wnt pathway is strongly activated.

Therefore, our current results suggest that a canonical Wnt pathway acts in the VPCs to influence cell fate specification, and that BAR-1 and POP-1 act positively and PRY-1 and APR-1 act negatively in this pathway (Fig. 3). Genetic evidence suggests that *mig-5*, which encodes a Dishevelled homolog (Antebi et al. 1997), may also act positively in this process; however, Wnt or Frizzled homologs regulating this pathway have not yet been identified (S. Peyrot and D. Eisenmann, unpubl.). All of these genes also act in a canonical Wnt pathway to control migration of the Q neuroblast descendants (Harris et al. 1996; Antebi et al. 1997; Korswagen et al. 2000, 2002; Herman 2001).

How does overactivation of this Wnt pathway lead to extra vulval induction, even when activation of the Ras pathway has been compromised? We believe that overexpression of one or more Wnt target genes causes these extra inductions. Currently, the only known target of the

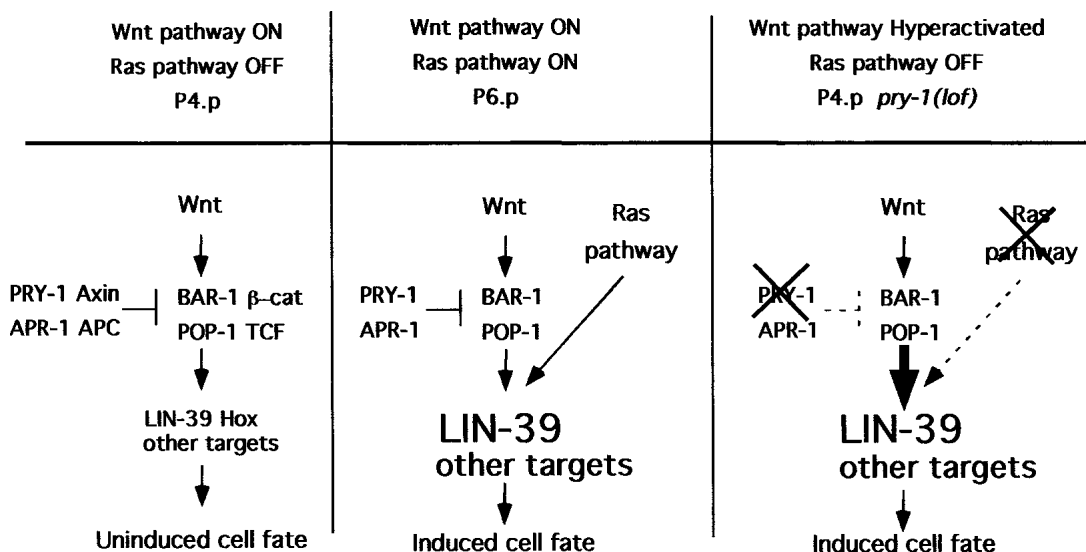


Figure 3. Model. We propose that Wnt signaling acts to maintain *lin-39* expression in all VPCs to prevent them from adopting the Fused fate. PRY-1 Axin and APR-1 APC act negatively to regulate the Wnt pathway, and BAR-1 β -catenin and POP-1 TCF act positively. In a cell in which the Ras pathway is inactive like P4.p, LIN-39 is required to adopt the 3^o uninduced cell fate. In a cell in which the Ras pathway is active such as P6.p, we propose that the activity of both pathways leads to higher levels of LIN-39 expression, as has been reported by Maloof and Kenyon (1998). We propose that this higher level of LIN-39 and/or other Wnt pathway targets is instructive in causing the VPC to adopt an induced cell fate. In a cell in which the Wnt pathway has been hyperactivated (e.g., *pry-1* loss-of-function), higher levels of LIN-39 and/or other Wnt targets allows adoption of an induced cell fate in the absence of input from the Ras pathway.

Wnt pathway in the VPCs is *lin-39* (Eisenmann et al. 1998). It is also known that LIN-39 protein levels increase in P6.p in a Ras pathway-dependent manner (Malooof and Kenyon 1998). This suggests that LIN-39 may function in the adoption of induced vulval fates when expressed at higher levels. Therefore, we propose that overactivation of the Wnt pathway may cause levels of LIN-39 to exceed some threshold in P3.p, P4.p, and P8.p, causing those cells to sometimes adopt induced cell fates (Fig. 3). In this model, the Wnt pathway normally plays a permissive role in the maintenance of LIN-39 expression in all VPCs to prevent these cells from adopting the Fused fate, but overactivation of the Wnt pathway can phenocopy a cell in which both the Wnt and Ras pathways are active. This would be consistent with the dependence of the Overinduced phenotype on *lin-39* activity in our experiments, and the independence of that phenotype on Ras signaling. Preliminary experiments have suggested that expression of high amounts of LIN-39 alone in L2/L3 larvae is not sufficient to phenocopy the Overinduced phenotype described here (Malooof and Kenyon 1998; J.E. Gleason and D.M. Eisenmann, unpubl.). This suggests that there may be additional targets of the Wnt pathway that contribute to the adoption of induced fates when Wnt signaling is overactivated. Future experiments will attempt to identify target genes regulated by overactivation of the Wnt pathway, as well as to determine the functional relationship between factors acting downstream of Wnt signaling, such as LIN-39, and transcription factors known to act downstream of Ras signaling during vulval induction, such as the winged helix protein LIN-31 (Miller et al. 1993) and the Ets domain protein LIN-1 (Beitel et al. 1995).

Materials and methods

Genetic methods and alleles

Methods for culture and genetic manipulation of *C. elegans* were as described (Brenner 1974). Wild-type animals were *C. elegans*, variety Bristol, strain N2. Experiments were performed at 20°C unless otherwise indicated. The reference for genes and alleles used in this work is as follows (Riddle et al. 1997) unless otherwise indicated: (LGI) *pop-1(hu9)* (Korswagen et al. 2002), *pry-1(mu38)* (Malooof et al. 1999); (LGII) *let-23(sy1)*, *mig-14(ga62)* (Eisenmann and Kim 2000); (LGIII) *lin-39(n709ts)*; (LGIV) *lin-45(n2018cs)*, *let-60(n1531dn)*, *n1046gf*, *dpy-20(e1282)*; (LG V) *him-5(e1490)*; (LGX) *bar-1(ga80)* (Eisenmann et al. 1998).

huls1 indicates the integrated array containing the *hs::delNTbar-1* construct, pHCK19 (50 ng/μL), and a *dpy-20(+)* marker plasmid, pMH86 (100 ng/μL). pHCK19 contains sequences downstream from the natural *NcoI* site in the 5' end of the *bar-1* cDNA driven from the heat-shock promoter in plasmid pPD49.78 (gift of A. Fire, Carnegie Institution, Baltimore, MD).

Heat-shock protocol

Synchronized L1 animals were seeded onto plates with food and incubated at 20°C for 22–24 h (except Fig. 2) in a Conviron MTR30 programmable incubator. Animals were subjected to

either a single heat shock at 38°C for 0.5 h or two heat shocks with the second occurring 3.5 h later. Following heat shock, animals were incubated at 20°C until the early L4 stage when vulval induction was examined.

RNA interference

The *apr-1* plasmid for RNAi by feeding was generated by PCR amplification of a 3.4-kb fragment from *apr-1* cDNA with primers ODE92 (CCATGGGGAATCTACTCACAACCTCGTG-CAGG) and ODE97 (CCATGGTTAGACTATTGTTACAAG). The *lin-39* plasmid for RNAi by feeding was generated by PCR amplification of *lin-39* cDNA using primers ODE115 (CAGCTCGAGAAGGAACGGGGGAACCTG) and ODE124 (CTCGA GATGACCACATCAACATCACC). Both PCR products were ligated into the feeding vector, pPD129.36, and transformed into the T7 polymerase-expressing *E. coli* strain HT115 (Timmons et al. 2001). Bacteria were grown at 37°C to an O.D. of 0.5–0.8, induced with 0.4 mM IPTG for 2.5 h, then concentrated and spread onto NGM plates containing 0.4 mM IPTG, 50 μg/mL carbenicillin and 12 μg/mL tetracycline. For feeding of P0 animals, adult hermaphrodites were placed directly on these plates at 20°C and their progeny analyzed. For larval feeding, eggs from strains to be tested were placed on NGM plates without food and the next day semi-synchronous L1 larvae were washed off and placed onto plates with the appropriate HT115 strain and allowed to develop at 20°C.

Scoring of vulval phenotypes

Most *pry-1(mu38)* animals burst at the vulva as adults, so we characterized the *pry-1* phenotype by examining vulval morphology at the L4 stage. For this reason, we use the term Overinduced (greater than three VPCs adopt induced fates) instead of Multivulva to describe the phenotype. To determine the extent of vulval induction, cell fates adopted by P3.p–P8.p were scored in living, early L4 hermaphrodites observed with Nomarski differential interference contrast optics by use of the criteria for designation of cell fate described in Sternberg and Horvitz (1986). A VPC was scored as having adopted an induced fate if 4–8 nuclei were found, and these cells had detached from the cuticle and invaginated toward the gonad. The presence of one or two nuclei was considered uninduced.

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