Evolution of Developmental Control Mechanism

Conserved mechanism of Wnt signaling function in the specification of vulval precursor fates in C. elegans and C. briggsae

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The C. elegans hermaphrodite vulva serves as a paradigm for understanding how signaling pathways control organ formation. Previous studies have shown that Wnt signaling plays important roles in vulval development. To understand the function and evolution of Wnt signaling in Caenorhabditis nematodes we focused on C. briggsae, a species that is substantially divergent from C. elegans in terms of the evolutionary time scale yet shares almost identical morphology. We isolated mutants in C. briggsae that display multiple pseudo-vulvae resulting from ectopic VPC induction. We cloned one of these loci and found that it encodes an Axin homolog, Cbr-PRY-1. Our genetic studies revealed that Cbr-pry-1 functions upstream of the canonical Wnt pathway components Cbr-bar-1 (β-catenin) and Cbr-pop-1(tcf/lef) as well as the Hox target Cbr-lin-39 (DFd/Scr). We further characterized the pry-1 vulval phenotype in C. briggsae and C. elegans using 8 cell fate markers, cell ablation, and genetic interaction approaches. Our results show that ectopically induced VPCs in pry-1 mutants adopt 2° fates independently of the gonad-derived inductive and LIN-12/Notch-mediated lateral signaling pathways. We also found that Cbr-pry-1 mutants frequently show a failure of P7.p induction. A similar, albeit low penetrant, defect is also observed in C. elegans pry-1 mutants. The genetic analysis of the P7.p induction defect revealed that it was caused by altered regulation of lin-12 and its transcriptional target lip-1 (MAP kinase phosphatase). Thus, our results provide evidence for LIN-12/Notch-dependent and independent roles of Wnt signaling in promoting 2° VPC fates in both nematode species.

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I n t r o d u c t i o n

Multicellular organisms have evolved complex cell communication machinery that enables cells to recognize and respond to a diverse range of extracellular signals. This interaction is crucial for the survival of organisms and their ability to function as coherent systems. Communication between cells and their environment is mediated by receptors that interact with specific ligands to transduce the signal into the cell. This leads to the activation of a cascade of intracellular proteins, many of which are components of a relatively small set of evolutionarily conserved signaling pathways, such as Ras, Notch, and Wnt (Bray, 2006; Eisenmann, 2005; Logan and Nusse, 2004; Sundaram, 2005). Among these, the Wnt signaling pathway has been shown to control diverse developmental processes including cell proliferation, cell polarity, and cell migration (Eisenmann, 2005; Widelitz, 2005). Studies on Wnt signaling have identified several genes that encode pathway components such as Wnt (ligand), Frizzled (receptor) and β-Catenin (transcriptional regulator). Analysis of their function has revealed that in normal cells, in the absence of Wnt ligands, β-Catenin is actively degraded by the action of a protein complex that contains scaffolding proteins Axin and APC and a serine/threonine kinase GSK3β (Logan and Nusse, 2004). The interaction of Wnts with Frizzled receptors activates the pathway leading to the dissociation of this complex, allowing cytoplasmic β-Catenin to translocate to the nucleus and interact with the TCF/LEF factor to regulate gene transcription.

The nematode C. elegans is a leading model organism to understand the mechanism of Wnt signaling function in development. Genetic studies in C. elegans have shown that Wnt signaling controls multiple processes including embryonic patterning, gonadogenesis, neuronal differentiation, male hook formation, and vulval development (Eisenmann and Kim, 2000; Eisenmann et al., 1998; Gleason et al., 2002; Maloof et al., 1999; Rocheleau et al., 1997; Salser and Kenyon, 1992; Siegfried and Kimble, 2002; Sternberg and Horvitz, 1988; Thorpe et al., 1997; Yu et al., 2009). The downstream targets of the pathway include three Hox genes, lin-39 (Deformed/Sex combs reduced (DFd/Scr) family), mab-5 (Antennapedia/Ultrabithorax/abdominal-A (Antp/Ubx/Abd-A) family) and egl-5 (Abdominal-B ( Abd-B) family) that are expressed in multiple tissues and control diverse cell fates (Eisenmann, 2005; Kenyon et al., 1997).

Due to its simplicity, the C. elegans vulva has been successfully used to study the regulation and function of Wnt signaling pathway...
components. The vulva develops from three of six ventral hypodermal cells (termed P3.p to P8.p) that escape fusion to the surrounding hypodermal syncytium, hyp7, during the L1 stage and become vulval precursor cells (VPCs). This process is mediated by lin-39 since all Pn.p cells in lin-39 mutants fuse to hyp7 in the L1 stage. The lin-39 activity is also required during the L2 stage to prevent VPCs from fusing to hyp7, and maintaining their competence to respond to patterning signals. The L2-stage expression of lin-39 is positively regulated by the BAR-1 (β-Catenin)-mediated canonical Wnt signaling pathway. In bar-1 mutants lin-39 activity is greatly reduced which causes VPCs to inappropriately fuse with hyp7 (Eisenmann et al., 1998). The other Wnt pathway components that regulate Wnt competence include 5 Wnt ligands (LIN-44, CWN-1, CWN-2, EGL-20, and MOM-2), 3 Frizzled receptors (LIN-17, MOM-5, and MIG-1), PRY-1 (Axin), and POP-1 (TCF/LEF) (Eisenmann, 2005; Gleason et al., 2002; Gleason et al., 2006; Inoue et al., 2004). The expression analysis of Wnt pathway genes has revealed that multiple tissues could act as sources of Wnt signals (including gonad, muscles, and many cells in the tail region) (Gleason et al., 2006; Herman et al., 1995; Inoue et al., 2004; Whangbo and Kenyon, 1999). The finding that Wnt ligands form antero-posterior gradient and pattern certain cell fates (Coudreuse et al., 2006) provides support to a model that similar signals originating from non-vulval tissues may differentially affect VPC fates.

In addition to Wnt, VPCs also respond to inductive signaling initiated by the LIN-3/Epidermal Growth Factor (EGF) ligand and lateral signaling via the LIN-12/Notch receptor (Greenwald, 2005; Sternberg, 2005). The LIN-3/EGF, secreted by a gonadal anchor cell (AC), interacts with the LET-23/EGF receptor and initiates a MKP-1/ MAP kinase-mediated signaling pathway in VPCs. This causes P6.p to adopt a 1° fate. The interactions between P(5-7).p, mediated by LIN-12/Notch lateral signaling, confers a 2° fate on P5.p and P7.p. The induced VPCs, P(5-7).p, divide during L3/L4 stages to generate 22 progeny that differentiate to form 7 different cell types (vulA to vulF) (Sharma-Kishore et al., 1999). The remaining uninduced VPCs (P3.p, P4.p and P8.p) adopt a 3° fate and fuse to hyp7. The presence of various regulators of the signaling pathways, genetic redundancies, as well as crosstalks between pathways, ensures that a 3°-3°-2°-1°-2°-3° spatial pattern is reproducibly generated.

The simplicity and ease of experimental manipulations of the vulva has facilitated comparative developmental analysis among nematode species. These findings have revealed similarities and differences in some of the underlying developmental mechanisms (Eizinger and Sommer, 1997; Felix, 2005, 2007; Sommer, 2005; Sommer and Sternberg, 1996; Tian et al., 2008; Zheng et al., 2005). For example, the lin-17/Frizzled receptor ortholog in Pristionchus pacificus represses vulval cell fates, whereas in C. elegans lin-17 promotes VPC competence and cell fates (Eisenmann, 2005; Zheng et al., 2005). The P. pacificus and Oscheius tipulae lin-39 orthologs represent another case of evolutionary conservation and divergence of gene function. While the O. tipulae lin-39 promotes VPC competence similar to lin-39 in C. elegans (Louvet-Vallee et al., 2003), the P. pacificus lin-39 prevents VPCs from undergoing programmed cell death (Eizinger and Sommer, 1997).

Among the species that are closely related to C. elegans, C. briggsae is used extensively in comparative studies (Gupta et al., 2007). Although the two species diverged roughly 30 million years ago (Cutter, 2008), morphologically they appear very similar. This provides a unique opportunity to study gene function and signaling pathways in specifying homologous processes. We are taking a forward genetics approach to study the mechanism of vulval development in C. briggsae. This work focuses on pry-1 (Axin family) and its interactions with Wnt and LIN-12/Notch signaling pathway components in VPC fate specification. We found that the Chr-pry-1 mutants display a unique pattern of vulval induction defect that is characterized by ectopically induced P3.p, P4.p, and P8.p, and an uninduced P7.p. The genetic analysis of Chr-pry-1 interaction with other genes revealed that Chr-bar-1 (β-Catenin)-Cbr-pop-1 (tcf/lef)-mediated canonical Wnt signaling plays an essential role in promoting VPC competence and cell proliferation in C. briggsae. The downstream targets of the pathway include the Hox gene Cbr-lin-39. To understand the mechanism of pry-1-mediated Wnt signaling function we used a combination of cell fate markers, laser microsurgery, and genetic interaction experiments. The findings show that ectopically induced VPCs in pry-1 mutants, in both C. elegans and C. briggsae, acquire 2° fate independently of the gonad-derived inductive signaling and LIN-12/Notch-mediated lateral signaling pathways. However, interestingly, in the case of P7.p our data suggests that pry-1 acts genetically upstream of lin-12 and its transcriptional target lip-1 to promote vulval cell fate. Taken together these findings reveal that Wnt signaling utilizes multiple mechanisms to specify the spatial pattern of VPC fates in C. elegans and C. briggsae.

Materials and Methods

Strains and general methods

The general methods for culturing and genetic manipulations have been previously described (Brenner, 1974). All experiments were carried out at 22 °C unless otherwise noted. The staging of animals was primarily based on the gonad morphology as described in Worman (Hall and Alltm, 2008). The gonad arms initiate turning during mid-L3 stage and by mid-L4 stage arms are in close proximity to the center of vulval invagination formed by P(5-7).p progeny. Various mutations used in this study are listed below in the alphabetical order. Where known, the locations of mutations and transgenic strains are indicated. The 'Chr' prefix denotes the C. briggsae ortholog of a C. elegans gene.

C. briggsae: AF16 (wild type), Cbr-pry-1 (sy5353) I, Cbr-pry-1 (sy5270) I, Cbr-pry-1 (sy5411) I, Cbr-unc-119 (sy20000) III, mfs5[Cbr-egf-17::GFP, myo-2::GFP], mfs8[Cbr-egf-17::GFP, myo-2::GFP], mfs29 [lip-1::GFP, myo-2::GFP], mfs33[dig-1::GFP, myo-2::dsRed], mfs42[sid-2(+)::myo-2::dsRed], bhEx59 [hsp::Cbr-pry-1::myo-2, myo-2::GFP], bhEx88[Cbr-pry-1-5 kb::GFP, unc-119(+)], bhEx84[Cbr-pry-1-3.8 kb::GFP, unc-119(+)].

C. elegans: N2 (wild type), lin-12(n137) III, lin-12(n952) III, lin-12(n676n909) III, lip-1(zh15) IV, pop-1(m18) I, pry-1 (mu38) I, unc-119 (ed4) III, afy-5[egf-17::GFP, dpy-20(+)], I, bhEx53[raf-6::GFP, myo-2::GFP], dels4[egf-20(+)], I, sly534[eh2::GFP, unc-119(+)], I, sly534[eh2::GFP, UNC-119(+)], I, sly534[eh2::GFP, UNC-119(+)], I, sly534[eh2::GFP, UNC-119(+)], I, wyEx3372[syg-2::GFP], zhs4[lip-1::GFP, unc-119(+)].

The transgenic animals carrying extrachromosomal arrays were generated by standard microinjection technique (Mello et al., 1991) using C. elegans unc-119 (Maduro and Pilgrim, 1995) and myo-2::GFP (pPD118.33) (S. Q. Xu, B. Kelly, B. Harfe, M. Montgomery, J. Ahnn, S. Getz and A. Fire, personal communication) as transformation markers. The concentrations of plasmids that were injected as part of this study are: hsp::Cbr-pry-1 125 ng/μl, Cbr-pry-1::GFP (each of 3.8 kb and 5 kb promoter fragment) 100 ng/μl, def-6::YFP 100 ng/μl.

The synchronized L1 stage hsp::Cbr-pry-1 animals were heat shocked at 31 °C for 24 hrs and subsequently grown at 22 °C until adulthood. Cell ablation experiments were performed as described (Avery and Horvitz, 1987). The gonad precursors (Z1 to Z4) were ablated during the L1 stage whereas VPCs were ablated during the L2 stage. Worms were recovered from slides and allowed to grow until L4 stage. Vulval phenotypes were examined using Nomarski optics.

Vulval phenotype and induction analysis

We scored VPC competence and induction during the L2-L4 stages. A VPC was considered induced if it gave rise to 4 or more progeny that had invaginated. With the exception of P7.p and P8.p in pry-1 mutants
that appear morphologically similar to P12,pa (referred as P12,pa-like fate), an uninduced VPC can adopt either an F fate (no division and fusion to hyp7 syncytium in L2) or a 3° fate (one division followed by fusion of both daughters to hyp7 in L3). In wild-type animals (C. elegans and C. briggsae) P4,p and P8,p always adopt a 3° fate, while P3,p does in ~20–50% of cases (F fate in the remainder). Statistical analyses were performed using InStat 2.0 (GraphPad) Software. Two-tailed P values were calculated in unpaired t tests and values less than 0.05 were considered statistically significant.

Unlike wild type animals in which P3,p, P4,p and P8,p fuse to hyp7, in pry-1 mutants these Pn,p cells can be ectopically induced to divide (termed ‘Overinduced’). Additionally, pry-1 mutants frequently show a failure of P7,p induction (termed ‘Underinduced’). Thus, the same animal can exhibit both Overinduced and Underinduced phenotypes.

**Isolation and mapping of C. briggsae pry-1**

The Cbr- pry-1 mutants were isolated in a genetic screen for animals that exhibit defects in vulval induction. Wild-type L4 stage AF16 animals were fed with 25 mM Ethyl Methanesulfonate (EMS; Sigma) for 3 hrs using standard procedures (Wood, 1988). More than 500,000 haploid genomes were screened and F2 animals showing multiple pseudo-vulvae were isolated. Putative lines that showed a reproducible phenotype were retained and backcrossed three to four times.

The complementation and linkage studies revealed that three mutants sy5270, sy5353 and sy5411 define a locus on L1 and map close to a levamisole-resistant mutant lev(sy5440) (zh15), and a close to a levamisole-resistant mutant lev(sy5440) (fh15), and pop-1 (GL184/GL185), 2.4 kb genomic fragment of Cbr-lin-39 (GL13/3GL314), and 1.5 kb genomic fragment of Cbr-lip-1 (GL46/G465). The heat-shock promoter (hs16-41) driven Cbr- pry-1 construct was made by subcloning the full-length Cbr- pry-1 cDNA into the Fire lab pPD49.83 vector.

**RNAi**

Since the wild type C. briggsae (AF16) is resistant to environmental RNAi, we used a transgenic strain mfs42 that carries wild type copy of the C. elegans sid-2 gene. mfs42 animals are sensitive to environmental RNAi similar to the wild-type C. elegans (Winston et al., 2007). RNAi was performed on plates containing 0.6% Na2HPO4, 0.3% KH2PO4, 0.1% NH4Cl, 0.5% Casamino Acids, 2% agar, 1 mM CaCl2, 1 mM MgSO4, 0.0005% cholesterol, 0.2% lactose, and 50 μg/ml Carbenicillin. Plates were seeded with 100 μl of overnight grown HT115 bacterial culture in LB and Carbenicillin media that produces dsRNA of the gene of interest. Three to 5 L4 stage worms were placed on RNAi plates and the phenotypes of F1 progeny were examined. For genes that caused sterility and early stage lethality, animals were subjected to RNAi treatment during L1 larval stage. All RNAi experiments were repeated 3–4 times and batches that produced consistent results were analyzed.

The C. briggsae genome contains two lin-12-like genes one of which appears to be a Cbr-lin-12 paralog (99% sequence identity) (www.wormbase.org). The Cbr-lin-12 RNAi construct used in this study is expected to inactivate both copies. This construct was described earlier and used to study lin-12 function in C. briggsae (Felix, 2007). The amplified genomic region lacks significant sequence similarity to Cbr-glp-1, a Cbr-lin-12/Notch family member, ruling out the possibility of an RNAi off-target effect. Furthermore, the Cbr-lin-12 and Cbr-glp-1 RNAi phenotypes are different and can be easily distinguished (Rudel and Kimble, 2001; Rudel and Kimble, 2002) (BPG, unpublished). Hence the Cbr-lin-12 RNAi results described below are likely to be specific to Cbr-lin-12 and its paralog.

**Results**

**IsoIation and molecular characterization of C. briggsae pry-1**

To identify genes involved in vulval development in C. briggsae, we carried out EMS mutagenesis screens and isolated mutants that exhibit ectopic vulval induction leading to the formation of multiple pseudo-vulvae in adults. Out of 10 such mutants that were recovered from the screen, three (sy5270, sy5353, and sy5411) failed to complement (Table 1) and defined a locus on chromosome 1. All three alleles showed high frequency of ectopically induced VPCs (Table 1). In many cases (40%; n = 25 for sy5353) the migration of gonad arms was also defective. The males also showed abnormal tail morphologies such as ectopic anterior rays, crumpled spicules and pseudovulvulvae-like structures (data not shown).

To understand the mechanism of sy5353 function in vulval development, we determined the molecular identity of the locus. A combination of phenotypic markers, polymorphism-based mapping,
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and C. briggsae genome sequence assembly (Hillier et al., 2007) enabled us to take a candidate gene approach. A search for the C. elegans orthologs in the vicinity of linked polymorphisms bhP1, bhP7 and bhP42 (Fig. 1A and Supplementary Fig. 1) revealed a Wnt pathway component pry-1 that is known to negatively regulate vulval induction (Gleason et al., 2002). The results of the following experiments indicate that sy5353 is an allele of C. briggsae pry-1. First, we found that the RNAi-mediated knockdown of Cbr-pry-1 phenocopies sy5353 (Table 1). Second, overexpression of Cbr-pry-1 full-length cDNA (using hsp16-41 heat-shock promoter, hsp::Cbr-pry-1) rescues the mutant phenotype in more than half of the sy5353 animals (53% wild type, n = 163, compared to 3% wild type, n = 39 in sy5353 animals without heat shock). Finally, we sequenced Cbr-pry-1 alleles and identified sy5353 and sy5411 mutations that introduce premature in-frame stop codons (see Materials and Methods and Supplementary Fig. 2), suggesting that both are likely to be Cbr-pry-1 hypomorphs.

A comparison of the pry-1 genomic regions between C. elegans and C. briggsae revealed the absence of one exon, as well as comparatively larger sizes for two introns in C. briggsae (Fig. 1B). We aligned protein sequences of Axin family members in nematodes and vertebrates to

Fig. 1. (A) The locations of various markers and lin(sy5353) on chromosome 1. The sy5353 mutation is tightly linked to indels bhP7, bhP1 and bhP42. (B) The open reading frames (ORFs) of pry-1 in C. briggsae and C. elegans. Major differences between the two ORFs include one less exon and two larger introns towards the 3’ end in C. briggsae compared to C. elegans. (C-J) Vulval induction defect in pry-1 mutants. Thick arrows mark the main vulva whereas arrowheads mark ectopic vulval invaginations. (C) The posterior VPCs are uninduced in a sy5353 animal. The inset panels show a wild-type (WT) vulva and the nuclei of P7.p, P7.pa and P12.pa in WT and sy5353 animals, and a sy5353 adult showing anterior pseudovulvae. (D) A pry-1(mu38) animal showing induced P4.p and uninduced P7.p. (E-I) Analysis of the cell fusion defect using dlg-1::GFP (mfEx33) in Cbr-pry-1(sy5353) and ajm-1::GFP (deIs4) in pry-1(mu38). Unlike the wild type (E, F) where the progeny of P(5-7).p fuse to form seven concentric toroids, in the sy5353 animal (G, H) P7.p and P8.p precursors remain unfused. (I, J) An unfused P7.p in pry-1(mu38) revealed by ajm-1::GFP expression. Anterior is to the left in all cases. The scale bars in C (same for E-J, except inset in C) and D are 10 μm.
identify domains in Cbr-PRY-1 that may facilitate homodimerization and interactions with C. briggsae homologs of APC, GSK-3β and β-Catenin (Behrens et al., 1998; Ikeda et al., 1998; Luo et al., 2005; Schwarz-Romond et al., 2007) (Supplementary Fig. 3). The C. briggsae PRY-1 is 70% identical to its counterpart in C. elegans with various domains being 70-85% conserved (Supplementary Fig. 4). This level of identity is close to APR-1 (APC homolog; 77% identical) but much lower than GSK-3 (GSK-3β homolog; 95% identical).

### Table 1

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*p: refers to wild-type genetic background (C. briggsae AF16 and C. elegans N2). *X denotes Pn.p cells that were ablated during the L2 stage. *Strains carry egl-17::GFP (ayIs4). VPC: vulva precursor cell, n: number of animals examined, nd: not done, na: not applicable.

pron-1 mutants exhibit both Overinduced and Underinduced phenotypes

The Cbr-PRY-1 alleles were isolated based on the presence of ectopic pseudo-vulvae in adults. The analysis of vulval phenotype in mid-L4 stage animals showed a unique defect in VPC induction pattern. Specifically, P3.p, P4.p, and P8.p were ectopically induced in most animals whereas P7.p remained uninduced (Table 1) (see Materials and Methods). The P5.p and P6.p fates were unaffected. We also looked at the placement of AC in such animals and found that it was always located on the top of P6.p and its descendents (data not shown). Frequently individual animals exhibited both Overinduced and Underinduced phenotypes (Fig. 1C). A similar, albeit less penetrant, defect was also observed in C. elegans pron-1 mutants (Fig. 1D, Table 1). In some cases only P5.p and P6.p were induced, a phenotype that has previously been reported in C. elegans (Gleason et al., 2002). The vulval cell lineages of mutant animals further supported these findings (Table 2). Thus, pron-1 appears to play a similar role in both species.

A careful examination of Pn.p cells in Cbr-PRY-1 mutants revealed an additional defect in that all P(7-11).p cell nuclei were significantly smaller in size compared to wild type, appearing similar to P12.pa (Fig. 1A inset). It remains to be determined whether such a morphological change is caused by transformation to P12.pa-like cell fate. This phenotype is distinct from C. elegans pron-1(mu38) which do not show an obvious change in the morphology of posterior Pn.p cells (except P11.p) (see P7.p in Fig. 1D, data not shown). Considering that mutations in C. elegans Wnt pathway genes cause cell fate transformation due to alterations in Hox gene expression (Korswagen et al., 2002; Malo et al., 1999), it is tempting to speculate that similar changes may underlie the Pn.p transformation phenotype in Cbr-PRY-1 mutants.

We examined posterior VPCs in pron-1 mutants using cell junction-associated markers ajm-1 (in C. elegans) and dlg-1 (in C. briggsae) that identify epithelial cell boundaries. The AJM-1 (novel coiled-coil protein) and DLG-1 (Drosophila Disc Large family) are localized to cell junctions (Sharma-Kishore et al., 1999) (Fig. 1E,F for mec-7::GFP (muIs32). #Strains carry egl-17::GFP (ayIs4). VPC: vulva precursor cell, n: number of animals examined, nd: not done, na: not applicable.)
P8.p in *pry-1* mutants are frequently unfused (Figs. 1C–J), which indicates that their lack of proliferation may be due to other defects. We also observed some cases of unfused P(9-11).p in *pry-1* mutants, which is similar to that reported earlier (Myers and Greenwald, 2007). These results suggest that in addition to maintaining competence, Wnt signaling also plays an important role in promoting cell proliferation and differentiation.

To determine whether P7.p and P8.p induction in *pry-1* mutants is inhibited by some unknown signal from neighboring VPCs we carried out VPC lineage analysis in *pry-1* mutants (Table 1). Overall, the RNAi-mediated knock-down of *pry-1* caused abnormal vulval morphology in 25% of animals examined. The cells attached to cuticle are underlined. In all cases, anchor cell was located on the top of the P6.p progeny.

**Chbr-1 is expressed in vulval precursors and their progeny**

The molecular cloning of *Chbr-1* facilitated the analysis of its expression pattern during development. We designed transcriptional GFP reporter plasmids using the *S. cerevisiae* genomic region of *Chbr-1* (3.6 kb and 5 kb) and generated transgenic lines in *C. briggsae*. The *Chbr-1::GFP* was expressed throughout the development, a finding similar to the *C. elegans* *lin-39* expression pattern (Table 1), although animals exhibited severe defects in gonad morphology and were frequently sterile (data not shown). However, a similar RNAi experiment involving another *β-Catenin*, *Cbr-bar-1*, strongly suppressed the Overinduced phenotype of *Chbr-1* (sy5353) (Table 1), suggesting that *Cbr-bar-1* is likely to act genetically downstream of *Chbr-1*. We also found that comparison to control animals P7.p in *Chbr-1* (sy5353): *Cbr-bar-1* (RNAi) was significantly more induced (p = 0.0002, Table 1).

Next, we examined the role of the *tcfl/lef* family member *Chbr-pop-1* in *Chbr-1*-mediated vulval development. The RNAi-mediated knock-down of *Chbr-1* suppressed ectopic VPC induction in *Chbr-1* (sy5353) (54% induced, 10% P12.pa-like, 36% 3°, and no F fates, n = 59 animals compared to 38% induced, 62% P12.pa-like and no 3°, or F fates, n = 50 animals in control) (Table 1). Similar to *Cbr-bar-1* RNAi, we also found an increased number of induced P7.p in *Chbr-1* (sy5353) *Chbr-pop-1* (RNAi) animals (Table 1). Interestingly, *Chbr-pop-1* (RNAi) animals alone showed severe defects in vulval cell proliferation (no P12.pa-like, 55% 3° and 29% F fates, n = 25) (Fig. 3A, Table 1), suggesting its essential role in regulating VPC competence and induction. Although no such defect was observed in *C. elegans* *pop-1* (RNAi) and *pop-1* (null) (a viable hypomorphic) animals, both strongly suppressed the *pry-1* (mu38) phenotype (Table 1 and data not shown). These results demonstrate that *bar-1*-pop-1-mediated canonical Wnt signaling plays a conserved role in vulval development in *C. elegans* and *C. briggsae*.

Studies in *C. elegans* have identified transcriptional targets of Wnt signaling that include *Hox* genes with functions (sy5353) and *mab-5* (Antp Hox family) (Eisenmann, 2005). *lin-39* is required at multiple times in vulval development. During the L2 stage, *lin-39*-mediated canonical Wnt signaling prevents VPCs from fusing to the hyp7 syncytium (Eisenmann et al., 1998). Later on, during the L3 stage, *lin-39* is involved in the specification of VPC fates (Eisenmann, 2005). Unlike *lin-39*, *mab-5* appears to play a limited role in vulval development, *mab-5* is expressed in P7.p and P8.p and regulates the responsiveness of these two cells to inductive signal (Clandinin et al., 1997; Salser et al., 1993). We found that the RNAi-mediated knock-down of *Chbr-mab-5* had no effect on VPC induction in either wild type or *Chbr-1* (sy5353) animals (Table 1). By contrast, *Chbr-1* RNAi caused abnormal vulval morphology due to cell fusion and cell fate specification defects (37% F and 33% 3° fates, respectively; n = 46 Pn.p cells) (Fig. 3B, Table 1). We also examined the *Chbr-1* (sy5353): *Chbr-lin-39* (RNAi) animals and found that of the 54% of P(3-8).p that were unfused in L2 (i.e., did not adopt an F fate), one-third fused to hyp7 in L3 (3° fate) whereas the remaining cells were induced giving rise to vulval progeny that invaginated during L4 stage. Overall, the RNAi-mediated knock-down of *Chbr-lin-39* strongly suppressed the *Chbr-1* (sy5353) animals (Table 1). These results demonstrate that, similar to *C. elegans*, *Chbr-lin-39* acts downstream of *Chbr-1* to regulate VPC competence and cell fate in *C. briggsae*.

**Ectopically induced VPCs in *pry-1* mutants acquire 2° fates**

homeodomain family) and two in *C. briggsae* (*Cbr-zmp-1* – zinc metalloproteinase family and *Cbr-egl-17*). These reporter genes are expressed in subsets of 1° and 2° lineage vulval cells and serve as faithful markers to assess induced VPC fates (Burdine et al., 1998; Felix, 2007; Gupta et al., 2003; Inoue et al., 2002; Perens and Shaham, 2005; Shen et al., 2004). In the case of *pry-1(mu38)* we found that four 2° lineage markers, namely *egl-17::GFP (ayIs4)*, *lin-11::GFP (syIs80)*, *ceh-2::GFP (syIs54)* (all mid/late-L4 stage) and *dhs-31::GFP (syIs101)* (old adult stage), were expressed in the progeny of all induced VPCs, P6.p excepted (Fig. 4A). Consistent with this, the expression of 1° lineage markers *egl-17::GFP (ayIs4)* (early/mid-L3 stage), *daf-6::YFP (bhEx53)* and *syg-2::GFP (wyEx3372)* (both mid/late-L4 stage) was localized to P6.p progeny (Fig. 4B). A similar phenotype was observed in *Cbr-pry-1(sy5353)* animals. Thus, *Cbr-egl-17::GFP (mfIs5)* (a2 ° lineage marker during mid-L4 stage) was expressed in the progeny of all induced VPCs, except P6.p, suggesting that these cells had...
adopted 2° fates (Fig. 4C). In no case was Cbr-egl-17::GFP expression observed in P6.p progeny. This agrees with Cbr-zmp-1::GFP expression (mfIs8; a 1° lineage marker during late-L4 stage) that was restricted to P6.p progeny (Fig. 4D). Taken together, these results provide evidence that activated Wnt signaling confers 2° fate on VPCs in both species and that this mechanism is evolutionarily conserved.

VPCs in pry-1 mutants can adopt 2° fates in a gonad-independent manner

Previous studies have shown that the gonad plays an important role in vulval induction. Overinduced phenotype. On the contrary, we observed a significant increase in induced VPCs in pry-1(mu38) (92% vs. 31%) (Table 1). In these animals pry-1(mu38); lip-1(zh15) failed to suppress the pry-1 Overinduced phenotype. We also examined the interaction of pry-1 with lip-1 (MAP kinase phosphatase), a transcriptional target of lin-12, that promotes 2° VPC fate by inhibiting MAP kinase activity and a 1° cell fate (Berset et al., 2001). The RNAi-mediated knockdown of Cbr-egl-17::GFP in both C. elegans and C. briggsae pry-1 mutants had no obvious effect on vulval induction except that P7.p was almost always induced (Table 1). In these animals 98% of ectopically induced VPCs (n = 140) adopted a 2° fate as judged by the expression of egl-17::GFP (ayIs4).

VPCs in pry-1 mutants can adopt 2° fates in the absence of LIN-12/Notch signaling

Since the LIN-12/Notch-mediated lateral signaling pathway is required for 2° fate induction in C. elegans (Greenwald, 2005; Sternberg, 2005), we examined its role in pry-1-mediated VPC fate specification. Previous studies have shown that Cbr-lin-12 is involved in C. briggsae vulval development (Felix, 2007; Rudel and Kimble, 2002). We took the RNAi approach to examine Cbr-lin-12 interaction with Cbr-pry-1 in vulval cells. Although the RNAi-mediated knockdown of Cbr-lin-12 in control animals caused a subtle Overinduced phenotype due to rare ectopic induction of P4.p (5%, n = 44) (Table 1) and an abnormal vulval morphology (Fig. 5C), similar to that reported earlier (Felix, 2007) (also see Materials and Methods), it did not suppress ectopic vulval induction in Cbr-pry-1(sy5353) animals (Fig. 5D, Table 1).

Similar to C. briggsae the lin-12 RNAi in C. elegans also had no effect on the pry-1(mu38) vulva phenotype (Table 1). We further examined the lin-12-independent role of pry-1 using a null allele, n676n909. In lin-12(n676n909) animals, P5.p, P6.p, and P7.p each adopt a 1° fate (Greenwald et al., 1983), resulting in an abnormally large vulval protrusion (termed protruding vulva or Pvl) in adults. We generated pry-1(mu38); lin-12(n676n909) double mutant animals carrying egl-17::GFP (ayIs4) and found that such animals exhibit a combination of Overinduced and Pvl phenotypes (Fig. 5E) and show GFP fluorescence in ectopically induced VPCs (Fig. 5F). In these animals (n = 12) P5.p and P7.p were always induced but had no detectable level of GFP fluorescence. These results demonstrate that vulval development in pry-1 mutants can occur in the absence of lin-12 function and ectopically induced VPCs are capable of adopting a 2° fate.

We also examined the interaction of pry-1 with lip-1 (MAP kinase phosphatase), a transcriptional target of lin-12, that promotes 2° VPC fate by inhibiting MAP kinase activity and a 1° cell fate (Berset et al., 2001). The RNAi-mediated knockdown of lip-1 in both C. elegans and C. briggsae pry-1 mutants had no obvious effect on vulval induction except that P7.p was almost always induced (Table 1). The lip-1 hypomorph (deletion allele zhi5) also failed to suppress the pry-1 Overinduced phenotype. On the contrary, we observed a significant increase in induced VPCs in pry-1(mu38); lip-1(zhi5) double mutants compared to pry-1(mu38) (92% vs. 31%) (Table 1). In these animals 98% of ectopically induced VPCs (n = 140) adopted a 2° fate as judged by the expression of egl-17::GFP (ayIs4).
While the above results suggest that pry-1 function does not depend upon LIN-12/Notch pathway activity in both C. elegans and C. briggsae, these data do not rule out the possibility that lin-12 may act upstream of Wnt signaling to promote 2° fates. To address this possibility we performed experiments with a weak gain-of-function C. elegans lin-12 allele n952 that causes ectopic vulval induction in roughly two-thirds of animals (Table 1) (Greenwald et al., 1983). The incomplete penetrance of lin-12(n952) provides a sensitized genetic background to test the impact of alterations in Wnt pathway effectors pop-1 and lin-39 on vulval induction. We found that although lin-39 (RNAi) strongly suppressed lin-12(n952) vulval phenotype, pop-1 (hu9) and pop-1(RNAi) had no such effect (Table 1). These results suggest a simple model in which Wnt and LIN-12/Notch signaling pathways function independently via lin-39 to specify 2° VPC fates in C. elegans.

Table 3

<table>
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<th>Genotype</th>
<th>Induced VPCs (Fraction of induced VPCs showing GFP fluorescence)</th>
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n, number of animals examined.

Inhibition of lin-12/Notch signaling in pry-1 mutants promotes P7.p induction

Although the RNAi-mediated knock-down of Cbr-lin-12 did not suppress ectopic vulval induction in Cbr-pry-1(sy5353) animals, we noted that it increased P7.p induction significantly (P = 0.0003, Table 1) and gave rise to a wild-type vulva in most animals (Fig. 5D). A similar trend was also observed in pry-1(mu38); lin-12 (RNAi) animals but the difference was statistically not significant (Table 1). This suggested that persistent activity of lin-12/Notch signaling in pry-1 mutants might interfere with P7.p induction and cell fate specification. To examine this, we analyzed lip-1 reporter expression that, in response to a lin-12 signal, is upregulated in presumptive 2° VPCs in C. elegans (Berset et al., 2001). The expression of lip-1::GFP in C. briggsae (mfs29) was first observed in all six VPCs during the L2 stage (50%, n = 14) (Figs. 6A,B). This pattern was dynamic, such that by the early/mid-L3 (Pn.p) stage the fluorescence could be seen in only P5.p and P7.p and was undetectable in other Pn.p cells (48%, n = 21) (Figs. 6C,D and data not shown). This indicates that while lip-1 expression in C. briggsae is maintained in 2° precursors it is rapidly downregulated in 1° and other cells. An analogous pattern was observed in mid-L3 (Pn.px) stage animals although in few cases (27%, n = 11 animals) faint GFP fluorescence was also detected in P4.p and P8.p daughters. At later stages no fluorescence was seen in P(5-7).p progeny.

The analysis of lip-1 reporter expression in Cbr-pry-1(sy5353) animals revealed a similar profile but the fluorescence was much higher in P7.p and P8.p cells. Thus, in L2 stage animals all six VPCs were seen fluorescing, with P7.p and P8.p being the brightest in half of the animals (n = 6) that showed GFP fluorescence (Figs. 6E,F). By the
early/mid-L3 (Pn.p) stage, fluorescence had rapidly faded in P6.p and other anterior VPCs, however, P7.p and P8.p continued to fluoresce brightly (90%, n=11 GFP expressing animals) (Figs. 6G,H). After the mid-L3 (Pn.px) stage, other than P7.p and P8.p, no VPC progeny showed detectable level of fluorescence (71%, n=14 GFP expressing animals) (Figs. 6I,J). Similar to *C. briggsae* we found that lip-1::GFP expression in *C. elegans* pry-1(mu38) L2 stage animals was also higher in P7.p compared to anterior VPCs (37%, n=19 GFP expressing animals) (Figs. 6K,L). These results show an abnormal pattern of lip-1 expression in pry-1 mutants and provide a molecular basis for P7.p induction defect in both species.

To further investigate genetic interaction between lip-1 and pry-1, we examined P7.p phenotype in pry-1 mutants by reducing lip-1 activity. Consistent with our lip-1 expression data, we found that RNAi-mediated knock-down of Cbr-lip-1 in *Cbr- pry-1(sy5353)* animals caused a significant increase in P7.p induction (P = 0.0034) (Table 1). A similar, but weak, phenotype was also observed in *C. elegans* pry-1(mu38); lip-1(RNAi) and pry-1(mu38); lip-1(zh15) animals (P = 0.0163 and 0.1175, respectively) (Table 1). Furthermore, induced P7.p in pry-1(mu38); lip-1(zh15) animals showed egl-17::GFP (ayIs4) expression (100%, n=60), suggesting that they adopted a 2° fate. Taken together, these findings provide evidence for a conserved interaction between Wnt and LIN-12/Notch signaling pathways to specify the 2° fate of P7.p in *C. elegans* and *C. briggsae*.

**Discussion**

Due to their apparent morphological similarity, *C. elegans* and *C. briggsae* offer unique advantages in comparative analysis of gene function and signaling networks. To study how homologous tissues are patterned in these two species, we are focusing on the vulva, a reproductive organ necessary for mating and egg laying. We carried out genetic screens in *C. briggsae* and isolated mutants that show defects in vulval development. In this study we report characterization of three of the mutants that exhibit an Overinduced phenotype and show that they are alleles of *Cbr-pry-1*. Our genetic experiments...
have revealed that Cbr‐pry‐1 functions in Cbr‐bar‐1‐Cbr‐pop‐1-mediated canonical Wnt signaling pathway to regulate VPC induction and fate specification. We have also identified Cbr‐lin‐39 Hox gene as a downstream target of this pathway. These findings demonstrate that, similar to C. elegans, canonical Wnt signaling pathway is involved in C. briggsae vulva formation. Both over- and under-activation of Wnt signaling causes vulval abnormalities thereby highlighting its key role in development. A recent study has shown that changes in environmental conditions have significant effects on Wnt signaling-mediated vulval induction (Braendle and Felix, 2008). This serves to further demonstrate the physiological importance of this pathway in patterning the vulva.

**Wnt signaling confers 2° fate on VPCs**

Previous studies have demonstrated the essential role of Wnt signaling in maintaining VPC competence (Eisenmann et al., 1998; Myers and Greenwald, 2007). During the L2 stage bar-1-mediated Wnt signaling promotes lin-39 activity in P(3-8).p and allows these cells to respond to patterning signals in the L3 stage. Our work has revealed that in addition to its role in VPC competence, Wnt signaling also promotes a specific cell fate in C. elegans and C. briggsae. Previous work by Gleason and colleagues (Gleason et al., 2002) showed that activated Wnt signaling causes excessive vulval cell proliferation. However it was unclear whether vulval progeny adopted a specific fate. Subsequently, Myers and Greenwald (Myers and Greenwald, 2007) argued that ectopic vulval progeny in pry-1 mutants could arise from spurious cell divisions due to high lin-39 levels. To address this issue we analyzed cell fates in pry-1 mutants using established molecular markers. We found that induced VPCs, except P6.p, in pry-1 mutants adopted a 2° fate as judged by a panel of 8 GFP-based markers (6 in C. elegans and 2 in C. briggsae). The use of wide range of reporters used in our assay (i.e., ligands, cell surface receptors, metalloproteases, and transcription factors) serves to demonstrate that Wnt signaling orchestrate expression of many important genes needed to confer a 2° fate. In these animals the P6.p fate remained unaltered. Further experiments revealed that 2° VPC fates are specified independently of the gonad-derived inductive signal since induced VPCs in gonad-ablated animals showed 2° marker expression similar to that seen in intact animals. These results significantly extend our understanding of pry-1-mediated Wnt signaling function in two nematode species and suggest that Wnt signaling plays both permissive role (in maintaining competence) and instructive role (in specifying cell fate) in vulval development.

The 2° fates of induced VPCs in pry-1 mutants prompted us to examine the relationship between Wnt and LIN-12/Notch signaling in vulval development. We found that alterations in the activities of LIN-12/Notch pathway receptor lin-12 and its transcriptional target lip-1 did not suppress ectopic vulval induction defect in pry-1 mutants. Thus, activated Wnt signaling in C. elegans and C. briggsae is sufficient to promote vulval development in the absence of lateral signaling. This raises the question of evolutionary roles of Wnt and LIN-12/Notch pathways in 2° fate specification. Perhaps the two pathways evolved as redundant mechanisms to robustly specify a 2° VPC fate. The completed genome sequences of Elegans group species (such as C. remanei and C. brenneri) provide opportunities to address such questions.

**Wnt signaling and P7.p fate specification**

Our work has uncovered novel roles of the Wnt signaling pathway in C. elegans and C. briggsae vulval development that involve both positive and negative regulation of cell fates. We found that P3.p, P4.p, and P8.p in pry-1 mutants are frequently induced to adopt a 2° fate whereas P7.p remains largely uninduced. This is likely to be caused by activated Wnt signaling since RNAI knock-downs of Cbr-bar-1 and Cbr-pop-1 promoted P7.p induction in Cbr-pry-1 mutants. Since LIN-12/Notch-mediated lateral signaling specifies the 2° fate of P7.p, we examined its role in mediating pry-1 function. Our results showed that the expression of lip-1 in pry-1 mutants was significantly higher in P7.p compared to P5.p and persisted in L4 stage animals. This pattern differs from wild type where lip-1 is dynamically regulated in P(5-7).p and is not observed beyond mid-L3 stage. This suggests that a failure to downregulate lip-1 in Cbr-pry-1 mutants may be the basis of the P7.p induction defect. Considering that LIP-1 is a MAP kinase phosphatase, one model is that the persistence of high level of LIP-1 activity in P7.p (directly or indirectly induced by Wnt signaling) abnormally antagonizes mpk-1 (MAP kinase, Ras pathway component) function such that the Ras pathway response in P7.p falls below the minimum threshold needed to promote an induced fate. It is equally possible that misregulation of lip-1 interferes with the expression of lin-12 target genes that in turn causes P7.p to remain uninduced. Consistent with these possibilities, we found that lowering lip-1 activity (either by directly targeting lip-1 or its upstream activator lin-12) in pry-1 mutants suppressed the P7.p induction defect.

While our results provide evidence for a genetic interaction between Wnt and LIN-12/Notch signaling pathways, more work is needed to understand the mechanism of interaction and its biological role in P7.p development. In this respect, reverse genetics and genomics approaches could prove valuable in dissecting the roles of Wnt and LIN-12/Notch pathway genes and their downstream targets. These studies may uncover the function of new genes thereby revealing their mechanism of function and signaling crosstalk. Ultimately, the findings will help understand how changes in gene expression and interactions are regulated to generate tissue morphology.

**Acknowledgments**

We are indebted to Marie-Anne Félix (Institut Jacques Monod) for generously providing some of the transgenic strains and RNAI bacterial clones. The genetic screen was carried in the laboratory of Paul Sternberg (Caltech). We thank Shaha Gharib for isolating sy5270, Bavithra Thillainathan for help in mapping sy5253, Kang Shen (Stanford University) for providing syg-2::GFP (wyEx3372) strain, and Zhongying Zhao (University of Washington, Seattle) for the C. briggsae unc-119 deletion allele sy32000. We also thank Nathan Farrar for helpful comments on the manuscript. Some of the strains were obtained from the Caenorhabditis Genetics Center. This work was supported by funds from the Natural Sciences and Engineering Research Council of Canada and Canada Research Chairs Program to BPG.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.07.003.

**References**

Berset, T., Hoier, E.F., Battu, G., Canevascini, S., Hajnal, A., 2001. Notch inhibition of RAS (MAP kinase, Ras pathway component) function such that the Ras pathway response in P7.p falls below the minimum threshold needed to promote an induced fate. It is equally possible that misregulation of lip-1 interferes with the expression of lin-12 target genes that in turn causes P7.p to remain uninduced. Consistent with these possibilities, we found that lowering lip-1 activity (either by directly targeting lip-1 or its upstream activator lin-12) in pry-1 mutants suppressed the P7.p induction defect.

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