A NOVEL NON-CANONICAL WNT PATHWAY REGULATES THE ASYMMETRIC B CELL DIVISION IN *CAENORHABDITIS ELEGANS*

by

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B.S., Jiangxi Agricultural University, 1997
M.S., China Agricultural University, 2000

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ABSTRACT

The polarities of several cells that divide asymmetrically during *C. elegans* development are controlled by Wnt signaling. LIN-44/Wnt and LIN-17/Fz control the polarities of cells in the tail of developing *C. elegans* larvae, including the male-specific blast cell, B, which divides asymmetrically to generate a larger anterior daughter and a smaller posterior daughter. We determined that the canonical Wnt pathway components are not involved in the control of B cell polarity. However, POP-1/Tcf is involved and asymmetrically distributed to B daughter nuclei. Aspects of the B cell division are reminiscent of the divisions controlled by the planar cell polarity (PCP) pathway that has been described in both *Drosophila* and vertebrate systems. We identified *C. elegans* homologs of Wnt/PCP components and have determined that many of them appear to be involved in the regulation of B cell polarity and POP-1 asymmetric distribution to B daughter nuclei. Thus a non-canonical Wnt pathway, which is different from other Wnt pathways in *C. elegans*, but similar to the PCP pathways, appears to regulate B cell polarity.

Molecular mechanisms of this PCP pathway were also investigated. We determined that LIN-17/Fz is asymmetrically distributed to the B cell cortex prior to, during, and after, division. Furthermore, the asymmetric localization of LIN-17::GFP is controlled by LIN-44/Wnt and MIG-5/Dsh. The cysteine rich domain (CRD), seven trans-membrane domain and KTXXXW motif of LIN-17 are required for LIN-17 to rescue *lin-17*, while only seven trans-membrane domains and KTXXXW motif are required for LIN-17 asymmetric localization. MIG-5::GFP asymmetrically localized to the B cell prior to and after division in a LIN-17/Fz dependent manner. We examined the functions of these MIG-5 domains. The DEP domain is required for MIG-5 membrane association, while the PDZ domain is responsible for different levels of MIG-5 in the B daughters. The DEP and PDZ domain are required to rescue B cell polarity defect of

mig-5 males, while the DIX domain is not that important. In summary, a novel PCP-like pathway, in which LIN-17 and MIG-5 are asymmetrically localized, is conserved in *C. elegans* and involved in the regulation of B cell polarity.

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Approved by:

Major Professor

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DEDICATION

To my wife Bei Liu and My son Joshua Wu.

Chapter 1

NONCANONICAL WNT SIGNALING PATHWAYS IN *C. ELEGANS* CONVERGE ON POP-1/TCF AND CONTROL CELL POLARITY

Michael A. Herman and Mingfu Wu

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Abstract

In the nematode *Caenorhabditis elegans,* a canonical Wnt signaling pathway controls a cell migration whereas noncanonical Wnt pathways control the polarities of individual cells. Despite the differences in the identities and interactions among canonical and noncanonical Wnt pathway components, as well as the processes they regulate, almost all *C. elegans* Wnt pathways involve the sole Tcf homolog, POP-1. Intriguingly, POP-1 is asymmetrically distributed between the daughters of an asymmetric cell division, with the anterior sister cell usually having a higher level of nuclear POP-1 than its posterior sister. At some divisions, asymmetric distribution of POP-1 is controlled by noncanonical Wnt signaling, but at others the asymmetry is generated independently. Recent experiments suggest that despite this elaborate anterior-posterior POP-1 asymmetry, the quantity of POP-1 protein may have less to do with the subsequent determination of fate than does the quality of the POP-1 protein in the cell. In this review, we will embark on a quest to understand Quality (1), at least from the standpoint of the effect POP/Tcf quality has on the control of cell polarity in *C. elegans.*

Introduction

Wnt signaling pathways are conserved from mammals to nematodes and function in diverse developmental processes, such as cell proliferation, cell differentiation, cell fate determination, synaptogenesis, cell migration, and cell polarity (reviewed in refs. 2-4). At least three major conserved Wnt signaling pathways are now recognized: Wnt/beta-catenin, Wnt/calcium and Wnt/JNK or planar cell polarity (PCP). In canonical or Wnt/beta-catenin pathways, Wnt signals function to stabilize beta-catenin level in the cell, allowing beta-catenin to

translocate to the nucleus and form a complex with Tcf/Lef factors to activate or repress expression of specific genes. Beta-catenin is not involved in the noncanonical Wnt/calcium and PCP pathways, although in some cases noncanonical Wnt signaling interferes with Wnt/beta-catenin signaling (reviewed in ref. 5).

Wnt signaling pathways control several aspects of *C. elegans* development, including cell fate decisions, cell migrations and cell polarity (reviewed in refs. 6, 7). Both canonical and noncanonical Wnt pathways function during *C. elegans* development. A canonical Wnt pathway controls the migrations of the descendants of the QL neuroblast, collectively called the QL.d (reviewed in refs. 6-8). This canonical pathway includes *egl-20/Wnt, mig-5/Dsh, sgg-1/GSK-3, bar-1/beta-catenin, pry-1/Axin* and *pop-1/Tcf* and functions to control the expression of *mab-5/Hox*, which controls the migration of the QL.d (9-14). There are three beta-catenin homologs in *C. elegans:* BAR-1, HMP-2 and WRM-1. Interestingly, it appears that the adhesion and signaling functions that are performed by a single beta-catenin molecule in other species have been distributed among the three *C. elegans* homologs (12, 15). BAR-1 is the only beta-catenin homolog that interacts strongly with the sole Tcf homolog, POP-1, which has been shown to function as a canonical Tcf (12). WRM-1 also participates in signaling, however it interacts weakly with POP-1 (15, 16) and appears to do so in the absence of the POP-1 amino-terminal beta-catenin binding site (15), indicating that the WRM-1-POP-1 interaction is different than the BAR-1-POP-1 interaction. Furthermore, WRM-1 only functions in pathways that also involve LIT-1, a nemo-like kinase that is involved in a mitogen-activated protein kinase (MAPK)-like pathway. Together, this suggests that Wnt signaling pathways in which WRM-1 participates are noncanonical. Although POP-1 also participates in these pathways, it is regulated differently than it is in the canonical pathways. HMP-2 appears to function only in adhesion: it does not

interact with POP-1, but does interact with HMR-1/cadherin (12, 15). Based upon the distinct functions for the three *C. elegans* beta-catenin homologs, signaling pathways that involve BAR-1 appear to be canonical, while those involving WRM-1 are noncanonical. A Wnt/calcium pathway has not been described in *C. elegans* and a PCP-like pathway appears to control the polarity of at least one cell that divides asymmetrically (M.W. and M.A.H., unpublished). Interestingly, in *C. elegans,* all Wnt pathways, canonical or noncanonical, appear to converge on POP-1/Tcf.

Some canonical pathway components, including BAR-1, have also been shown to be involved in controlling the fates of the P12 ectoblast and the vulval precursor cells. However, in these cell fate decisions the full pathway has not been shown to be active and Ras signaling is also involved. Thus, it is not clear whether these cell fates are controlled by the interaction of the canonical Wnt pathway and a Ras pathway or whether the pathway is also noncanonical in some respect.

WRM-1 and POP-1 function in noncanonical Wnt signaling that is almost exclusively involved in controlling the orientations, or cell polarities, of several cells that divide asymmetrically during *C. elegans* development; specifically the EMS blastomere, the T cells in the tail and the Z1 and Z4 cells in the developing gonad. In each of these asymmetric cell divisions (except for the Z1 and Z4 cells), as well as many others, the nuclear levels of POP-1 are asymmetric with one sister cell, usually the anterior cell, having a higher level of nuclear POP-1 than it's sister. Furthermore, it appears that the function of the WRM-1/LIT-1 noncanonical pathway is to lower the nuclear level of POP-1 in one sister cell. The mechanisms responsible for this regulation, as well as the consequences of cells having different POP-1 nuclear levels, are being intensely investigated. However, while much has been learned, many

questions remain. The control of POP-1 nuclear level appears to be fundamental to the control of cell polarity by noncanonical Wnt signaling in *C. elegans,* however modification of POP-1 also seems to be important. This review will focus on the progress that has been made in understanding POP-1/Tcf regulation and role in the control of cell polarity and discuss the remaining questions.

Nocanonical Wnt signalings control the polarity of the EMS blastomere

At the four-cell stage of *C. elegans* embryogenesis, the EMS blastomere divides asymmetrically to produce an anterior MS cell, that subsequently divides to generate mesodermal precursors, and a posterior E cell, that subsequently divides to generate all the endoderm. Blastomere isolation and reconstitution experiments demonstrated that the P2 blastomere polarizes the EMS blastomere by inducing the nucleus and centrosomes to rotate 90°, reorienting the spindle along the anterior-posterior axis, as well as inducing the E cell fate at the point of contact between P2 and EMS (17, 18). Forward and reverse genetic approaches have identified many genes that when mutated or inactivated by RNAi affect EMS polarity and endodermal cell fate specification. These include several Wnt pathway components: *mom-2/Wnt, mom-1/Porc, mom-5/Fz*, *wrm-1/beta-catenin, sgg-1/GSK3, apr-1/APC* and *pop-1/Tcf* (19-21). Two of the three *C. elegans* Dishevelled homologs, *mig-5* and *dsh-2,* are enriched in oocytes (22) and appear to be involved in endoderm induction (23). In addition, MAPK pathway components *lit-1/NLK* and *mom-4/TAK1*, which encodes a MAP kinase kinase kinase protein similar to mammalian TAK1, were also identified (16, 24). The molecular identity of an additional gene, *mom-3,* has not yet been determined.

Mutations in each of the above genes, except *pop-1,* leads to the loss of endoderm, whereas mutation of *pop-1* leads to the loss of mesoderm. Furthermore, upstream components *mom-1, mom-2, mom-5, mig-5/dsh-2* and *sgg-1* are involved in both EMS spindle orientation and endoderm specification, whereas downstream components *apr-1, wrm-1,* and *pop-1* affect only endoderm specification. This suggests that the spindle orientation and endoderm specification pathways branch at *sgg-1* (21, 23). These and other considerations suggest that the spindle orientation pathway may interact directly with the cytoskeleton (25).

Wnt/MAPK signaling represses of POP-1 function

The Wnt pathway that specifies endoderm involves WRM-1 and is noncanonical. The involvement of LIT-1/NLK and MOM-4/TAK1 also make this Wnt pathway unusual. Both the Wnt and MAPK pathways converge on POP-1/Tcf. In the absence of a signal from P2, POP-1/Tcf represses E cell fate. Thus, endoderm induction is achieved by inhibition of a repressor. POP-1 represses endoderm fate in the MS cell by recruiting a complex that contains the histone deacetylase (HDAC), HDA-1 and UNC-37/Groucho (26). This appears to occur by repressing the transcription of the endoderm-specific GATA-like transcription factor genes, *end-1* and *end-3,* in the MS cell. Two other redundant, zygotically expressed GATA factors, MED-1 and MED-2 bind to the *end-1* and *end-3* promoters and are required for *end* gene expression in the E cell (27). Even in the unstimulated MS cell, MED-1, and presumably MED-2, bind to the *end* gene promoters, but so does POP-1, which somehow inhibits *end* gene expression by the MEDs (28). Interestingly, recent data indicate that the *C. elegans* p300 histone acetyltransferase homolog CBP-1 interacts with POP-1 and acetylates it (29). Furthermore, acetylation of POP-1 is required for POP-1 function in MS, as a *pop-1* construct in which the three acetylated lysine

residues (K185, K187 and K188) were changed to either alanine (GFP::POP-1MutAAA) or arginine (GFP::POP-1MutRRR) failed to rescue a *pop-1* mutant, while the control GFP::POP-1 construct was able to rescue (29). Thus, both HDA-1/HDAC and CPB-1/p300 are required in the unsignalled MS cell to block *end* gene expression and repress E cell fate. Interestingly, CBP-1 function is also required for *end-1* expression and endoderm fate (26). Thus, CBP-1 function is required both in MS to repress endodermal fate and in E to promote endoderm fate. One possibility is that the targets of CBP-1 acetylation in MS and E are different. For example, CBP-1 may acetylate POP-1 in MS in order to repress *end* gene expression and to acetylate histones in E to allow *end* gene activation.

In the E cell, the Wnt and MAPK pathways components function to repress POP-1 function. An unusual aspect of the Wnt pathway involved in endoderm induction is that APR-1/APC and SGG-1/GSK3 act positively in Wnt signal transduction to activate WRM-1, leading to the negative regulation of POP-1. This is very different from canonical Wnt pathways where APC and GSK3 function as part of a complex involved in the degradation of beta-catenin in the absence of Wnt signals and are inhibited in the presence of Wnt signals. WRM-1 and LIT-1 can interact and together phosphorylate POP-1 (16), leading to the inhibition of POP-1 function.

Wnt/MAPK signaling causes nuclear-cytoplasmic redistribution of POP-1

Recent studies have begun to illuminate how POP-1 function is inhibited (28, 29). The nuclear level of POP-1 is higher in the anterior MS cell than it is in the posterior E cell. However, the overall level of POP-1 in the MS and E cells is similar. Wnt/MAPK signaling appears to cause a nuclear-cytoplasmic redistribution of POP-1 in the E cell. The difference in POP-1 nuclear levels is apparent immediately after the division of EMS. This leads Maduro *et*

al. to suggest that the lowered nuclear POP-1 level is due to the inefficient import of POP-1 into the reforming E cell nucleus (28), although other mechanisms are possible. An internal 124 amino acid region of POP-1, that does not include the beta-catenin binding site in the amino-terminus of POP-1 nor the HMG box DNA-binding domain, is required for asymmetric nuclear accumulation (28). Acetylation of POP-1 also influences nuclear-cytoplasmic partitioning. Specifically, acetylation promotes nuclear localization, possibly by increasing nuclear import and blocking nuclear export. However, phosphorylation of POP-1 by WRM-1 and LIT-1 does not affect POP-1 acetylation state, but does cause POP-1 to accumulate in the cytoplasm. This is supported by the observation that both GFP::MutAAA or GFP::MutRRR are predominantly cytoplasmic, yet retain anterior-posterior asymmetric nuclear levels in MS and E (29). Thus, while acetylation is involved in nuclear-cytoplasmic distribution of POP-1, it does not appear to be involved in the anterior-posterior asymmetry. This suggests that phosphorylation of POP-1 is independent of acetylation and can override the promotion of nuclear localization by acetylation. One consequence of Wnt/MAPK signaling, then, appears to be phosphorylation of POP-1, resulting its redistribution from the nucleus to the cytoplasm.

Quality, not quantity of POP-1 is important

Despite the elaborate regulation of nuclear POP-1 level in the E cell, three experiments suggest that the absolute amount of nuclear POP-1 is not the most important consequence of Wnt/MAPK signaling on E cell fate. First, overexpression of a GFP::POP-1 construct in EMS and E had no effect on endoderm induction, although there was an estimated ten-fold increase in the amount of functional POP-1 in the E cell (28). Second, nuclear POP-1 levels in MS and E are equal in the *lit-1(t1534)* mutant, but endoderm is still properly specified. This demonstrates

that POP-1 asymmetric nuclear distribution can be uncoupled from endoderm specification, suggesting that there is a qualitative difference in POP-1 in signaled versus nonsignalled cells. Finally, although some nuclear POP-1 remains in the E cell, it does not bind to the *end* gene promoters, which allows for *end* gene activation by MED-1 and MED-2 (28). Something is different about POP-1 in the E cell nucleus after Wnt/MAPK signaling that prevents it from binding to the *end* gene promoters. This qualitative difference may be reflected in the intranuclear localization of POP-1 in nonsignalled cells. In MS, POP-1 has been observed to localize to puncta within the nucleus; whereas in the nucleus E, it is present in a low, but uniform pattern (28). Maduro *et al.* suggest the puncta observed in the MS nucleus may reflect POP-1's function as a repressor. Whether or not this turns out to be the case, it seems clear that a qualitative difference in POP-1 function, apart from the consequence that Wnt/MAPK signaling has on level of nuclear POP-1, does exist. The function of this qualitative difference and the role of POP-1 asymmetric nuclear distribution remains a puzzle, however.

A parallel pathway involving SRC-1 is also involved in EMS polarity

New players have recently entered the picture. Mutations in the Src Kinase homolog *src-1* caused defects in EMS spindle orientation reminiscent of Wnt pathway mutants (23). In addition, *src-1* mutants also caused defects in the germline precursor cells similar to those caused by *mes-1* mutations. MES-1 is a putative transmembrane protein that has a structure of a receptor tyrosine kinase, although it is not predicted to be an active kinase (23, 30). Although *src-1* and *mes-1* mutants do not display endoderm defects on their own, the similarity of the EMS spindle defects led Bei and colleagues to determine whether they might interact genetically

with mutations in Wnt pathway components. Double mutant combinations between *src-1* or *mes-1* and *mom-1/Porc, mom-2/Wnt, mom-5/Fz, sgg-/GSK3,* or *mom-3* displayed synergistic EMS spindle orientation and endoderm defects. In addition, the *src-1* or *mes-1* triple mutant combination with *dsh-2/Dsh* and *mig-5/Dsh* also displayed synergistic EMS spindle orientation and endoderm specification defects. This indicates that SRC-1 and MES-1 function in parallel with the noncanonical Wnt pathway to control both EMS spindle orientation and endoderm specification (23). These data were supported by observations that double mutants between Wnt pathway components did not display any genetic interactions. In addition, the nuclear levels of POP-1 were equal in MS and E in *src-1; mom-2* and *mes-1; mom-2* double mutants; indicating that these pathways converge on POP-1. A long-standing curiosity about the involvement of the Wnt pathway in these processes was that the upstream Wnt pathway mutants showed only a partial loss of P2-to-EMS signaling. Thus, the existence of a parallel pathway, in addition to the LIT-1/MAPK pathway, that also functions to control EMS spindle orientation and endoderm specification, makes sense. This was demonstrated by showing that a *src-1* mutant also genetically interacts with *mom-4* and with *apr-1* mutants. *apr-1* interacts genetically with both the Wnt pathway (19, 23) and *lit-1* (16). *mom-4* also interacts genetically with the Wnt pathway (23, 31). Thus evidence exists for three pathways as well as APR-1; which appears to have some function that is independent of all three pathways. It is not clear exactly where these pathways intersect, although for endoderm specification, it must be upstream of POP-1. There may also be multiple points of interaction among the pathways, leading to the idea that it is more of a network, than separate interacting pathways (23) (Figure 1).

Wnt- dependent and independent controls on POP-1 asymmetric distribution in other cells

POP-1 is asymmetrically distributed to anterior-posterior sister cells at many other divisions during *C. elegans* development (13, 32). What controls POP-1 asymmetry at these divisions? Park and Priess have recently addressed this question by examining the controls over POP-1 asymmetry within the AB cell lineage (33). The two-cell *C. elegans* embryo consists of an anterior blastomere, AB, and a posterior blastomere, P1. AB then divides along a skewed anterior-posterior plane to generate AB.a and AB.p (the AB^2 stage). Next, these cells divide transversely along a left-right plane to generate AB.al, AB.ar, AB.pl and AB.pr (the AB^4 stage). Each of these, and subsequent, AB descendants divide along an anterior-posterior plane (the AB^8, AB^{16}, AB^{32}, etc. stages). Within the AB lineage, POP-1 is not asymmetrically distributed until the third division (the AB^8 stage) (32). To determine what prevents POP-1 asymmetry during the first two AB divisions and what establishes it at the third, Park and Priess examined POP-1 levels in embryos and cultured embryonic cells. They first used RNAi to inhibit the function of the G-alpha proteins encoded by *goa-1* and *gpa-16* to randomize the EMS division plane (34). They observed that if EMS divided along a transverse (left-right) plane, POP-1 was not asymmetric in the daughter cell nuclei, whereas if EMS divided along the normal anterior-posterior plane, POP-1 was asymmetric. Normally, the AB^2 cells divide transversely, with both cells contacting P2, and there is no POP-1 asymmetry. In similar *goa-1(RNAi); gpa-16(RNAi)* embryos, POP-1 was asymmetric if the AB^2 cells divided along an anterior-posterior axis. Furthermore, the POP-1 asymmetry in these embryos was dependant upon MOM-2/Wnt. Isolated AB^2 cells also divided transversely. If an isolated P2 cell is placed perpendicular to the division axis, which is the normal configuration, POP-1 is not asymmetric in the daughter cell nuclei, as expected. However, if an isolated P2 cell is placed in line with the division axis, POP-1 is asymmetric in the daughter cell nuclei, with the distal daughter having a higher nuclear level

of POP-1. Thus, AB^2 cells can respond to polarity signals from P2 (which depend upon MOM-2), but the transverse division plane places both daughters in contact with P2, precluding asymmetric distribution of POP-1 in the daughter cell nuclei. As a result, POP-1 is not asymmetric in the AB^4 cells.

When the AB^4 cells divide to generate the AB^8 cells, POP-1 asymmetry is observed. How is POP-1 asymmetry established at this division? Isolated C, P3 (both P1 descendants), E and to some extent MS cells, are capable of inducing POP-1 asymmetry at the division of the AB^2, AB^4 and AB^8 cells. Furthermore, C and P2 require MOM-2 to induce POP-1 asymmetry, whereas MS does not. Among the multiple cells that are capable of inducing POP-1 asymmetry at the division of the AB^4 cells, blastomere recombination experiments established that P2, E and C have precedence over MS. This suggests that C, P3 and E function together to orient POP-1 asymmetry at the division of the AB^4 cells. In addition, the observation that the AB^8 cells display POP-1 asymmetry in *mom-2* mutants (24, 32) can be explained by a Wnt-independent polarity signal from MS.

After division of the AB^8 cells, the AB and EMS descendants can generate POP-1 asymmetry independent of MOM-2/Wnt signals. This was demonstrated by sequential isolation of embryonic AB daughter cells in culture. POP-1 was not asymmetric at the AB^4 or AB^8 stages, but became asymmetric at the AB^{16} stage. The acquisition of POP-1 asymmetry in these experiments was independent of MOM-2, yet still required MOM-5/Fz. This is reminiscent of planar cell polarity pathways in *Drosophila*, which are dependant upon Frizzled, but for which no Wnt signal has been identified (reviewed in ref. 35). Interestingly, when adjacent AB^{16} cells divided, POP-1 asymmetry was mirror symmetric. Instead of nuclear POP-1 levels being high/low, high/low, they were low/high, high/low. This is in contrast to what occurs in intact

embryos, where the POP-1 asymmetry is always anterior-posterior asymmetric high/low. This suggests the existence of two signaling pathways that generate POP-1 asymmetry, one that is Wnt-dependant and another that is not. However, it is not clear whether all the high/low POP-1 asymmetries that are generated during development (and there are many), do so in response to a Wnt signal, or whether in an intact animal, other cellular behaviors dictate the high/low POP-1 asymmetry independent of Wnt signals.

Role of POP-1 in the control of T cell polarity

Noncanonical Wnt signaling also controls the polarities of the B and T cells that divide asymmetrically in the tail of developing *C. elegans* larvae. Mutations in *lin-44/Wnt* cause the polarities of the B and T cells to be reversed, while mutations in *lin-17 /Fz* cause a loss of polarity in the same cells (review in refs. 6, 8). This suggests that LIN-17 is the LIN-44 receptor. LIN-44 is expressed in the epidermal cells at the tip of the developing tail, posterior to the T cells (36). The difference in polarity effects of *lin-44* and *lin-17* mutants (reversal versus loss of polarity) is curious and suggests the existence of a second Wnt signal that emanates from a source anterior to the T cells that serves to orient the T cell division in the absence of *lin-44* (37). Such an anterior signal has yet to be found, however. Other Wnt pathway components that have been shown to function in the control of T cell polarity include LIT-1 (16), WRM-1 (H. Takeshita and H. Sawa, personal communication) and POP-1 (13). Interfering with the function of these genes causes a loss of T cell polarity, rather than the reversal of polarity observed in *lin-44* mutants. Effects of this pathway may occur through the action of chromatin remodeling complexes as mutations in *egl-27*, which encodes a protein with similarity to a factor isolated as

a component of the NURD complex as well as mutations in *psa-1* and *psa-4,* which encode homologs of the SWI/SNF complex all cause a loss of T cell polarity (38, 39). *tlp-1* encodes a zinc-finger protein required for neural cell fates within the T.p and T.ap cell lineages and may be a target gene, as TLP-1 is localized specifically to T.p and responds to LIN-44/Wnt signals (40). The involvement of WRM-1 and LIT-1 suggests that the noncanonical Wnt pathway controlling T cell polarity may bear some resemblance to that involved in endoderm induction. However, the role POP-1 plays in the T cell divisions suggests that there might be some differences.

As in many other anterior-posterior asymmetric cell divisions, the nuclear level of POP-1 is higher in the anterior T cell daughter, T.a, than it is in the posterior daughter, T.p (13). However, in *lin-44* mutants, the nuclear level of POP is lower in T.a, reflecting the reversal of T cell polarity. Surprisingly, inactivation POP-1 by RNAi, mutation or expression of a dominant negative POP-1 construct that lacks the amino-terminal beta-catenin binding site causes a loss of polarity similar to that observed for *lin-17* mutants; the production of two epidermal cells, the anterior cell fate (13, 41). This is contrary to what one might expect if POP-1 functions similarly in the EMS and T cell divisions. Loss of POP-1 function in the EMS division leads to the production of two endodermal cells, the posterior cell fate. If the same were true of the T cell division, *pop-1* mutations should lead to the production of two neural cells, the posterior cell fate, rather than two epidermal cells. This suggests that POP-1 may play a positive role in specifying the neural T.p cell fate, despite the lower POP-1 nuclear levels observed in T.p. Similar to what is proposed for endoderm specification, it appears that it is the quality of POP-1, rather than the quantity of POP-1 that is important for determining neural cell fate and establishing cell polarity (13). If POP-1 is phosphorylated by WRM-1 and LIT-1 in T.p, as it is in E, the mechanism for POP-1 nuclear to cytoplasmic redistribution may be similar, while the

functional consequence of that modification on cell fate specification may be different (Figure 2). The current challenge is to determine which mechanisms T cell polarity shares with EMS polarity and which are different.

Other cell polarities tha involve noncanonical Wnt signaling

Noncanonical Wnt signaling also controls the asymmetric cell divisions of the Z1 and Z4 cell that generate the somatic gonad. The gonad primordium lies in the center of the animal, the Z1 and Z4 cells flank the two primordial germ cells Z2 and Z3. The hermaphrodite gonad develops an anterior and a posterior arm, each having a proximal-distal axis (reviewed in (42). The polarities of the Z1 and Z4 divisions are oriented along the proximal-distal axis of the gonad. Mutations in *mom-1/Porc, lin-17/Fz, wrm-1/beta-catenin, lit-1/NLK* and *pop-1/Tcf* cause the loss of polarity of the Z1 and Z4 divisions (41). Thus, this pathway is similar to that involved in the control of EMS and T cell polarities. In fact, it may be a bit more like the T cell pathway in that interfering with each of these genes has the same effect on Z1 and Z4 polarities, instead of pop-1 having the opposite effect as occurs with EMS polarity. It is also not known whether nuclear POP-1 levels are asymmetric at any of these asymmetric divisions, as POP-1 antibodies do not stain the gonads of L1 animals. Finally, the Wnt involved in specifying Z1 and Z4 polarity is also not known, as mutation or interference with each of the *C. elegans* wnt genes did not cause gonad defects. It is possible that two or more of the *C. elegans* Wnts function redundantly, however.

Polarity of the male specific blast cell, B, is also specified by Wnt signaling. In wild-type males the B cell divides asymmetrically generating a larger anterior daughter, B.a, and a smaller posterior daughter, B.p. In addition to the difference in size, these asymmetric daughter cells

have different fates, each producing different numbers of progeny. Mutations in *lin-44* cause both the difference in size and subsequent division pattern to be reversed (43). Mutations in *lin-17* cause a loss of polarity with two anterior cell fates being produced (44). It is not yet clear which other Wnt pathway components function to control B cell polarity. However, recent data suggest that components of the PCP pathway might play a role (M.W. and M.A.H., unpublished).

Conclusions and remaining questions

EMS spindle effects

Wnt signals that control asymmetric cell divisions and cell polarity affect the mitotic spindle and subsequent asymmetric cell fate determination. In the EMS blastomere, the spindle must rotate for the asymmetric division to occur. The rotation of the EMS spindle is controlled by Wnt pathway components upstream of SGG-1 that appear to function in parallel with a SRC-like pathway (23). The target of signals that control spindle rotation may be the cytoskeleton, perhaps mediated by Rho-like GTPases (23). This is reminiscent of PCP pathways that control cell polarity in the *Drosophila* wing that also appear to function through regulating Rho-like GTPases (review in ref., 35). Spindle rotation does not appear to be involved in the asymmetric division of the T, Z1 and Z4 cells, thus Wnt effects on the spindle are not absolutely required for asymmetric cell divisions and may be unique to the EMS division. However, displacement of the mitotic spindle must be important for asymmetric divisions that generate daughters of different sizes such as the division of the B cell and certain other asymmetric divisions that occur within the T cell lineage (43).

POP-1 and asymmetric cell fate determination

POP-1/Tcf is involved in most, if not all, Wnt signaling pathways in *C. elegans.* In particular, the noncanonical Wnt pathways that control cell polarities and asymmetric cell fate determination during *C. elegans* development converge upon POP-1, leading to modifications that affect the function and cellular distribution of POP-1. One important modification is acetylation, which affects the nuclear-cytoplasmic distribution of POP-1 by promoting nuclear retention. Acetylation is also important for POP-1 function, as a mutant POP-1 protein that cannot be acetylated is unable to repress expression of endodermal genes in the mesodermal precursor cell MS. However, the mechanism by which acetylation affects POP-1 function is not clear. Perhaps POP-1 must be acetylated to interact properly with HDA-1/HDAC and UNC-37/Groucho in order to inhibit MED induced expression of the *end* genes in MS (Figure 1). Other unidentified modifications may also prove to be important for POP-1 function in MS.

Of particular interest are the mechanisms that lead to the anterior-posterior difference in nuclear POP-1 levels between sisters cells of an asymmetric cell division. This appears to occur by affecting the nuclear-to-cytoplasmic ratios of POP-1 without affecting total cellular POP-1 levels (28), suggesting that degradation of POP-1 is not involved as was previously thought. What the mechanisms might regulate the differences in the nuclear-to-cytoplasmic POP-1 distribution in sister cells? POP-1 acetylation can affect its nuclear-to-cytoplasmic distribution, however there is no evidence for differential POP-1 acetylation between asymmetric sister cells. Wnt/MAPK/Src signaling can, however, override the influence of acetylation and cause a redistribution of POP-1 to the cytoplasm. This appears to occur by modification of POP-1, presumably phosphorylation by WRM-1 and LIT-1, without affecting the acetylation state of POP-1.

How does the redistribution of POP-1 from the nucleus to the cytoplasm occur? Some evidence indicates that redistribution only occurs after cell division, suggesting that modified POP-1 is excluded from the reforming nucleus in the signaled cell (28). However, nuclear export may also be involved. In either case, it is of interest to identify the proteins and mechanisms responsible for the exclusion or export of modified POP-1 from the nucleus, as well as to determine the role of SRC-1 signaling. Specifically, it is not yet clear exactly where the Wnt/MAPK and Src pathways intersect and how they interact.

Later in embryonic development, unidentified Wnt-independent mechanisms of POP-1 asymmetric nuclear distribution take over. It appears that MOM-5/Fz may play role, suggesting parallels with the PCP pathway (33). Much later in development, during the first larval stage, the asymmetric distribution of POP-1 nuclear levels again comes under the control of Wnt signals that control the polarity of the T cell divisions. What governs the transition from Wnt-independence to Wnt-dependence? The Wnt pathway that controls T cell polarity bears some resemblance to the EMS polarity pathway in that WRM-1 and LIT-1 are involved. It is not known whether SRC-1 signaling is also involved. Although this seems unlikely as, in contrast to EMS polarity where mutations in the upstream components *mom-2/Wnt* and *mom-5/Fz* cause a only a partial loss of cell polarity, mutations in *lin-44/Wnt* and *lin-17/Fz* cause highly penetrant, cell polarity defects. Thus in the T cell division, there does not seem to be a need for a parallel signaling pathway (Figure 2).

On the nature of POP-1 Quality

The functional consequence for the asymmetric distribution of nuclear POP-1 is still not clear. For example, it appears that it is not the difference in quantity of POP-1 between the MS and E cells that is important for endoderm induction, but instead the difference in quality of

POP-1 in each cell (28). For the MS cell, POP-1 has a quality that allows it to interact with HDA-1/HDAC and UNC-37/Groucho to inhibit MED induced expression of the *end* genes. This quality may be related to the observation of puncta of POP-1 accumulation in the MS nucleus. POP-1 puncta are also observed in other anterior sisters of asymmetric divisions during embryonic development, but they may not be involved in all anterior asymmetric sisters. For the E cell, POP-1 has a quality that allows it *not* to interact with the MEDs, which allows *end* gene expression and endoderm fate. This quality is likely to be the phosphorylation of POP-1 by WRM-1 and LIT-1. Perhaps phosphorylated POP-1 cannot interact with and inhibit the MEDs , allowing for *end* gene expression. Interestingly, the same quality of POP-1 may also lead to its redistribution to the cytoplasm. However, we will not know for sure until the sites on POP-1 that are phosphorylated are identified and the functional consequence of each phosphorylation determined. For the T cell division, POP-1 quality in the posterior daughter, T.p may have consequences different from that in the E cell. In the EMS cell division, the effect of removing POP-1 function on cell fate is the same as modifying and lowering the nuclear level of POP-1; the posterior endodermal cell fate is produced. In the T cell division, the effect of removing POP-1 function on cell fate is different than modifying and lowering the nuclear level of POP-1: the posterior T.p cell has a low nuclear level of POP-1 and generates neural cells whereas removal of POP-1 function causes the anterior epidermal cell fate to be produced. This suggests that POP-1 may have a quality that allows it to play a positive role in specifying the neural cell fate. Is the quality of POP-1 in E and T.p similar? If so, how can the consequences be different? If not, what are the differences in POP-1 qualities in these two cells and how are they generated? Finally, observations that the difference in POP-1 nuclear quantity plays little functional role in the specification of asymmetric cell fates, yet exists for many asymmetric cell divisions, remains

a major mystery to be resolved by further study. Hopefully, workers in this field will have a better go at defining quality than did Phædrus, who found that although “there is such a thing as Quality, but that as soon as you try to define it, something goes haywire. You can't do it." (1).

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Figure 1 Model of control of polarity in the EMS divisions.

Modified from refs. 22 and 27. Model for P2-to-EMS signaling (upper) and subsequent asymmetric division of EMS (lower). Red circles indicate phosphorylation of POP-1, presumably by WRM-1 and LIT-1. Blue rectangles indicate acetylation of POP-1, presumably by CBP-1. CBP-1 may also acetylate histones to modify chromatin structure allowing for *end* gene expression in the E cell. Acetylation promotes nuclear retention of POP-1, thus non-acetylated POP-1 may be found in the cytoplasm of MS. Phosphorylation of POP-1 overrides the acetylation of POP-1 causing redistribution of POP-1 to the cytoplasm in E, thus cytoplasmic POP-1 in E may be both acetylated and phosphorylated. See text for additional details.

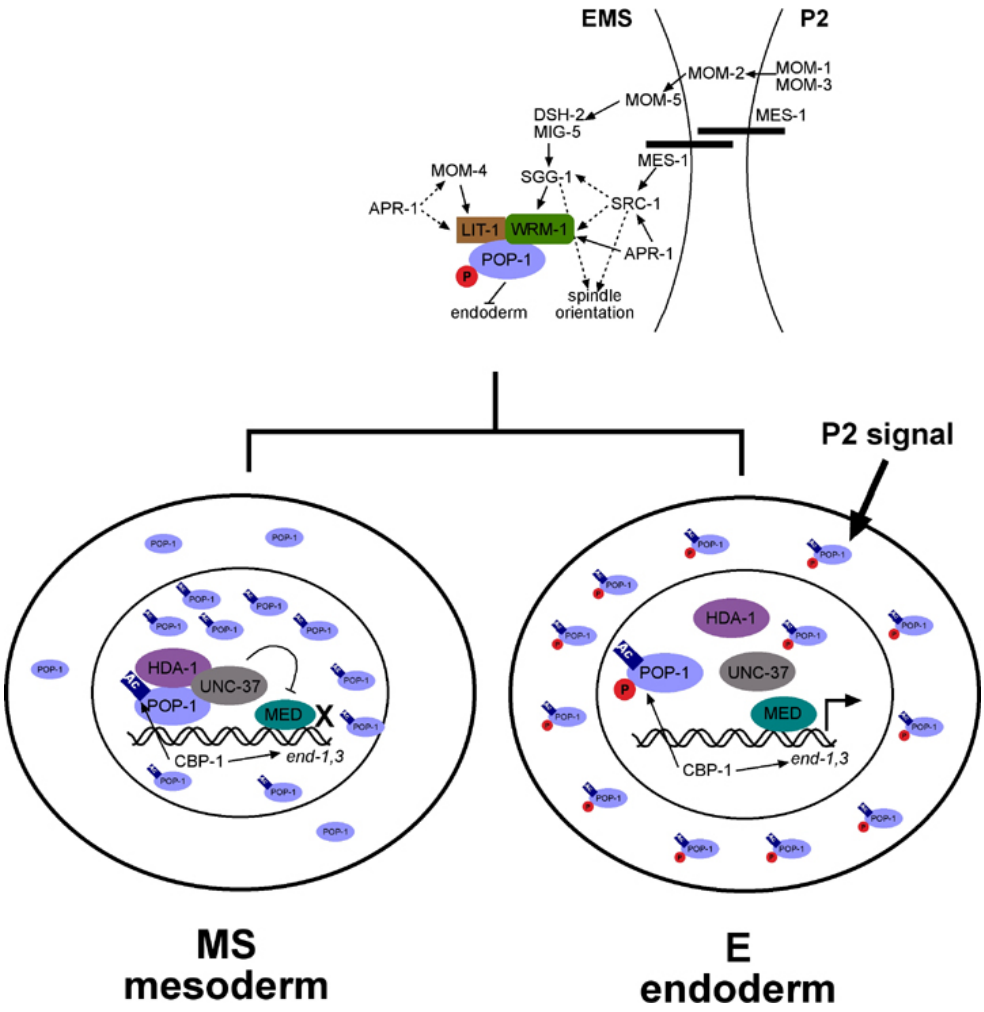
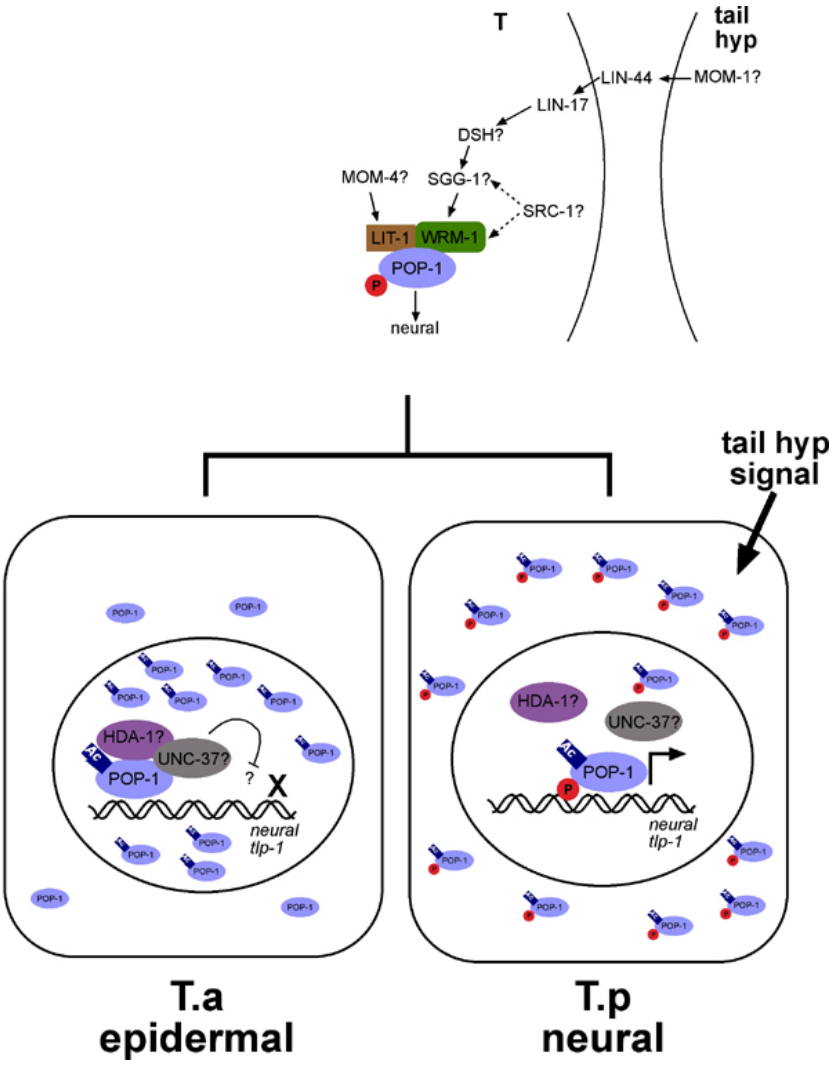


Figure 2. Model for LIN-44 signaling (upper) and control of T cell asymmetric division (lower).

Proposed interactions based upon results observed for EMS polarity (see Figure 1). ? indicates that a role for the component has not been reported. MOM-3 and APR-1 do not appear to play a role in T cell polarity (M.A.H., unpublished). See text for additional details.



Chapter 2

Introduction and objectives

Wnt signaling pathways

Wnt signaling pathways are conserved from nematodes to mammals and function in diverse developmental processes, such as cell proliferation, cell differentiation, cell fate determination, synaptogenesis, cell migration, and cell polarity. At least three major conserved Wnt signaling pathways are recognized: Wnt/β-catenin, Wnt/calcium and planar cell polarity (PCP). In the canonical Wnt or Wnt/β-catenin pathway, the presence of Wnt ligand antagonizes the degradation of β-catenin, and allows β-catenin to translocate to the nucleus and form a complex with Tcf/Lef factors to activate expression of specific genes (Cadigan and Nusse, 1997; Nelson and Nusse, 2004a; Veeman, Axelrod and Moon, 2003). The noncanonical Wnt/Calcium and PCP pathways do not signal through β-catenin (Veeman, Axelrod and Moon, 2003).

Canonical Wnt signaling initiation: the assembly of the membrane complex.

Wnt signal pathways function in almost all animals in diverse developmental processes (Cadigan and Nusse, 1997; Nelson and Nusse, 2004b; Veeman, Axelrod and Moon, 2003). Wnt signaling pathways are tightly regulated spatially and temporally during development and abnormal Wnt signal activation can cause disease, especially cancer (Logan and Nusse, 2004; Moon and Kohn, et al, 2004; Nusse, 2005). Signal initiation involves the interaction between the secreted Wnt glycoprotein and extracellular domains of both the Fz receptor (Bhanot and Brink, et al, 1996) and members of Low-density lipoprotein receptor-related protein (LRP) including LRP5 and LRP6 or Arrow in *Drosophila* (Cong, Schweizer and Varmus, 2004a; Mao and Wang, et al, 2001; Pinson

and Brennan, et al, 2000; Tamai and Semenov, et al, 2000a; Wehrli and Dougan, et al, 2000). The interactions among Wnt, Fz and LRP5 and LRP6 are regulated by many secreted antagonists that can bind to Wnt, such as the sFRP family, or to LRP5 and LRP6, such as the Dkk family (Reviewed by Kawano and Kypta, 2003). Ryk, a receptor protein tyrosine kinase (Halford and Stacker, 2001), was shown to be another Wnt co-receptor and is also involved in the interaction between Wnt and Fz, however, the mechanism by which Ryk physically interacts with Wnt and other receptors is still not clear (He, 2004; Inoue and Oz, et al, 2004; Lu and Yamamoto, et al, 2004). When Wnt ligand is absent, Axin and APC function as scaffold proteins to help the casein kinase (CK) and GSK-3 sequentially phosphorylate β-catenin (Liu and Li, et al, 2002). The phosphorylated β-catenin will be recognized by β-TrCP, targeted for ubiquitination and then degraded by the proteosome. Some amount of β-catenin is associated with the plasma membrane where it functions in the regulation of cell adhesion and is not degraded until it dissociates from the plasma membrane (Nelson and Nusse, 2004). However, the cytoplasmic and nuclear level of β-catenin is kept low and target gene expression is repressed by TCF. Upon signal initiation, by presence of Wnt ligand, Dishevelled/Dsh is phosphorylated, possibly in a LRP5- and LRP6-independent manner (Gonzalez-Sancho and Brennan, et al, 2004), and translocates to the membrane and binds to Fz (Rothbacher and Laurent, et al, 2000). LRP5 and LRP6 are also phosphorylated upon signal initiation, and the phosphorylated LRP5 and LRR6 provide a docking site for Axin. The binding of Axin to the phosphorylated Dsh and LRP5 and LRP6 causes the separation of the Axin from the β-catenin destruction complex and the disruption of this complex (He and Semenov, et al, 2004; Tamai and Zeng, et al, 2004) leading to the

elevation of β-catenin levles. β-catenin then translocates to the nucleus and activates the expression of target genes (Fig. 1) (Bienz and Clevers, 2003).

Function of Fz in Wnt signal transduction

There are 19 Wnt genes in the human and the mouse, seven in *Drosophila*, and five in *C. elegans.* There are 10 Fz receptors in the human or mouse, four in *Drosophila*, and three in *C. elegans* (Adams and Celniker, et al, 2000; Miller, 2002). Interaction between Wnt and Fz is limited by the insolubility of Wnt due to the palmitoylation of cysteine residues in Wnt, which is essential for Wnt signal initiation (Willert and Brown, et al, 2003). Structural and functional analysis of Fz is essential to understand the mechanism of how Wnt ligand or proto-cadherins signal through Fz receptors and activate the cytoplasmic protein Dsh. Such analysis will also lead to understanding of how Fz and Dsh are asymmetrically localized to the epithelial cell sheet. Each Fz receptor family member has an extracellular cysteine-rich domain (CRD), seven putative trans-membrane domains, a carboxyl cytoplasmic domain, three extracellular loops and three intracellular loops (Bhanot and Brink, et al, 1996). The CRD usually has 10 cysteine residues, and can physically interact with Wnt ligand in the cell culture assays (Hsieh and Rattner, et al, 1999), which was also confirmed by mutational analysis (Dann and Hsieh, et al, 2001). CRD also can bind to LRP6 in the presence of Wnt (Tamai and Semenov, et al, 2000). The affinity between CRD and Wingless determines Fz involvement in the canonical or PCP pathway (Rulifson, Wu and Nusse, 2000). However, over-expression of the CRD will cause it to dimerize which is sufficient to activate Wnt/ β-catenin signaling (Carron and Pascal, et al, 2003). An *in vivo* test showed that the CRDs of *Dfz* and *Dfz2* are not

essential for Wingless transduction and CRD deleted Fz and Fz2 can rescue *fz* mutant (Chen and Strapps, et al, 2004), which indicates that the seven trans-membrane domain (7TMD) might be important for the signal transduction. It is also possible that the CRD deleted N-terminal of Fz and Fz2 still possess the ability to capture Wnt to initiate signaling (Povelones and Nusse, 2005). Mutational analysis revealed that several residues in the Fz loops affect the strength of the Wnt/β-catenin signal (Cong, Schweizer and Varmus, 2004b). The length and similarity of the cytoplasmic domains of Fz members varies, but there is a conserved motif, Lys-Thr-X-X-X-Typ (KTXXXW), located two amino acids after the seventh transmembrane domain of most of the Fz receptors (Umbhauer and Djiane, et al, 2000). The KTXXXW motif is required for canonical signal transduction, as well as membrane localization and phosphorylation of Disheveled (Dsh) (Umbhauer and Djiane, et al, 2000). Consistent with this, the PDZ domain of Dsh was shown to bind to the KTXXXW motif directly (Wong and Bourdelas, et al, 2003), however, the PDZ is not required for Dsh membrane localization, while the DEP domain is required (Rothbacher and Laurent, et al, 2000). The KTXXXW motif might interact with Dsh to regulate canononical Wnt pathway (Boutros and Mlodzik, 1999; Li and Yuan, et al, 1999) and function in the PCP to recruit Dsh to the plasma membrane.

Since the insolubility of Wnt ligands limits the use of biochemical assays to study the interaction between Wnt and Fz in mammals, we have taken the advantage of the powerful genetic and molecular tools in *C. elegans* to study the functions of LIN-17/Fz and the interaction between LIN-44/Wnt and LIN-17/Fz.

The Planar Cell Polarity (PCP) pathway is conserved from *Drosophila* to vertebrates.

PCP pathway

Epithelial cells often polarize along the apical-basal axis, and perpendicular to this axis is the planar axis, which is defined as PCP (Fig. 2A). During *Drosophila* wing and eye development, the epithelial cell sheet in the wing and eyes possess PCP, and also in vertebrates, skin and inner ear epithelia possess PCP (Veeman, Axelrod and Moon, 2003). PCP is established by the Wnt/PCP pathway and was first described in *Drosophila* to regulate the orientation of hairs on the wing and dorsal thorax, as well as the specification of photoreceptor 3 and 4 (R3 and R4) in the eye (Mlodzik, 1999; Tree, Ma and Axelrod, 2002). The Wnt/PCP pathway in *Drosophila* contains a cassette of six core genes, including *Frizzled (Fz)*, *Dishevelled (Dsh)*, *Flamingo (Fmi)*, *Van Gogh or Strabismus (Stbm)*, *Diego (Dgo) and Prickle (Pk)*. Disruption of any of these six genes caused PCP defects (Fig. 2B). These proteins were also found to function in the regulation of gastrulation movements during embryogenesis and the body hair orientation in vertebrates (Guo, Hawkins and Nathans, 2004; Fanto and McNeill, 2004; Mlodzik, 2002; Veeman, Axelrod and Moon, 2003)), which suggests that a PCP pathway is conserved from *Drosophila* to vertebrates. In different tissues and species, PCP pathways may contain different signaling initiation factors and their outcome also varies, for example, the PCP pathway functions to specify cell fate in the eye, regulates cytoskeletal organization in wing in *Drosophila*, regulates cell migration in vertebrate gastrulation movements. PCP pathways share six conserved PCP core genes (Klein and Mlodzik,

2005; Mlodzik, 2002). In the following, the asymmetric localization of the six core proteins, the initiation of PCP signaling, and differences between the PCP and the Wnt/β-catenin pathway will be discussed.

The asymmetric localization and interactions of the six PCP core proteins

Prior to PCP signal initiation, PCP components are uniformly localized around the apical cell membrane in ommatidial and wing epithelia cells, and during PCP signal initiation, these components relocalize in an interdependent manner, as loss of any one of the six will disrupt the asymmetric localization of the other five proteins. In individual wing epithelial cells, Fz and Dsh accumulate at the distal membrane and Stbm and Pk accumulate at the proximal membrane, while Fmi and Diego accumulate at both ends (Fig. 2C) (Axelrod, 2001; Bastock, Strutt and Strutt, 2003; Feiguin and Hannus, et al, 2001; Shimada and Usui, et al, 2001; Tree and Shulman, et al, 2002; Usui and Shima, et al, 1999). Functionally, the proximal membrane-localized Stbm and Pk appear to antagonize Fz/Dsh activity, while at both ends localized Diego might promote Fz/Dsh activity (Feiguin and Hannus, et al, 2001) and Fmi might promote or inhibit Fz/Dsh activity depending on its localization (Usui and Shima, et al, 1999). In cells of the ommatidium, the six PCP core proteins display a similar asymmetric localization pattern in the apical cell membrane of the preR3/ preR4 cell in an interdependent manner (Reviewed by Adler, 2002; McNeill, 2002; Strutt, 2003). The asymmetric localization of the six proteins is required to amplify Fz activity difference between two adjacent cells (Tree and Shulman, et al, 2002). While asymmetric localization of the six proteins is

required for the PCP signaling, the asymmetric localization of Fz/Dsh to distal cortical cell membrane, where the hair forms, is essential for PCP signaling, while the other four proteins help Fz/Dsh to be localized correctly and also regulate the activities of Fz/Dsh. Unipolar membrane localization of MOM-5/Fz has also been observed during *C. elegans* embryogenesis (Park, Tenlen and Priess, 2004) and of DSH-2/Dsh during asymmetric neuron division (Hawkins and Ellis, et al, 2005). This indicates that a similar regulation pattern of PCP components might exist in *C. elegans*.

Cadherin proteins initiate the PCP signaling

Unlike mammals, in which Wnt ligands are involved in the signal initiation of the PCP pathway that regulates cell movements during gastrulation (Heisenberg and Tada, et al, 2000; Tada, Concha and Heisenberg, 2002; Weidinger and Moon, 2003), in *Drosophila*, a Wnt ligand for PCP pathway has not been identified. None of the *Drosophila* Wnts is expressed in the patterns that fit functions in PCP. In addition, neither mutation nor over-expression of any of the six Wnt genes has displayed a PCP defect. How is the PCP signal initiated in *Drosophila*? It appears that signaling molecules, which display long-range gradient expression pattern across the wing or eyes, might regulate PCP. The signaling molecules appear to be proto-cadherins Dachsous (Ds), four jointed (Fj) and Fat (Ft) that might cooperate to regulate Fz signaling in the eyes and wing hair polarity (Ma and Yang, et al, 2003; Rawls, Guinto and Wolff, 2002; Simon, 2004; Yang, Axelrod and Simon, 2002). Loss of either *ft* or *ds* or *fj* function disrupted PCP in both the eyes and the wing. Both Ds and Fj are expressed in reciprocal gradients across the eyes or wing

(Figure 3A), with a low level of Ds and a high level of Fj at distal end of the wing, and a high level of Ds and a low level of Fj in the proximal end of the wing (Fig. 3A). In the eye disc, there is a low level of Fj and a high level of Ds in the equatorial region and there is a high level of Fj and a low level of Ds in the polar region (Adler, Charlton and Liu, 1998; Casal, Struhl and Lawrence, 2002; Ma and Yang, et al, 2003; Yang, Axelrod and Simon, 2002). The Ds and Fj protein gradients regulate the activity of Ft. Ds promotes Ft activity by binding to Ft and recruiting it to the cell surface of the more proximal or more equatorial neighboring cell (Ma and Yang, et al, 2003; Matakatsu and Blair, 2004; Simon, 2004). Fj might regulate the Ds function or modify Ft directly (Strutt and Mundy, et al, 2004). Overall, Ds and Fj regulate Ft activity to form a gradient activity across the wing or eye, with proximal high and distal low activities in the wing, or equatorial high and polar low in the eye. The gradient Ft activity is responsible for the bias of Fz activity in the neighboring cells, and causes the distal end of a proximal cell to have higher Fz activity than the proximal end of a distal neighboring cell in the wing, or polar end of a equatorial cell to have higher Fz activity than the equatorial end of a polar neighboring cell in the ommatidium.

The PCP and Wnt/β-catenin pathways branch at Dsh

The PCP and Wnt/β-catenin pathways share the Fz receptor and the cytoplasmic transduction molecule Dsh, but are activated by different Wnts or initiation factors and signal through different downstream components. Although Dsh is involved in both the PCP and the Wnt/β-catenin pathways, there is domain specificity within the Dsh

molecule. Deletion experiments showed that the PDZ domain is required for both pathways, and the DIX domain is required only for the Wnt/β-catenin pathway, while the DEP domain is required only for PCP (Fig. 3B) (Axelrod and Miller, et al, 1998; Boutros and Paricio, et al, 1998). The DEP domain is required for membrane localization, which is a requisite for PCP (Park and Gray, et al, 2005), while the PDZ domain was shown to interact with Fz (Wong and Bourdelas, et al, 2003). The DEP domain might also interact with Daam1 to regulate RhoA activity to regulate cytoskeletal changes and gene transcriptions in *Xenopus* (Habas, Kato and He, 2001; Habas, Dawid and He, 2003). Spatial localization of Fz receptors appears to explain how Wnt ligands do not simultaneously activate PCP and Wnt/β-catenin pathways. When PCP signal is initiated in *Drosophila*, Fz is primary localized to the apical side of the epithelia cell, where its activity is regulated by Ft. Fz2, which only serves as the receptor for Wg and has higher affinity for Wg than does Fz, is evenly distributed to the epithelial cell, and its activity is regulated by Wnt (Fig. 4) (Bejsovec, 2005; Wu, Klein and Mlodzik, 2004).

Asymmetric localization of Wnt signaling components contribute to the asymmetric cell division

Asymmetric cell division, in which a mother cell produces two daughter cells that posses different developmental potential via unequal inheritance of cell fate determinants or interaction with other cells or the environments, is essential to generate cellular diversity

during development (Reviewed by Horvitz and Herskowitz, 1992; Jan and Jan, 1999; Jan and Jan, 2000). The asymmetric division of *C. elegans* embryos has been intensively studied and the asymmetric localization of PAR proteins prior to division is required for this asymmetric cell division (Reviewed by Cowan and Hyman, 2004; Nance, 2005; Pellettieri and Seydoux, 2002a)). The functions of PAR proteins are conserved (Pellettieri and Seydoux, 2002b) and also link to Wnt signaling. PAR-5 is involved in the subcellular localization of POP-1/Tcf in *C. elegans* (see below) (Lo and Gay, et al, 2004) and PAR-1 functions both in the canonical Wnt and the PCP pathways in vertebrates (Ossipova and Dhawan, et al, 2005; Sun and Lu, et al, 2001). In *C. elegans*, Wnt signaling is involved in the regulation of asymmetric cell divisions (Chamberlin and Sternberg, 1995; Chang, Lloyd and Zarkower, 2005; Herman, 2001; Herman and Horvitz, 1994; Herman and Vassilieva, et al, 1995; Sawa, Lobel and Horvitz, 1996) and mitotic spindle orientation (Bei and Hogan, et al, 2002; Rocheleau and Downs, et al, 1997; Siegfried and Kimble, 2002). Interestingly, Wnt signaling components POP-1/Tcf, WRM-1/β-catenin, LIT-1/NLK-1 and MOM-5/Fz are also asymmetrically localized both prior to and after division.

Wnt/MAPK regulates asymmetric nuclear localization of POP-1 in most but not all asymmetric cell divisions in *C. elegans*

Noncanonical Wnt signaling pathways converge on POP-1, the sole Tcf in *C. elegans* (See Chapter One). In the following, I will address the new discoveries about POP-1 in the last two years. At the four-cell stage of *C. elegans* embryogenesis, P2-EMS signaling or Wnt/MAPK signaling from the P2 blastomere is involved in the regulation of EMS

asymmetric cell division (Fig. 5). EMS divides asymmetrically to produce an anterior MS cell, which will differentiate to mesoderm, and a posterior E cell, which will differentiate to endoderm. The two daughters have different nuclear levels of POP-1, with the E cell being lower level than that of MS. The asymmetric localization of POP-1/Tcf in the MS and E cells is controlled by Wnt/MAPK and the daughter cell E proximal to Wnt/MAPK signaling, has lower nuclear level (Lin, Thompson and Priess, 1995; Lin, Hill and Priess, 1998a). Wnt/MAPK promotes the phosphorylation and acetylation of POP-1 and causes POP-1 to be exported to the the cytoplasm in the E cell (Gay and Calvo, et al, 2003; Lo and Gay, et al, 2004). The qualitatively changed POP-1 in the nucleus of the E cell might inhibit the retention of POP-1 in the E nucleus (Herman and Wu, 2004). Wnt/MAPK also might directly regulate the nuclear exporting and importing machine. The Wnt/MAPK-induced change in the level and quality of POP-1 in the E cell will induce the expression of *end-1* and *end-3*, which are required to specify E cell fate (Maduro, Lin and Rothman, 2002a). The asymmetric localization of POP-1 to sister cells, defined as POP-1 asymmetry (Kidd and Miskowski, et al, 2005), was also found to be common in larvae stages, as POP-1 is asymmetrically localized to the daughter cells of the T cell (Herman, 2001), somatic gonad precursor (SGP) cells (Siegfried and Kidd, et al, 2004b), P cells (Deshpande and Inoue, et al, 2005; Inoue and Oz, et al, 2004), B cell (Wu and Herman, Submitted). Wnt/MAPK pathways are involved in the regulation of asymmetric localization of POP-1 to the daughter cells of EMS blastomere, T cell and SGP, but not the B cell (Wu and Herman, Submitted) {{4132 Rocheleau, C. E. 1999; 4838 Takeshita, H. 2005; 4861 Siegfried, K. R. 2004;}}. Asymmetric localization of POP-1 to the B daughters is lost in *lin-17* mutants and reversed in *lin-44* mutants, but is not changed in

both *wrm-1* and *lit-1* mutants. Instead, POP-1 asymmetry in B daughters is lost in *rho-1/RhoA* and *let-502/Rock* mutants, which strongly suggested that a PCP-like pathway, a novel noncanonical Wnt pathway in *C. elegans*, might be involved in the regulation of B cell asymmetric cell division (Wu and Herman, submitted).

Nuclear export of POP-1 in the signal-responding cell requires PAR-5/CRM-1 and WRM-1/LIT-1

Phosphorylation of POP-1 by LIT-1/WRM-1 regulates POP-1 nuclear cytoplasmic redistribution (Herman and Wu, 2004). Although WRM-1 does not interact with POP-1 directly, it is required for LIT-1 to phosporylate POP-1 and is indirectly involved in the POP-1 nuclear cytoplasmic redistribution, which was shown both in *C. elegans* (Lin, Hill and Priess, 1998b; Rocheleau and Yasuda, et al, 1999) and in tissue culture cells (Rocheleau and Yasuda, et al, 1999). Phosphorylation of POP-1 by LIT-1/WRM-1 is not sufficient for exporting POP-1 from the nucleus and Lo et al (2004) indicated that the nuclear exportation is also involved in this POP-1 asymmetry. PAR-5/14-3-3 and CRM-1/exportin are required for POP-1 asymmetry, as POP-1 asymmetry in the sister cells is lost in *par-5* and *crm-1* mutants. In summary, in the signal-responding cell, WRM-1/LIT-1 will phosphorylate POP-1, most likely on serines 118 and 127. The phosphorylation of POP-1 has stronger affinity for PAR-5 and will be exported to the cytoplasm by CRM-1 causing the signal responsive cell to display a lower level of nuclear POP-1 (Lo and Gay, et al, 2004). However, the quality of POP-1 in response to Wnt/MAPK is more important. When all five potential phosphorylation residues were mutated to alaine, POP-1 nuclear

asymmetric localization was lost, but it could partially rescue *pop-1* (Lo and Gay, et al, 2004). These results indicate that Wnt/MAPK regulates POP-1 nuclear cytoplasmic redistribution via phosphorylation of POP-1 and the exportation of POP-1 from the nucleus, and also changes POP-1quality by an unknown mechanism.

A new β-catenin sheds light on how POP-1 functions in cell specification.

As discussed above, a lower level of POP-1 in the posterior EMS daughter cell in response to Wnt/MAPK is required to specify E cell fate. Disruption of Wnt/MAPK will cause both daughter cells to take the MS cell fate, while loss of POP-1 will cause both anterior daughter cells to take the E cell fate, which indicates that POP-1 represses E cell fate (Herman and Wu, 2004). The Wnt/MAPK pathway also regulates the asymmetric cell division of SGP and T cells in L1 larvae, but unlike the Wnt/MAPK in the regulation of EMS asymmetric division, disruption of Wnt/MAPK or loss of POP-1 caused both daughter cells to take anterior daughter cell fate (Herman, 2001), which indicates a positive role for the POP-1 in the specification of the posterior daughter cell (Herman and Wu, 2004). The paradox of POP-1 function was resolved by the identification of a new β-catenin, SYS-1 (Bowerman, 2005; Kidd and Miskowski, et al, 2005; Siegfried and Kidd, et al, 2004a). SYS-1 was identified by functional criteria rather than sequence. SYS-1 can replace BAR-1, the only β-catenin that function in the Wnt canonical pathway, and interact with POP-1 directly to transform POP-1 to a transcriptional activator in vitro and in vivo. Unlike the upstream components of Wnt/MAPK, loss of SYS-1 does not affect POP-1's asymmetric localization to SGP daughters but causes the two daughters to take anterior cell fate, which indicates that SYS-1 functions downstream or parallel with POP-

1 to specify SGP daughter cell fates. Kidd et al hypothesized that SYS-1 transforms POP-1 to an activator in a dosage-dependent manner, indicating that SYS-1 is a limiting co-activator for POP-1 (Fig. 6). Both daughters have a limited amount of SYS-1 and an equal amount of POP-1, and when Wnt/MAPK signaling is initiated, POP-1 will be exported from the nucleus of the posterior daughter cell. The reduction of POP-1 in the nucleus of posterior cell will enable SYS-1 bind to POP-1, which will co-activate the expression of posterior cell specific genes.

Although the discovery of SYS-1 function as a limiting activator of POP-1 sheds light on the model (Fig. 6), many questions still need to be resolved: what is the expression pattern of SYS-1 in the two daughter cells, what are the dynamics of SYS-1 expression in response to Wnt/MAPK, how *sys-1* interacts genetically with components of Wnt/MAPK, and what is the function of *sys-1* in the T cell and other asymmetric cell divisions.

Asymmetric localization of WRM-1 and LIT-1 is regulated by Wnt signaling

Since LIT-1 and WRM-1 are responsible for the differential phosphorylation of POP-1 in the two daughters and for POP-1 nuclear asymmetry, are LIT-1 and WRM-1 also asymmetrically localized to the two daughter cells? Recent work demonstrated that they are (Nakamura and Kim, et al, 2005, Takeshita and Sawa, 2005). A *wrm-1::gfp* transgenic line rescued *wrm-1* was constructed and the time course expression pattern of WRM-1::GFP in the EMS was examined. At the beginning of telophase, during the EMS division, WRM-1::GFP enters the nucleus in all cells in a MOM-4 and LIT-1 dependent manner. Some WRM-1::GFP was associated with the cell cortex in all cells in a MOM-

5/Fz dependent manner. In the E cell, when Wnt/MAPK was initiated at late stage of the cell cycle, WRM-1 is phosphorylated and disassociated from the cell cortex to be located in the cytoplasm. WRM-1 in the cytoplasm might complex with LIT-1 and be transported to the E nucleus by an unknown mechanism or a default mechanism in a LIT-1 and MOM-4 dependent manner. While in the MS cell distal to Wnt/MAPK signal, WRM-1 that associates with the cell cortex still associates with the cortex, while nuclear WRM-1 in the MS, accumulates by an unknown mechanism or a default mechanism at the beginning of telophase, will be exported by the IMB-4/CRM-1 complex (Nakamura and Kim, et al, 2005). WRM-1 in the E nucleus can also be exported to the cytoplasm by IMB-4/CRM-1, but the nucleus of the E cell has a stronger ability to retain WRM-1::GFP than the MS nucleus (Nakamura and Kim, et al, 2005) by inhibiting CRM-1 activity or by disassociating WRM-1 from cortex, which is still unknown. This model gives a clear idea of the correlation between the WRM-1 expression pattern and the EMS cell polarity as well as how Wnt signaling controls the dynamic expression pattern of WRM-1, but how WRM-1 is asymmetrically localized prior to and during the EMS cell division and how WRM-1 is involved in the EMS cell spindle orientation are unknown. A similar expression pattern of LIT-1 is observed during the EMS cell division (Lo and Gay, et al, 2004). GFP::LIT-1 accumulates to the nucleus of the signal-responding cell at interphase at early stage of embryogenesis (Lo and Gay, et al, 2004; Maduro, Lin and Rothman, 2002). Surprisingly, this interphase nuclear enrichment of GFP::LIT-1 was also regulated by the Wnt/MAPK pathway(Lo and Gay, et al, 2004), which indicates the Wnt/MAPK regulates LIT-1 activity and also its spatial localization. Similar expression patterns of LIT-1 and WRM-1 are also observed in larval stages. LIT-1 and WRM-1 are

asymmetrically localized to the V, P, QL and QR daughter cells, with the posterior nucleus having a higher level than that of anterior (Takeshita and Sawa, 2005), however we could not exclude the possibility that the expression pattern of WRM-1 and LIT-1 in these cells is due to the heterologous promoter.

Overall, the expression patterns of WRM-1, LIT-1 and POP-1 in EMS, V, P, QL and QR are regulated by Wnt and also correlate with cell polarity to some extent. How does Wnt signaling establish the asymmetric localization of WRM-1, LIT-1 and POP-1? The direction and source of Wnt ligand might provide a hint. It seems that in all asymmetric cell divisions, the daughter cell that is proximal to Wnt signaling has a higher level of WRM-1 and LIT-1 in the nucleus than the distal cell, while the distal cell has a higher level of WRM-1 and LIT-1 associated with the cell cortex than the proximal cell. So, what are the expression patterns of Fz and Dsh that function upstream of LIT-1/WRM-1 in these asymmetric cell divisions? Half of this thesis investigates the asymmetric localization of LIN-17/Fz and MIG-5/Dsh. The asymmetric localization of LIN-17 and MIG-5 prior to and after B cell division might illuminate how LIN-17 and MIG-5 are involved in the regulation of WRM-1 and LIT-1 and how asymmetric localization of LIN-17 and MIG-5 contribute to B cell asymmetric division.

Objective: Is the PCP pathway conserved in *C. elegans*?

The Wnt/β-catenin pathway is conserved from nematodes to mammals, and PCP is conserved from *Drosophila* to vertebrates (Fanto and McNeill, 2004). However, it is not yet clear whether the PCP pathway is also conserved in *C. elegans*. It is also not clear

whether *C. elegans* has epithelia-like tissues whose planar polarity might be regulated by PCP or other pathways. In *C. elegans*, the male specific blast cell B, divides asymmetrically to generate a larger anterior-dorsal daughter cell B.a and a smaller posterior-ventral daughter B.p, while in hermaphrodites, the B cell does not divide. B.a divides to produce 40 cells and generates male copulatory spicules, and B.p divides to produce 7 cells (Sulston, Albertson and Thomson, 1980). LIN-44/WNT and LIN-17/Fz regulate the asymmetric B cell division. In *lin-44* mutant males, B cell polarity is reversed: the B cell divides to produce a smaller anterior B.a cell and a larger posterior B.p cell (Herman and Horvitz, 1994; Herman and Vassilieva, et al, 1995). LIN-17, a protein with seven putative transmembrane domains, is a homolog of the *Drosophila* Frizzled receptor. In *lin-17* mutant males, B cell polarity is lost and the B cell divides to produce two cells with equal size and the same fate (Herman and Horvitz, 1994; Sawa, Lobel and Horvitz, 1996; Sternberg, 1988). LIN-44/Wnt is expressed in the tail hypodermal cells, and regulates cell polarities of the TL, TR, B, U and F cells in the *C. elegans* tail. These are adjacent ectodermal blast cells, and for an animal that has only about one thousand cells, it is somewhat reminiscent of an epithelia sheet. However, the pathway that is activated by LIN-44 and LIN-17 to regulate B cell polarity has not been elucidated. This thesis identifies the PCP homologs in *C. elegans* and examines their functions in the control of B cell polarity. Dynamic expression patterns of some of the PCP homologs were investigated and the molecular mechanism that is involved in the control of asymmetric localization of those components was also investigated.

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Figure 1 Wnt signal inition

(A) Without Wnt, the scaffolding protein Axin assembles a protein complex that contains Apc, Gsk3, Ck1 and ß-catenin. In this complex, ß-catenin is sequentially phosphorylated by Ck1 and Gsk3. Phosphorylated ß-catenin is recognized by ß-Trcp, which is a component of an ubiquitin-ligase complex that conjugates ß-catenin with ubiquitin. Poly-ubiquitinated ß-catenin is degraded by the proteosome. (B) In the presence of Wnt, ß-catenin phosphorylation and degradation is inhibited. Accumulated ß-catenin forms a nuclear complex with the DNA-bound TCF/LEF transcription factor, and together they activate Wnt-responsive genes. (He et al. 2004)

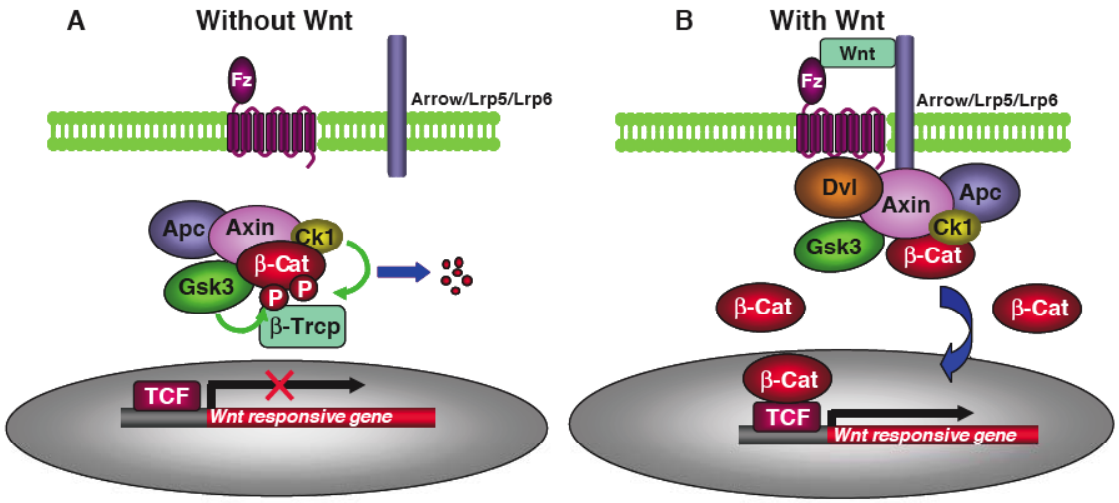


Figure 2 PCP phenotype is controlled by PCP components and the six PCP core proteins are asymmetrically localized.

(A) The planar axis is perpendicular to the apical-basal axis. Epithelia cells polarize along apical basal axis (Adapted from Mlodzik 1999). (B) In the *Drosophila* wing, the wing hairs are distally oriented, but when the PCP pathway was disrupted, the orientation of wing hairs is disrupted. (C) In individual wing epithelia cell, Fz and Dsh accmulate to the distal cortex, and Stbm and Pk accumulate to the proximal cortex, while Fmi and Dgo accumulate at both proximal and distal cortex (Adapted from Fanto, et al 2004).

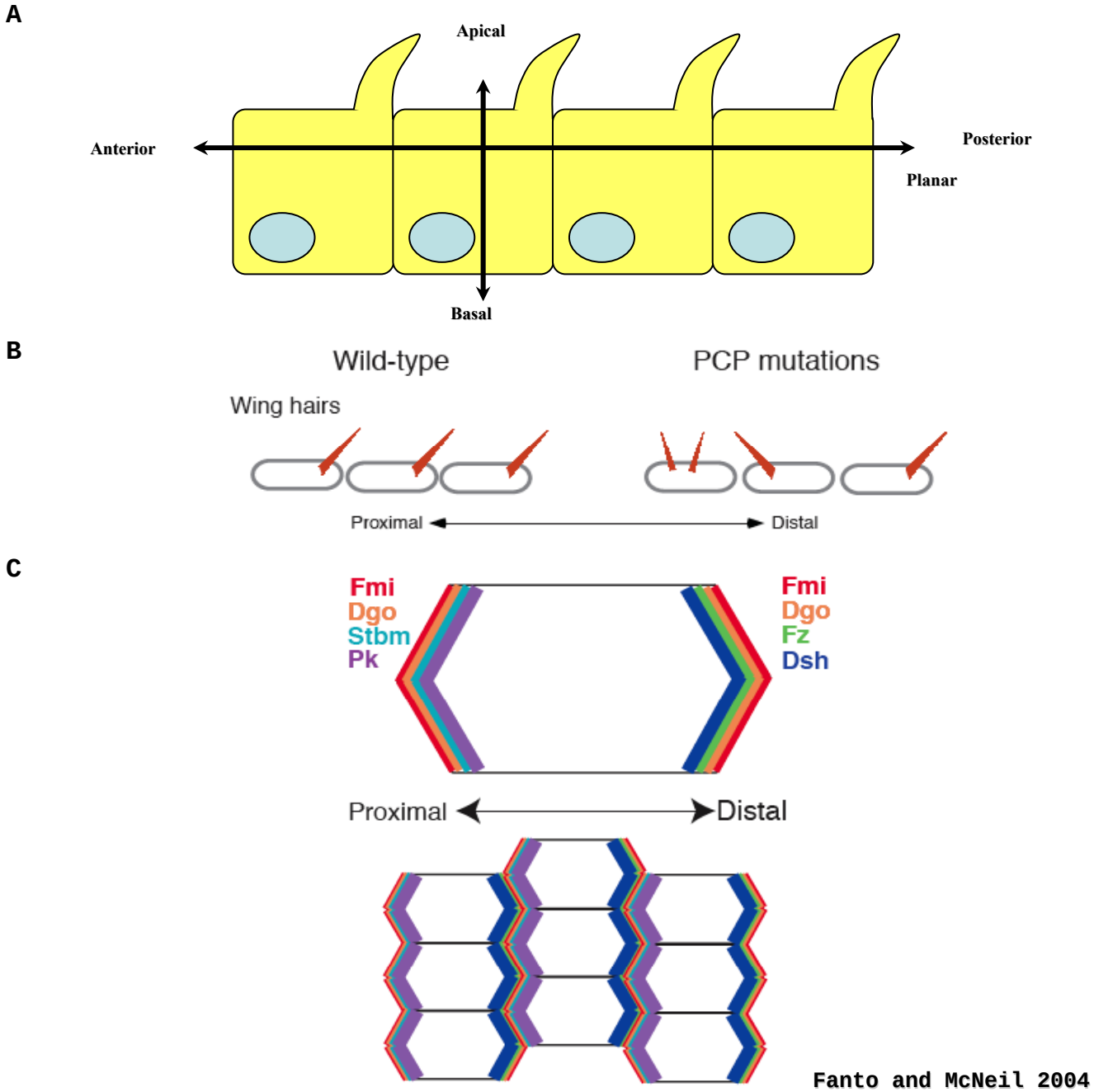
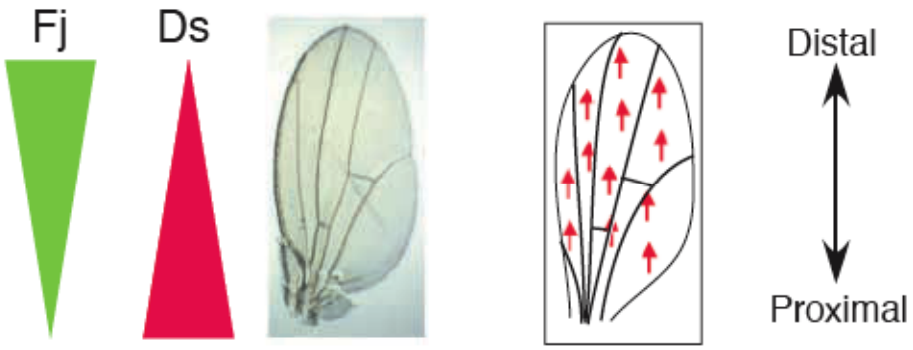


Figure 3 Fj and Ds are expressed in a gradient expression pattern in the wing and PCP pathway and canonical Wnt pathway branch at Dsh.

(A) Fj and Ds display a reciprocal gradient expression pattern across the wing, with a lower level of Fj or a higher level of Ds at proximal of the wing, while a higher level of Fj or a lower level of Ds at distal of the wing. (B) PCP and canonical Wnt pathways share Fz and Dsh, but their signal initiation and downstream components are different.

A



Fanto, et al 2004

B

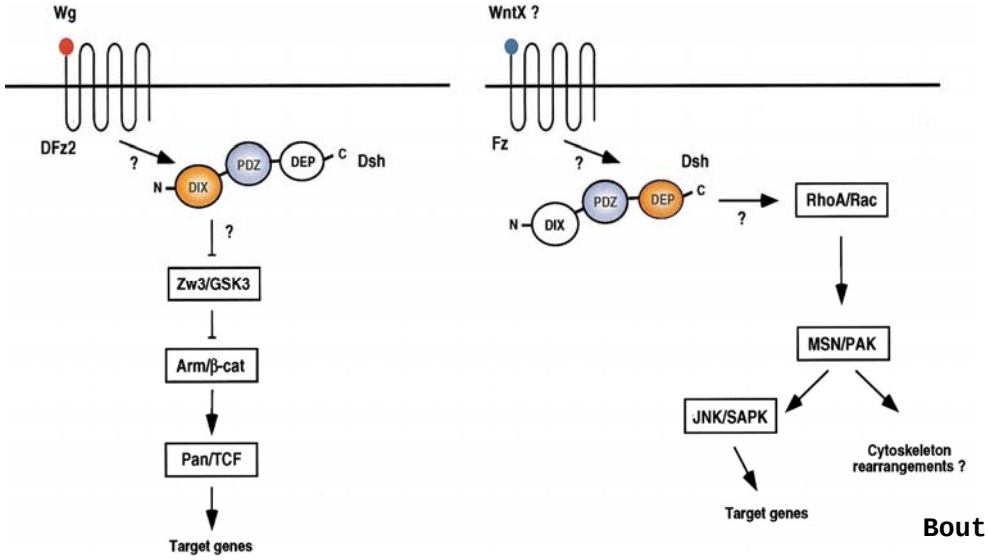


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Figure 4 PCP and canonical Wnt pathway are spatially separated.

(A) Fz (pink) accumulates at the distal side of the apical region of an epithelia cell, where PCP pathway is activated, G-protein pathway (brown) is activated in the proximal at the apical region of the cell, while Fz2 (purple) is evenly distributed to the proximal and distal in the basal region of the cell. (B) PCP pathway activation in the apical region of an epithelia cell. (C) Canonical pathway is activated at the basal region of an epithelia cell.

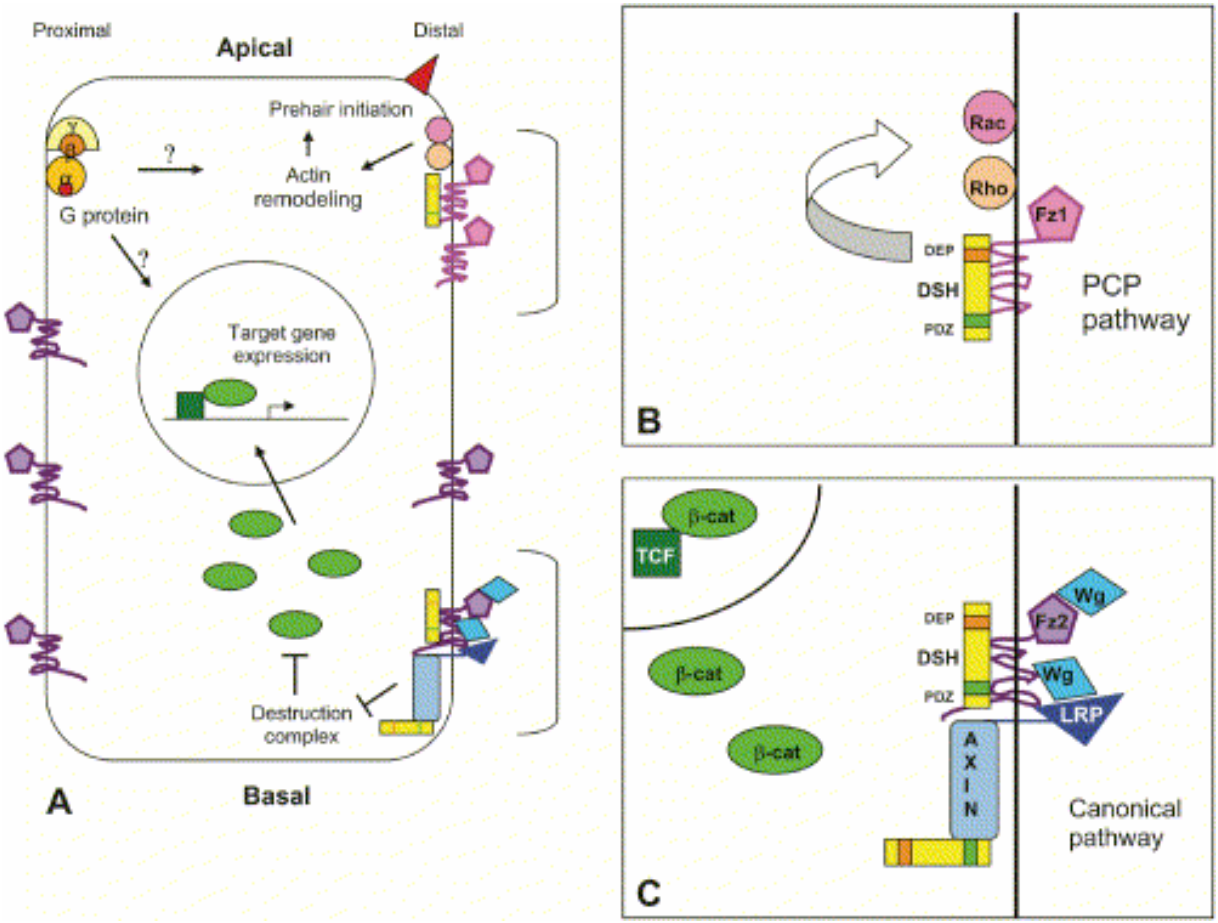


Figure 5 Three interacting pathways that mediate the P2-EMS signaling are shown.

The identification of MIG-14/MOM-3 locus has not been done yet, but acts in P2. Active kinases are shown in red. The two horizontal lines indicate the plasma membrane of the signaling cell P2 (top line) and of EMS (bottom line). Question marks indicate that the point at which the MES-1/SRC-1 pathway interacts with the Wnt pathway is not yet known. A dashed arrow indicates that Wnt binding may also activate the MOM-4/LIT-1 pathway. (Adapted from Eisenmann DM. 2005)

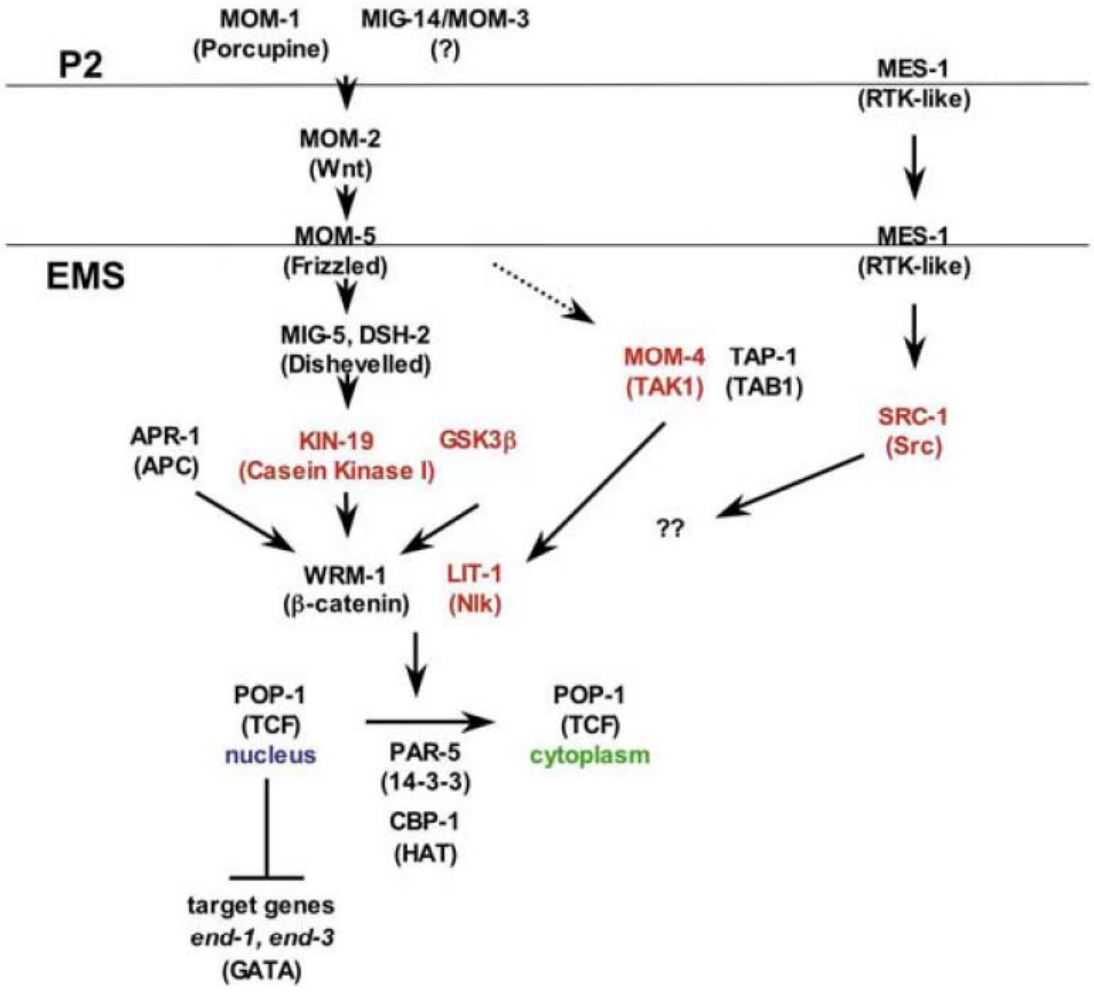
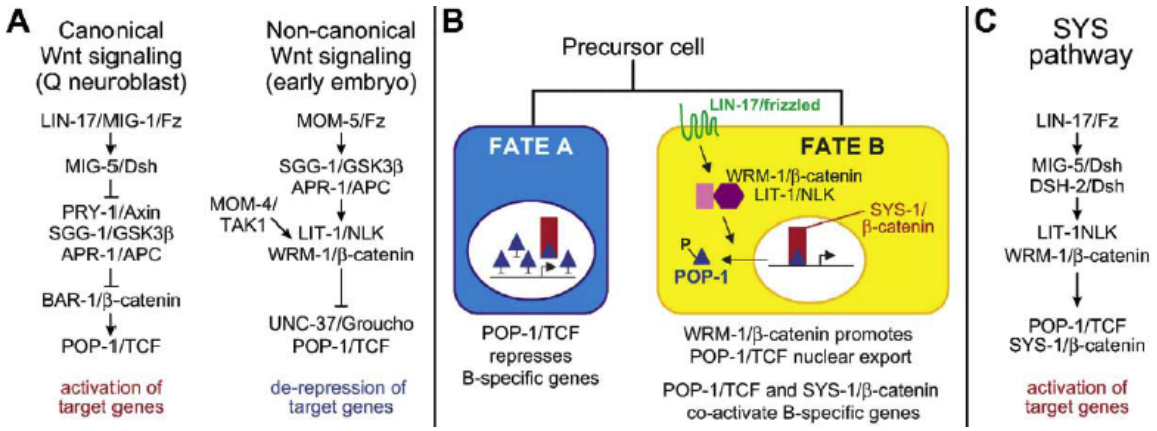


Figure 6 SYS, a limiting coactivator of POP-1, sheds light in the paradox functions of POP-1 in the Wnt signaling.

(A) Two Wnt signaling pathways in *C. elegans*. Left, canonical Wnt pathway. Right, Wnt/MAPK pathway. Q neuroblasts employ the classical β-catenin, BAR-1, to coactivate POP-1/TCF. The early embryo employs a divergent β-catenin, WRM-1, to relieve POP-1/TCF-mediated repression. (B) SYS variant of Wnt signaling. The precursor cell divides asymmetrically to generate two distinct daughters. Without Wnt signaling, Fate A is specified by either POP-1 repression of B-specific target genes as shown or by lack of POP-1 activation of B-specific genes. With Wnt signaling, Fate B is specified by SYS pathway. WRM-1/β-catenin and LIT-1/NLK promote POP-1 exit from the nucleus, and SYS-1/β-catenin and POP-1 coactivate B-specific target genes. SYS-1/β-catenin, red tooth; POP-1, blue triangle; WRM-1/β-catenin, pink rectangle; LIT-1, purple hexagon; LIN-17, green membrane protein. (C) SYS signaling unifies the canonical and Wnt/MAPK forms of Wnt signaling. (Kidd et al. 2005)



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Chapter 3

A novel noncanonical Wnt pathway is involved in the regulation of the asymmetric B cell division in *C. elegans*

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Abstract

The polarities of several cells that divide asymmetrically during *C. elegans* development are controlled by Wnt signaling. LIN-44/Wnt and LIN-17/Fz control the polarities of cells in the tail of developing *C. elegans* larvae, including the male-specific blast cell, B, that divides asymmetrically to generate a larger anterior daughter and a smaller posterior daughter. We determined that WRM-1 and the major canonical Wnt pathway components: BAR-1, SGG-1/GSK-3 and PRY-1/Axin were not involved in the control of B cell polarity. However, POP-1/Tcf is involved and is asymmetrically distributed to the B daughter nuclei, as it is in many cell divisions during *C. elegans* development. Aspects of the B cell division are reminiscent of the divisions controlled by the planar cell polarity (PCP) pathway that has been described in both *Drosophila* and vertebrate systems. We identified *C. elegans* homologs of Wnt/PCP signaling components and have determined that many of them appear to be involved in the regulation of B cell polarity. Specifically, MIG-5/Dsh, RHO-1/RhoA and LET-502/ROCK appear to play major roles, while other PCP components appear to play minor roles. We conclude that a non-canonical Wnt pathway, which is different from other Wnt pathways in *C. elegans*, regulates B cell polarity.

Key words: B cell polarity, Planar Cell Polarity, *C. elegans*, Asymmetric cell division, Wnt signaling; Rho

Introduction

Wnt signaling pathways function in almost all animals in diverse developmental processes (Cadigan and Nusse, 1997; Veeman et al., 2003; Nelson and Nusse, 2004). At least three major conserved Wnt signaling pathways have been recognized: Wnt/β-catenin, Wnt/calcium and Wnt/planar cell polarity (PCP) (Nelson and Nusse, 2004). In the canonical, or Wnt/β-catenin pathway, Wnt ligands act through Frizzled (Fz) receptors and Dishevelled (Dsh) to antagonize the degradation of β-catenin, allowing β-catenin to translocate to the nucleus and complex with Tcf/Lef factors to activate or repress the expression of specific genes. The noncanonical Wnt/calcium and PCP pathways do not signal through β-catenin (Veeman et al., 2003; Nelson and Nusse, 2004). In *Drosophila*, the Wnt/PCP pathway regulates the orientation of hairs on the wing and dorsal thorax as well as the polarity of ommatidia in the eye (Mlodzik, 1999; Tree et al., 2002). In addition, Wnt/PCP has been found to regulate cell movements during vertebrate gastrulation and other biological processes (Veeman et al., 2003; Fanto and McNeill, 2004). The PCP pathway contains six core genes, *Fz*, *Dsh*, *Flamingo/Fmi*, *Van Gogh or Strabismus/Stbm*, *Diego/Dgo and Prickle/Pk*. PCP pathways that control bristle, hair and ommatidial polarity in *Droshophila* share these six molecules, but each tissue has its own specific downstream components and an unknown upstream signal (Tree et al., 2002). The PCP and Wnt/β-catenin pathways share the Fz receptor and the cytoplasmic transduction molecule Dsh, but are activated by different Wnts or unknown factors and signal through different downstream components. Although Dsh is involved in both PCP and Wnt/β-catenin pathways, domains within the Dsh molecule display different specificities (Axelrod et al., 1998; Boutros et al., 1998). The asymmetric localization of

the core PCP molecules is critical to planar polarity and the inhibition of Wnt/β-catenin signaling. In cells of the *Drosophila* pupal wing, Fz and Dsh are localized to the distal membranes, where the hair forms, whereas the Stbm and Pk are found in the proximal membranes and Fmi and Dgo are found in both (Wodarz and Nusse, 1998; Jenny et al., 2003; Strutt, 2003).

The Wnt/β-catenin and Wnt/PCP pathways are conserved throughout the animal kingdom (Fanto and McNeill, 2004). Recent work by Park et al. (2004) demonstrated that during *C. elegans* ventral closure, MOM-5/Frizzled is localized within cells in a manner similar to *Drosophila* Frizzled during planar polarity and dorsal enclosure. This suggests that the PCP pathway might also be conserved in *C. elegans*. During *C. elegans* larval development, LIN-44/Wnt is expressed in the tail hypodermal cells, and regulates cell polarities of the TL, TR, B, U and F cells which lie further anterior in the tail. In males, the B cell divides asymmetrically to generate a larger anterior-dorsal daughter cell, B.a, and a smaller posterior-ventral daughter, B.p. B.a divides to produce 40 cells and generates male copulatory spicules, and B.p divides to produce 7 cells (Sulston et al., 1980). In *lin-44* mutant males B cell polarity is reversed (Herman and Horvitz, 1994; Herman et al., 1995), while in *lin-17* mutant males B cell polarity is lost (Sternberg, 1988; Sawa et al., 1996). While genes specifically involved in T cell polarity have been isolated and studied (Sawa et al., 2000; Zhao et al., 2002; Zhao et al., 2003), the pathway that is activated by LIN-44 and LIN-17 to regulate B cell polarity has not been elucidated.

Here, we begin to define the Wnt pathway that controls B cell polarity. We determined that β-catenin homologs WRM-1 and BAR-1 as well as SGG-1/GSK-3, PRY-1/Axin and

DSH-2/Dsh did not appear to be involved in the control of B cell polarity. However POP-1, the sole *C. elegans* TCF homolog, is involved and GFP::POP-1 (Siegfried et al., 2004) is asymmetrically distributed to the B.a and B.p cell nuclei. We also identified putative *C. elegans* homologs of Wnt/PCP signaling components and have determined that many of them appear to be involved in the regulation of B cell polarity. We show that, in addition to LIN-44 and LIN-17, MIG-5/DSH, RHO-1/RhoA and LET-502/Rock play major roles in the control of B cell polarity, and other PCP components play minor roles. In addition, we show that LIN-17/Fz is expressed in the T and B cells, whereas MIG-5/Dsh is expressed in the B cell. We conclude that a non-canonical Wnt or a PCP-like pathway, which is different from other Wnt signal pathways in *C. elegans*, regulates B cell polarity.

Materials and methods

General methods and strains

Nematodes were cultured and manipulated by standard techniques (Brenner, 1974). N2 was used as the wild-type strain. The following mutations were used:

Linkage Group I (LGI): *tag-15(gk106), pry-1(mu38), lin-17(n671), pop-1(q645, q624), lin-44(n1792), let-502(h392), dpy-5(e61), unc-29(e1072)*;

LGII: *mig-5(ok280), dsh-2(or302), mIn1[mIs14 dpy-10(e128)], rrf-3(pk1426)* (Simmer et al., 2002);

LGIII: *wrm-1(ne1982ts), cdh-3(pk77, pk87), unc-32(e189), Y48G9A.4(ok460), lit-1(or131ts), unc-119(e2498), cyk-1(t1611)*;

LGIV: *unc-44(e1260, e1197), jnk-1(gk7), unc-43(n498), him-8(e1489), cyk-4(t1689)*;

LGV: *unc-42(e270), rde-1(ne219), cdh-6(tm306), him-5(e1490)*;

LGX: *qIs74[gfp::pop-1], B0410.2a(ok1142), jkk-1(km2), bar-1(ga80).*

Strains were obtained from the *C. elegans* Genetics Center (University of Minnesota), or from *C. elegans* Gene Knockout Consortium. *qIs74*, which contains *gfp::pop-1* (Siegfried et al., 2004), was used to observe POP-1 expression.

RNAi

RNAi was performed according to (Fire et al., 1998). dsRNA was synthesized using MEGAscript® (Ambion) using cDNA clones (provided by Dr. Yuji Kohara, NIG, Mishima, Japan) or genomic DNA templates. PCR primers used genomic templates for dsRNA synthesis are available upon request.

To bypass the RNAi maternal lethal effects of *pop-1, rho-1, dsh-2*, *hmr-1*, *hmp-2, lit-1*, *wrm-1*, *mlc-4*, *apr-1* and *sgg-1*, a zygotic RNAi scheme was used (Herman, 2001). Similarly, *rho-1* or *cyk-4* dsRNA was injected into *unc-42(e270) rde-1(ne219); mhIs9* to determine their effect on cytokinesis.

Expression constructs

A *lin-17::gfp* construct that fused *gfp* to the end of *lin-17* coding sequence, similar to the functional *Drosophila* Fz-GFP (Strutt, 2001), was constructed from three fragments: a 14,450 bp *Hind*III-*Kpn*I fragment from pSH6 (Sawa et al., 1996), a 4,373 bp *Kpn*I-*Hind*III fragment from pPD95.75 (a gift from A. Fire, Stanford University, CA), which includes *gfp*, and the last 705 bp coding sequence of *lin-17* cDNA amplified from yk1130b08.

gfp was amplified from pPD95.75 and inserted between the *mig-5* coding sequence and the *mig-5* stop codon to generate the *mig-5::gfp* construct with 5,520 bp upstream and

1,035 bp downstream regulatory sequences. The *mig-5* coding sequence (2,499 bp) and upstream sequence was amplified from genomic DNA T05C12, so was the downstream sequence.

The *lin-17::gfp* and *mig-5::gfp* constructs were microinjected at a concentration of 10 ng/µl and 15 ng/µl respectively, with the co-injection marker pPDMM0166 [*unc-119* (+)] at a concentration of 40 ng/µl, into *unc-119(e2498); him-5(e1490)* or *mig-5(ok280); unc-119(e2498); him-5(e1490)* hermaphrodites (Maduro and Pilgrim, 1995). Transgenic extrachromosomal arrays containing *lin-17::gfp* were integrated into the genome using a UV irradiation-based method (Mello et al., 1991) to generate *mhIs9*. The integrated array was backcrossed five times before phenotypic analysis.

Cell lineage and polarity analysis

Living animals were observed using Nomarski optics; cell nomenclature and cell lineage analysis were as previously described (Sulston and Horvitz, 1977). N.x refers to both daughters of cell N. Fates of the T and B cell descendants were determined by nuclear morphologies and size; orientation to the body axis (Herman and Horvitz, 1994) was used as an indicator of T and B cell polarities, as previously described (Herman et al., 1995). Phasmid dye-filling was also used as an indicator of T cell polarity (Herman and Horvitz, 1994).

Orientation of the spindle during the division of the B cell was determined using the rectum as a reference. Micrographs of the B.x nuclei were analyzed by measuring the angle formed between the rectum and a line that bisected the B.x nuclei.

Results

LIN-17/Fz is localized to the membranes of the B and T cells

mhIs9 males contain a *lin-17::gfp* construct that was expressed in the membranes of the T, B cells and their descendants as well as the F, P11, P12 and vuval precursor cells (Fig.1A-C, data not shown). *mhIs9* rescued the *lin-17* T and B cell polarity defects. Only 4% (n=54) of T cells and 7% (n=54) of B cells displayed polarity defects in *lin-17; mhIs9* animals, while 99% (n=70) of T cells and 79% (n=58) of B cells displayed polarity defects in *lin-17* animals. Only 2% (n=101) of *mhIs9* males showed a B cell polarity defect. Thus the *lin-17::gfp* construct was functional.

Neither AJM-1::GFP nor MH27 antibody staining (Mohler et al., 1998) visualized the B cell membrane (data not shown). However, as LIN-17::GFP appeared to be localized to the cell membrane, we used it to mark the membranes of the B cell and its descendants (data not shown).

POP-1/Tcf is involved in the regulation of B cell polarity and functions downstream of LIN-44/Wnt and LIN-17/Fz

The relative difference in B daughter cell nuclear size was used to determine the polarity of the B cell division. Wild type males exhibit normal polarity with B.a being larger than B.p, while *lin-44* males primarily display reversed polarity with B.p being larger than B.a and *lin-17* males display a loss of polarity with B.a and B.p being equal size. We examined the relative sizes of B daughters to determine that 32% (n=25) of *pop-1(q645)* and 21% (n=39) of *pop-1(q624)* males displayed a loss of B cell polarity (Table 1 and Fig. 2A). The penetrance of the B cell defect caused by these non-null *pop-1* alleles is comparable to the T cell defects of 40% and 19% for *pop-1(q645)* and *pop-1(q624)*, respectively (Siegfried and Kimble, 2002). The *pop-1(q624)* mutation alters a conserved amino acid in the HMG box DNA binding domain and causes many defects at low penetrance, as one expects of a typical partial loss-of-function allele. However, the *pop-*

1(q645) mutation alters a conserved amino acid in the ß-catenin binding domain and causes a highly penetrant gonad defect, but other defects at low penetrance, suggesting that it may alter residues specifically involved in hermaphrodite gonadogenesis (Siegfried and Kimble, 2002). The low penetrance of the *pop-1(q645)* B cell defect might also be explained by the observation that *C. elegans* ß-catenin homologs *bar-1* and *wrm-1* are not involved in the control of B cell polarity (see below).

We constructed a *lin-44 pop-1* double mutant to determine the functional order of POP-1 and LIN-44 in the regulation of B cell polarity. The B cell defect of *lin-44 pop-1* males is similar to that of *pop-1* males, and 51% displayed a loss of polarity (Table 2 and Fig. 2B). Thus, *pop-1*/*Tcf* functions downstream of *lin-44 /Wnt* in the control of B cell polarity, as it does in the control of T cell polarity and other Wnt signaling pathways (Lin et al., 1998; Herman, 2001; Herman and Wu, 2004).

To determine the effect of *pop-1* B cell polarity defects on B cell fate, we followed the B cell lineage of 18 *pop-1* males (Fig. 2F-H). While B.a was larger than B.p 25 minutes after the B cell division in all the wild-type males examined (n=7), in 6/18 *pop-1* males the B daughters were of equal size (loss of polarity) and in another 3/18 B.p was larger than B.a even one hour after the B cell division (polarity reversal). However the relative nuclear sizes of the B.a and B.p nuclei sometimes changed just before B.a or B.p divided. In the three *pop-1* males with reversed B cell polarities, the B.a and B.p nuclei become equal in size before they divided, the B.p cell divided with abnormal pattern and abnormal timing and the B.axx cells were abnormally oriented (Fig. 2F, G). In the six worms that displayed a loss of polarity, B.p divided earlier than in wild-type males (Fig. 2F, H). Although not severe, the B cell lineage defect in *pop-1* males suggests that the

B.p cell fate is abnormal. Consistent with this, 40% (n=55) of *pop-1* males had crumpled or shortened spicules. However, the B cell itself is likely to be normal in *pop-1* mutants as the pattern of B.a divisions was normal (Fig. 2G, H) and LIN-17::GFP is expressed in the B cell (data not shown).

POP-1 is asymmetrically distributed to anterior-posterior daughters of most asymmetric cell divisions during *C. elegans* development. At several cell divisions, the asymmetric distribution of POP-1 is controlled by Wnt signaling (Lin et al., 1998; reviewed by Herman and Wu, 2004). To examine whether POP-1 is asymmetrically localized to the nuclei of the B daughter cells, we used an integrated array, *qIs74*, that contains a *gfp::pop-1* construct (Siegfried et al., 2004). In *qIs74* males, GFP::POP-1 is asymmetrically distributed to the nuclei of the B.a and B.p cells (Fig. 2C), with the level of GFP::POP-1 being higher in the B.a nucleus than in the B.p nucleus (100%, n=27). In order to confirm that POP-1 asymmetric distribution to the B cell daughters is regulated by Wnt signaling, we examined GFP::POP-1 localization in *lin-44* and *lin-17* mutants. The levels of GFP::POP-1 in the nuclei of the B.a and B.p cells were equal in *lin-17; qIs74* males (82%, n=17) (Fig. 2D), but in *lin-44; qIs74* males, the level of GFP::POP-1 in B.p. nucleus was higher than that in B.a. nucleus (67%, n=33) (Fig. 2E). The regulation of GFP::POP-1 levels in the B cell daughters is similar to that of T cell daughters in which POP-1 was also regulated by *lin-44* and *lin-17* (Herman, 2001).

MIG-5/Dsh is expressed in the B cell and its descendants and is involved in the regulation of B cell polarity

Of the three *C. elegans* Dsh homologs, *mig-5* plays the larger role in the control of B cell polarity (Table 1, 3); 50% of *mig-5(ok280)* males segregating from *mig-5(ok280)/mIn1* mothers displayed a loss of B cell polarity and 5% (n=117) displayed a reversal of B cell polarity (Table 1 and Fig. 3A), while 85% (n=60) of *mig-5(ok280)* animals displayed normal T cell polarity. In addition, *mig-5 (RNAi)* increased the loss of B cell polarity of *lin-44* males from 12% to 48% (n=54) (Fig. 3B) and 70% (n=46) of *lin-44; mig-5(ok280)* males displayed loss of B cell polarity (Table 2), indicating that *lin-44* functions upstream of *mig-5*.

In order to determine whether MIG-5 is expressed in the B cell and its descendants, a *mig-5::gfp* construct was made. *mig-5* animals bearing an extrachromosal array containing *mig-5::gfp* can grow to adulthood, whereas non-array containing *mig-5* animals arrested as L1 or early L2 larvae, indicating that the construct is functional. Animals that contained *mig-5::gfp* expressed GFP in the B, QL cell and several cells in the nerve ring (Fig. 3C and data not shown). MIG-5::GFP was expressed strongly in the B cell and its descendants (Data not shown). *mig-5::gfp* also rescued the *mig-5(ok280)* B cell polarity defect; 85% (n=33) of *mig-5* animals that contained *mig-5::gfp* showed normal B cell polarity.

In order to determine whether *mig-5* functions upstream of *pop-1*, a *mig-5(ok280)/mIn1; qIs74* strain was constructed. Interestingly, the levels of GFP::POP-1 in the B cell and its descendants was dramatically reduced in *mig-5; qIs74* males, as well as in the Z1, Z4 and P11/P12 cells (data not shown). GFP::POP-1 was distributed equally to the B daughter nuclei in 31% (n=29) of *mig-5; qIs74* males in which the sizes of the B.a and B.p nuclei were equal, suggesting that MIG-5 functions upstream of POP-1.

However it is also possible that other Dsh orthologs function redundantly with MIG-5 to control B cell polarity and regulate the asymmetric distribution of POP-1 to B.a and B.p nuclei.

lit-1 mutants weakly affect B cell polarity, but _wrm-1_ mutants may not; and neither affect the asymmetric distribution of POP-1 to the B cell daughters

WRM-1 is one of three *C. elegans* β-catenin homologs (Natarajan et al., 2001) and functions with LIT-1/Nemo like kinase to control the polarities of the EMS blastomere and the Z1 and Z4 somatic gonad precursor cells. WRM-1 interacts with and activates LIT-1 kinase, which phosphorylates POP-1 and regulates its subcellular localization (Rocheleau et al., 1999; Maduro et al., 2002). *wrm-1* and *lit-1* mutations also affect the asymmetric distribution of POP-1 in the Z1 and Z4 cells (Siegfried et al., 2004) and *lit-1* mutations caused a loss of T cell polarity (Rocheleau et al., 1999). We used phasmid dye-filling (Herman and Horvitz, 1994) as well as nuclear morphologies of the T cell granddaughters (Herman and Horvitz, 1994) to assess the effectiveness of *lit-1(RNAi)* and *wrm-1(RNAi)* as well as *lit-1(or131ts)* and *wrm-1 (ne1982ts)* mutations. In each case T cell polarity was defective (Table 3). In addition, only 15% (n=34) of T cell daughter nuclei displayed the asymmetric distribution of GFP::POP-1 in *lit-1(ts); qIs74* animals and only 18% (n=28) of *wrm-1(RNAi)* animals (Fig. 4A-C).

Next, we investigated whether LIT-1 and WRM-1 were also involved in the regulation of B cell polarity and the asymmetric distribution of POP-1 to the B daughter cell nuclei. Disruption of *lit-1* function caused a minor B cell polarity defect, but disruption of *wrm-1* caused little or no defect (Table 3). Surprisingly, *lit-1* did not affect the asymmetric

distribution of GFP::POP-1 to the B.a and B.p cells. All of the *lit-1(RNAi); qIs74* males (n=26) and *lit-1(ts); qIs74* males (n=29) displayed a normal asymmetric distribution of POP-1 to B.a and B.p nuclei (Fig. 4D), even though two of the *lit-1(RNAi); qIs74* males and four of *lit-1(ts); qIs74* males showed a loss of B cell polarity. In addition, the asymmetric distribution of GFP::POP-1 to the B.a and B.p cell nuclei was normal in *wrm-1(RNAi)* (n=33) males (Fig. 4E). Thus, while *wrm-1* and *lit-1* play major roles in the control of T cell polarity and the asymmetric distribution of POP-1 to the T daughters, they played lesser roles in the control of B cell polarity and the asymmetric distribution of POP-1 to the B daughters. This suggests that the pathways that control T and B cell polarities are different.

Canonical Wnt signaling does not control T or B cell polarity

A canonical Wnt pathway has been shown to be involved in the migration of QL neuroblast descendants and other processes during *C. elegans* development (Korswagen et al., 2000), but not in the control of T cell polarity (Herman, 2001). As the pathways that control the polarities of B and T cells appeared to differ, we wanted to know whether other canonical Wnt signaling components might be involved in the control of B cell polarity. We checked B cell polarity defect of *dsh-2*, *bar-1* and *pry-1* mutants and found they did not affect B cell polarity (Table 3). Since either mutation or RNAi of *hmp-2* and *apr-1* caused embryonic lethality, we used zygotic RNAi to determine that they were not involved in the regulation of B cell polarity. This was also true for *sgg-1* (Table 3). Since zygotic RNAi has been shown to be effective for genes involved in T cell polarity (Herman, 2001), these negative RNAi results are likely to be informative. Also

mutations in *bar-1*, the major β-catenin in canonical Wnt signaling in *C. elegans* (Eisenmann, 2005), did not cause a B cell polarity defect, we conclude that the canonical Wnt pathway does not appear to control B or T cell polarity.

RHO-1/RhoA and LET-502/ROCK are involved in the control of B cell polarity and the asymmetric distribution of POP-1 to B cell daughters

RhoA and RhoA Associated Kinase (Rock) have been shown to be part of the PCP pathway that regulates vertebrate gastrulation and the actin cytoskeleton in *Drosophila* (Habas et al., 2001; Habas et al 2003; Winter et al., 2001). The *C. elegans* RhoA ortholog, *rho-1*, has been shown to be involved in cytokinesis (Jantsch-Plunger et al., 2000) and P cell migration (Spencer et al., 2001). Since *rho-1(RNAi)* caused embryonic lethality we used zygotic RNAi to determine that in 25% (n=68) of *rho-1(RNAi)* males, the size of B.a nucleus was equal to that of B.p (Fig. 5A), while in 47% of males, B.a was slightly larger than B.p, however the size difference was not as great as that observed in wild-type males (Fig. 5B). We also observed a cytokinesis defect in *rho-1(RNAi)* males. Using *lin-17::gfp* to visualize the B cell membrane, we observed two, three and ten B daughter nuclei within a common cytoplasm (Fig. 5C, D). Despite the lack of normal cytokinesis, 15/22 of *rho-1(RNAi)* B cells produced ten nuclei by the late L2 stage (as occurred in wild-type males), and 7/22 animals produced six or seven nuclei, suggesting that B cell fate is somewhat normal. However, the orientation of the B daughter nuclei was abnormal. In wild-type males the B.al/raa and B.al/rpp cells migrate to assume the Bα, Bβ, Bγ and Bδ fates, respectively (Sulston and Horvitz, 1977; Chamberlin and Sternberg, 1993). However these cell migrations did not occur in 12 of the 15 *rho-*

1(RNAi) males (Fig. 5D), possibly due to the secondary effect of cytokinesis. RHO-1 is also involved in spindle orientation during the B cell division. In wild-type males, the B cell spindle is orientated almost parallel to the rectum, with the angle between the spindle axis and the rectum being less than nine degrees (n=15), while in 82% of *rho-1(RNAi)* (n=70) males, the angle between the B cell spindle and the rectum varied from 10° to 45° (Fig. 5E-G).

We performed *rho-1* zygotic RNAi in a *lin-44* mutant background to determine that RHO-1 functions downstream of LIN-44 (Table 2 and Fig. 5H). In addition, GFP::POP-1 was symmetrically distributed to the B.a and B.p nuclei in all *rho-1 (RNAi)* males in which B.a was equal to or slightly larger than B.p (n=23) (Fig. 5A, B). That GFP::POP-1 was localized to the nuclei in *rho-1 (RNAi)* animals (Fig. 5A, B) indicated that *rho-1* does not affect the ability of POP-1 to localize to the nucleus.

The *C. elegans* Rock homolog, LET-502, has been shown to be involved in the *C. elegans* embryonic elongation (Wissmann et al., 1997) and P cell migration (Spencer et al., 2001) in a RHO-1 dependent manner. The *Drosophila* Rock homolog, Drok, links Frizzled-mediated PCP signaling to the actin cytoskeleton (Winter et al., 2001). In budding yeast, Pkc1p, which is structurally and functionally related to mammalian Rock, is localized to sites of polarized growth in a Rho1p dependent manner (Andrews and Stark, 2000). Thus Rock functions downstream of RhoA and is involved in the regulation of polarization in several systems. *let-502(RNAi)* animals had severe body morphology and cytokinesis defects as well as B cell polarity defects (Table 2 and Fig. 5I). *lin-44(n1792) let-502(RNAi)* males also displayed loss of B cell polarity (Fig. 5J, Tables 1,2), which indicated that LET-502/Rock functions downstream of LIN-44/Wnt. Like

rho-1(RNAi), let-502(RNAi) also caused GFP::POP-1 to be symmetrically distributed to the B.a and B.p cell nuclei (data not shown). *mlc-4/Sqh (RNAi)* males displayed a loss of B cell polarity, although at a lower penetrance (Table 1), and symmetric distribution of GFP::POP-1 to B.a and B.p nuclei (Figure 5L).

To determine whether the B cell polarity defect was secondary to the cytokinesis defect we observed in *rho-1(RNAi)* and *let-502(RNAi)* males, we examined the polarity and cytokinesis of the B cell division in *cyk-4(RNAi)* males. CYK-4 is a GTPase activating protein (GAP) and functions with RHO-1 in the completion of cytokinesis (Jantsch-Plunger et al., 2000). 55% (n=58) of *cyk-4(RNAi)* males displayed a B cell cytokinesis defect, while showed normal B cell polarity with B.a. nucleus being larger than the B.p nucleus (Fig 5K), suggesting that polarity was normal. However, 63% (n=32) of the *cyk-4(RNAi)* animals, that displayed cytokinesis defect, showed an equal distribution of GFP::POP-1 to the B daughter nuclei (Fig 5K). This suggests that while the cytokinesis defect caused by *cyk-4(RNAi)* does not affect the polarity of the B cell nuclear division, it may interfere with the differential nuclear distribution of the GFP::POP-1.

Other PCP components weakly affect B cell polarity

We identified *C. elegans* homologs of several conserved PCP signaling components. It was sometimes difficult to determine the *C. elegans* ortholog by sequence analysis alone, thus we investigated several candidate orthologs. In other cases, there did not appear to be a clear *C. elegans* homolog, thus we investigated the most similar *C. elegans* protein,

for example the *C. elegans* protein most similar to Dgo is ankyrin-related UNC-44 (Table 1). However in that case it is likely that UNC-44 is not an ortholog or homolog of Dgo.

Mutations of the other four PCP core genes, *B0496.8/Pk* (Gubb et al., 1999), *B0410.2a/Stbm*, *cdh-6/Fmi* and *unc-44*, each showed weak B cell polarity defects (Table 1). There does not appear to be a homolog of Four-jointed in the *C. elegans* genome. Dachsous (Ds) and Fat (Ft), two other components that function upstream of the six PCP core genes in *Drosophila*, are cadherin-like proteins and Ds is required for Wg-dependent pattern formation in the *Drosophila* wing disc. It was difficult to assign Ds and Ft orthologs in *C. elegans*, however, *hmr-1*, *cdh-1*, *cdh-3* and *cdh-4* are clear homologs, thus we investigated the role of each in the control of B cell polarity. *hmr-1(RNAi)*, *cdh-1, cdh-3* and *cdh-4(RNAi)* also showed weak B cell polarity defects (Table 1) and CDH-3 was expressed in the B cell (Chamberlin et al., 1999). Downstream of the PCP core components, the molecules that are responsible for polarity are tissue specific (Tree et al., 2002). In *Drosophila*, c-Jun N-terminal kinase (Jnk) and c-Jun kinase kinase (Jkk) function downstream of PCP core components to control ommatidal polarity. Mutations in the *C. elegans JNK* and *JKK* orthologs, *jnk-1* and *jkk-1,* did not cause a B cell polarity defect. We also tested a *C. elegans* homolog of the formin-homolog protein dishevelled associated activator of morphogenesis 1 (Daam1), reported to be involved in the regulation of gastrulation in *Xenopus* and to physically interact with both Dsh and RhoA (Habas et al., 2001). However, these *C. elegans* proteins are also similar to Diaphanous (Dia), which is involved in cell division in *Drosophila* (Castrillon and Wasserman, 1994) and vertebrate homologs function with RhoA to regulate cell polarity (reviewed by Fukata et al., 2003). RNAi of the two conserved *C. elegans* Daam1 homologs, Y48G9A.4

and F56E10.2, weakly affected B cell polarity. Finally, the *C. elegans* ortholog of Ca^{2+}/calmodulin-dependent protein kinase II (CamKII), which has been shown to be involved in the Wnt/Ca^{2+} pathway, *unc-43*, did not affect B cell polarity (Table 1).

Discussion

The Wnt pathway that regulates B cell polarity is different from the other known *C. elegans* Wnt pathways

Wnt signaling controls the polarities of several cell divisions during *C. elegans* development including the EMS, T, Z1, Z4, B, U and F cells. LIN-44/Wnt (Herman and Horvitz, 1994; Herman et al., 1995) and LIN-17/Fz (Sawa et al., 1996) are involved in the regulation of T and B cell polarities. The pathway that regulates T cell polarity also includes WRM-1, LIT-1 and POP-1, which is asymmetrically distributed to T cell daughters (Herman, 2001; Herman and Wu, 2004). Our results confirmed that mutations in *lit-1* and *wrm-1* caused defects in T cell polarity and the asymmetric distribution of GFP::POP-1 to the nuclei of T daughter cells. WRM-1 and LIT-1 are also involved in a branched Wnt/MAPK pathway that controls the polarity of the EMS division, with one branch controlling spindle orientation and the other specifying endodermal cell fates (Rocheleau et al., 1997; Thorpe et al., 1997; Schlesinger et al., 1999). In addition, a *C. elegans* Src kinase homolog, SRC-1, and MES-1 also function in the control of EMS polarity (Bei et al., 2002). All these pathways interact to control the nuclear levels of POP-1/Tcf in the EMS daughter nuclei and repress POP-1 function in the posterior E cell. Although WRM-1, LIT-1 and POP-1 all are involved in the control of T, Z1 and Z4 cell polarities, *pop-1* might play a positive role in specifying the posterior neural T.p and the

distal Z1.a and Z4.p cell fates (Herman, 2001; Herman and Wu, 2004; Siegfried et al., 2004), which is different from its role in the specification of posterior EMS fate. Interestingly, we observed that mutations in *lit-1* have a minor effect and *wrm-1* have little or no effect on either the asymmetry of the B cell division or the asymmetric distribution of GFP::POP-1. Thus, the pathway that controls B cell polarity is different from those that control EMS and T cell polarities. We also determined that mutations of components of canonical Wnt pathway, which regulates the migration of QL descendants (Korswagen et al., 2000), did not affect B cell polarity. This suggests that the Wnt pathway that regulates B cell polarity is different from the known *C. elegans* Wnt pathways.

Rho-1/RhoA and LET-502/Rock links Wnt/Fz signaling to the actin cytoskeleton to control B cell polarity

How Wnt/PCP signaling pathways relay information to the cytoskeleton and lead to cytoskeleton reorganization is still not clear. Recent findings suggest that the small GTPase RhoA may function as a critical link. RhoA and Rock are important regulators of cytoskeletal architecture. Expression of either the *Xenopus* Fz7, rat Fz1, or *Drosophila* Wnt1 activated RhoA in a Dsh-dependent, but β-catenin-independent manner in both human 293T cells and *Xenopus* embryos, suggesting that Wnt activation of RhoA may be a mechanism by which the cytoskeleton is regulated (Habas et al., 2001; Habas et al., 2003). During eye development in *Drosophila*, RhoA functions within Fz/PCP signaling to regulate transcription and ommatidial polarity (Strutt et al., 1997). In *Drosophila*, *Drok/Rock* mutations cause the formation of multiple hairs in one cell and ommatidial

orientation defects (Winter et al., 2001). Thus, RhoA might function downstream of Wnt/PCP to regulate the actin cytoskeleton in many systems. Homologs of RhoA and Rock also function in yeast to regulate polarization (Andrews and Stark, 2000). In *C. elegans*, Wnt signaling is involved in alignment of the mitotic spindle in the EMS cell along the anterior-posterior body axis, indicating that Wnt signaling can polarize the cytoskeleton (Thorpe et al., 2000). Thus Wnt signaling in *C. elegans* may function through RhoA and Rock to regulate aspects of cell polarity. We demonstrated that *rho-1*/*RhoA* and *let-502*/*Rock* affected the sizes of the B.a and B.p nuclei, the orientation of the spindle during the B cell division and function downstream of *lin-44*.

In addition to the loss of polarity, we also observed that *rho-1* and *let-502* mutations blocked cytokinesis at the B cell division. This raised the question as to whether the *rho-1* cytokinesis defect was the primary cause of the polarity defect. RHO-1 is required for cytokinesis in the first and/or second cell cycle as RHO-1 is likely to be the critical target for CYK-4, the RHO-1 GAP that functions in central spindle formation and cytokinesis (Jantsch-Plunger et al., 2000), and 90% of *rho-1(RNAi)* embryos displayed a cytokinesis defect. It was possible that the effect of *rho-1* on the relative sizes of the B daughter nuclei was nonspecific and that RHO-1 was either required for all the cell divisions that occur during *C. elegans* development or involved in cytokinesis of several cells, including the B cell, and the effect was non-specific. We do not believe this to be the case because both *cyk-4(RNAi)* and *rho-1(RNAi)* animals displayed B cell cytokinesis defects, but *cyk-4(RNAi)* males still display an asymmetric nuclear division. Also, inactivation of RhoA GTPase disrupted the formation of cortical actin structures and the contractile ring, but not chromosomal separation or nuclear envelope reformation (Kishi et al., 1993;

O'Connell et al., 1999; Jantsch-Plunger et al., 2000), suggesting that the symmetric nuclear division in *rho-1(RNAi)* mutants occurred before the cytokinesis defect. Thus, although both CYK-4 and RHO-1 are involved in cytokinesis, RHO-1 may be also involved in the asymmetric B cell nuclear division. In addition, *rho-1(RNAi)* animals only displayed weak T cell polarity defects, demonstrating the difference between the control of B and T cell polarities and suggesting that the *rho-1* B cell polarity defect is specific.

A PCP-like pathway might regulate B cell polarity

Disrupting the functions of *C. elegans* homologs of *Drosophila* PCP genes caused defects of B cell polarity. Although it was not possible to identify *C. elegans* orthologs in all cases, such as Ft, Ds and Pk, several components had clear orthologs, such as RhoA, Rock, Fmi and Stbm. The PCP pathway functions to control the polarities of epithelial cells that lie in a tissue. Are there any analogous tissues in *C. elegans*? We think that there might be. LIN-44/Wnt is expressed in the tail hypodermal cells, and regulates cell polarities of the T, B, U and F cells in the *C. elegans* male tail. These are adjacent ectodermal blast cells that have an epithelial character. For an animal that has only about one thousand cells, these cells might comprise a kind of epithelia sheet. Killing the B cell with a laser microbeam caused polarity defects in the F and U cells, indicating that F and U cells polarities are dependent upon the B cell (Herman and Horvitz, 1994). The behavior is somewhat reminiscent of the directional nonautonomous effect caused by *Frizzled* mutant clones in *Drosohpila* (Strutt and Strutt, 2002). Thus it is possible that the pathway that regulates B cell polarity could be similar to the *Drosophila* PCP pathway. Different components function downstream of the six core PCP genes in the *Drosophila*

wing and eye. In the eye, Jnk and Jkk mediate ommatidial polarity, whereas RhoA and Drok/Rock function in the wing hair cells. We did not observe a B cell polarity defect when we interfered with the functions of *jnk-1* and *jkk-1*, but did, when interfered with the functions of *rho-1/RhoA*, *let-502/Rock* and *mlc-4/Sqh*. This suggests that the pathway that regulates B cell polarity is more similar to the PCP pathway that regulates polarity in the *Drosophila* wing.

Although there appear to be many differences between the pathway that regulates B cell polarity and the pathway that regulates *Drosophila* wing cell polarity, much is conserved. Based upon our results and the PCP pathway that regulates *Drosophila* wing hair polarity, we propose a model for a PCP-like pathway that might function to regulate B cell polarity (Fig. 6). A major difference was that mutation or RNAi of the *C. elegans* homologs of PCP core proteins Pk, Dgo, Stbm and Fmi only cause a minor B cell polarity defect. Similarly, homologs of the two global proteins Ds and Fat only play a minor role, suggesting that these proteins might function redundantly in the control of B cell polarity. However, determination of the degree of this potential redundancy is beyond the scope of this study. Another difference is the involvement of LIN-44/Wnt in B cell polarity, whereas no Wnt has been shown to be included in Drosophila PCP. Some of our results are based on RNAi experiments and whether null mutants might have higher penetrance is unknown. However, based upon results from mutants alone, it is clear that *lin-44/Wnt, lin-17/Fz, mig-5/Dsh, rho-1/RhoA* and *let-502/Drok* play large roles; Ds or Fat homologs *cdh-3* and *cdh-4*, *cdh-6/Fmi* and *tag-15/Pk* play lesser roles; and *B04102.a/Stbm, jnk-1/Jnk* and *jkk-1/Jkk* do not appear to be involved in the control of B cell polarity. It is also possible that the cadherin-like proteins Ds and Fat and trans-membrane proteins Fmi

and Stbm function nonspecifically and are involved in many cell-cell interactions. We also cannot exclude the possibility that all the homologs may not be orthologs.

It is possible that multiple Wnt pathways may function downstream of the LIN-44 signal to control B cell polarity, for example, the WRM-1/LIT-1 pathway that controls the polarities of the EMS and T cells may function redundantly with PCP components to control B cell polarity (Fig 6).

Function of POP-1 in the control of B cell polarity

There are at least three aspects of *pop-1* function during the B cell division that need to be explained: first, the relative sizes of the B cell daughters are equalized in *pop-1* mutants, which we interpret as a loss of B cell polarity; second, *pop-1* mutations cause a small, but significant B.p cell fate defect, which can also be explained by a loss of B cell polarity; third, the asymmetric distribution of POP-1 to the B daughter nuclei is controlled by LIN-44 and LIN-17, although the roles of RHO-1 and LET-502 are less clear. Let us consider each of these in turn.

It is curious that POP-1, a member of the TCF/LEF transcription factor family, is involved in the control of cell size during an asymmetric cell division. The difference in relative sizes of the B cell daughters is apparent during and immediately after the B cell division. Thus the processes that function to control the cell size difference must function prior to the completion of cytokinesis and separation of the B cell daughters. These processes are controlled by *lin-44*, as mutations in *lin-44* cause reversals of the relative sizes of the B daughters. We have shown that *lin-17*, *mig-5, rho-1, let-502* and even *pop-1* function downstream of *lin-44* in this process. The speed of the

establishment of B daughter cells of different sizes suggests that the process occurs without new gene transcription. How can a transcription factor control such an early event? One possibility is that *pop-1* mutations cause a change in B cell fate prior to its division, such that it often does not undergo an asymmetric division and cannot respond to *lin-44*. However, our data suggest that B cell fate is normal in *pop-1* mutants: LIN-17::GFP is expressed in the membrane of the B cell and the B.a divides with a normal pattern. While it is possible that the B cell could be transformed to another *lin-17*-expressing cell in *pop-1* animals, the lack of effect on B.a fate suggests that B cell fate is fairly normal. It is likely that the establishment of cells of different sizes involves regulation of cytoskeletal elements, including the asymmetric positioning of the mitotic spindle during the B cell division, perhaps by small GTPases of the Rac or Rho family. POP-1 might interact with some of these cytoskeletal regulators, such as RHO-1 and LET-502. In support of this idea, Esufali and Bapat (2004) recently demonstrated that Rac1 GTPase binds to ß-catenin and TCF and affected the intracellular distribution of ß-catenin, leading to changes in target gene expression. Perhaps interaction of POP-1 with RHO-1, LET-502 and other cytoskeletal regulators is required for the asymmetric B cell division. Further experimentation beyond the scope of this study will be required to investigate these interactions and possible mechanisms.

The role that POP-1 plays in the control of B cell fate and how this relates to the control of cell polarity is also unclear. Our cell lineage analysis of *pop-1* males with a loss of B cell polarity showed no obvious defects in B.a cell fate and minimal, but clear, defects in B.p cell fate. In addition, we observed crumpled spicules consistent with cell fate defects among the B cell descendants. The effect of *pop-1* mutations on fates of the B cell

descendants could be caused by defects in B cell polarity. One possibility is that *pop-1* plays a minor role in the control of B cell polarity and subsequent fates of the B cell descendants and may function in parallel with another pathway, such as the PCP pathway (Fig. 6).

The asymmetric distribution of POP-1 to the B daughter nuclei is controlled by *lin-44* and *lin-17*. Recent work has shown that the asymmetric distribution of POP-1 to nuclei in the early embryo is controlled by differential nuclear export mediated by the 14-3-3 protein PAR-5 and nuclear exportin homolog IMB-4/CRM-1 (Lo et al., 2004; Nakamura et al., 2005). Furthermore, LIT-1 modification of POP-1 was shown to be required for its asymmetric nuclear distribution (Lo et al., 2004). Along with LIT-1, WRM-1, whose function is also required for asymmetric nuclear distribution of POP-1, was also localized differentially, with higher levels in the posterior E cell nucleus (Lo et al., 2004; Nakamura et al., 2005). WRM-1 was also localized to the anterior cortex of the anterior MS cell in a process that required MOM-5/Fz. Nakamura et al. (2005) proposed that Wnt and Src signaling leads to the phosphorylation and retention of WRM-1 in the posterior E nucleus, where it phosphorylates POP-1 in a LIT-1 dependent manner. Thus asymmetric nuclear retention of WRM-1 appears to drive the control of cell polarity during the EMS division. A similar process may also occur during the T cell division and other larval cell divisions (Takeshita and Sawa, 2005). In this work, we have shown that neither *lit-1* nor *wrm-1* mutations affected the asymmetric nuclear accumulation of POP-1 in the B cell daughters (Fig. 4). This suggests that another mechanism may function to control asymmetric POP-1 nuclear accumulation in the B cell daughters. We have also shown that *rho-1* and *let-502* function specifically in the control of B cell polarity. However,

their role in the asymmetric nuclear accumulation of POP-1 is less clear. While POP-1 was symmetrically distributed to the B daughter nuclei in 72% of *rho-1(RNAi)* males, cytokinesis was also blocked, so that the nuclei were in a common cytoplasm. Thus, we cannot rule out the possibility that the effect of *rho-1* and *let-502* on POP-1 distribution is a secondary consequence of the cytokinesis defect. However, POP-1 was symmetrically distributed to the B daughter nuclei in only 35% of *cyk-4* males, the despite the cytokinesis defect. This leaves open the possibility that RHO-1 and LET-502 might also function in the asymmetric nuclear localization of POP-1 in a process distinct from that which functions at the EMS and T cell divisions. Further experimentation will be required to elucidate this potential mechanism. However, it should be noted that after the 28-cell stage, POP-1 nuclear asymmetry becomes independent of Wnt/MAPK signaling, yet still requires MOM-5/Fz (Park and Priess, 2003). The mechanism that regulates these POP-1 nuclear asymmetries has not been elucidated, however it has been suggested that it may be similar to PCP signaling (Herman and Wu, 2004; Lo et al., 2004) and it may be this sort of mechanism that functions in the B cell.

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Table 1. B cell polarity defects

Wnt component[a]	*Genotype*	Relative nuclear sizes of B.a and B.p (%)			
		N	Ba>Bp (Normal)	Ba=Bp (Loss)	Ba<Bp (Reversed)
None	*wild-type*	many	100	0	0
Wnt	*lin-44(n1792)*	49	16	12	71
Fz	*lin-17(n671)*	58	21	69	10
Dsh	*mig-5(ok280)*	117	45	50	5
	mig-5(RNAi)	107	76	23	1
	mig-5(ok280)dsh-1(RNAi)	24	42	50	8
	mig-5(ok280)dsh-1(RNAi)	32	37	50	13
	dsh-1dsh-2mig-5(zygotic)[b]	35	61	29	0
Tcf	*pop-1(q645)*	25	64	32	4
	pop-1(q624)	39	77	21	2
Nlk	*lit-1(zygotic RNAi)*	57	91	9	0
	lit-1(or131) at 25ºC	86	79	21	0
Ds/Fat[c]	*hmr-1*	49	84	16	0
	cdh-1(RNAi)	69	97	3	0
	cdh-3 (pk77)	45	89	11	0
	cdh-3 (pk87)	42	86	14	0
	cdh-4 (RNAi)	78	87	13	0
Stbm	*B0410.2a*	62	81	19	0
Fmi	*rrf-3; cdh-6 (RNAi)*	40	87	13	0
	cdh-6(tm306)	39	93	7	0
Pk	*B0496.8 (RNAi)*	88	93	7	0
	rrf-3; ZK381.5(RNAi)	66	83	17	0
	tag-15 (gk106)	45	80	20	0
	tag-15 (gk210)	60	88	12	0
RhoA	*rho-1 (zygotic RNAi)*	68	28	72	0
Rock	*let-502*	50	48	52	0
	let-502; rrf-3(RNAi)	32	30	70	0
Daam1/Dia[c]	*Y48G9A.4*	55	85	15	0
	rrf-3; F56E10.2 (RNAi)	41	87	13	0
	cyk-1	32	94	6	0
	mlc-4(or253)	64	72	28	0
Jnk	*jnk-1(gk7)*	52	94	4	2
Jkk	*jkk-1(km2)*	40	97	3	0
CamKII	*unc-43(n498)*	50	92	8	0
	unc-43(n498n1179)	30	97	3	0
	unc-43(n498n1186)	45	93	7	0

The relative sizes of daughter nuclei of B cell division, B.a and B.p, of late L1 or early L2 stage males were scored using Nomarski microscopy. N, number of males scored. B.a>B.p, the B.a nuclear size was larger than that of B.p; B.a=B.p, the nuclear size of B.a was the same as that of B.p; B.a<B.p, the nuclear size of B.a was smaller than that of B.p. [a] Wnt components: homolog in *Drosophila* or vertebrate systems. [b] dsRNA of the three were mixed and injected to wt or *rde-1* mutants, and the wt progeny displayed severe embryonic lethality (84%, n=108). [c] It was difficult to tell the difference between the homologs of Daam1 and Dia or Ds and Fat in *C. elegans*, so their homologs are listed together. In some cases, it was difficult to identify *C. elegans* orthologs; so several potential homologs were tested. The genes tested showed three levels of involvement based upon the penetrance of their defects. Genes that showed a penetrance of 30% or greater were defined as playing major roles in the control of B cell polarity, genes with a penetrance of between 10 and 30% as playing minor roles and genes with less than 10% were considered to play little or no role.

Table 2. Pathway analysis

Components	Genotype	Relative nuclear sizes of B.a and B.p (%)			
		N	Ba>Bp (Normal)	Ba=Bp (Loss)	Ba<Bp (Reversed)
wt	*wild-type*	Many	100	0	0
Wnt	*lin-44(n1792)*	49	16	12	71
Fz	*lin-17(n671)*	58	21	69	10
	lin-17lin-44	48	17	77	6
Dsh	*mig-5(ok280)*	117	45	50	5
	lin-44;mig-5	46	13	70	17
RhoA	*rho-1 (zygotic RNAi)*	68	28	72	0
	lin-44 rho-1 (zygotic RNAi)	40	27	68	5
Drok	*let-502(RNAi);rrf-3*	32	30	70	0
	lin-44; let-502(RNAi)	27	26	70	4
Tcf	*pop-1(q645)*	25	64	32	4
	pop-1lin-44	47	11	51	39

The relative sizes of daughter nuclei of B cell division, B.a and B.p, of late L1 or early L2 stage males were scored as in Table 1.

Table 3. Canonical Wnt pathway components are not involved in the control of B cell polairy

Genotype	T cell defects (%)		Relative nuclear sizes of B.a and B.p cells (%)			
	N	phasmids filled with dye (%)	N	Ba>Bp (Normal)	Ba=Bp (Loss)	Ba<Bp (Reversed)
wild-type	many	100	many	100	0	0
wrm-1(zygotic RNAi)	114	38	115	95	5	0
wrm-1(ne1982ts) at 25ºC	60	3	48	92	8	0
lit-1(zygotic RNAi)	78	12	57	91	9	0
lit-1(or131) at 25ºC	88	45	86	79	21	0
dsh-1(RNAi)	136	100	37	97	3	0
dsh-2	ND	ND	59	90	10	0
bar-1(ga80)	114	87	46	93	7	0
hmp-2(zygotic RNAi)	ND	ND	51	92	8	0
pry-1(mu38)	192	99	45	91	9	0
sgg-1(zygotic RNAi)	114	100	48	94	6	0
apr-1(zygotic RNAi)	86	90	56	96	4	0

Phasmid dye-filling was used as an indicator of normal T cell polarity (Herman and Horvitz, 1994). There is one phasmid on each side of the animal. The relative sizes of daughter nuclei of B cell division, B.a and B.p, were scored as in Table 1. ND, not determined.

Figure 1 LIN-17::GFP is expressed in and localized to the membranes of the B and T cells and their descendants.

Anterior is left and ventral is down, bars are 10 micrometers in all subsequent figures. Fluorescence is shown above and corresponding DIC images below. LIN-17::GFP is expressed in the membrane (arrows) of the (A) wild-type B cell, (B) wild-type B.a and B.p cells and (C) wild-type T cell descendants.

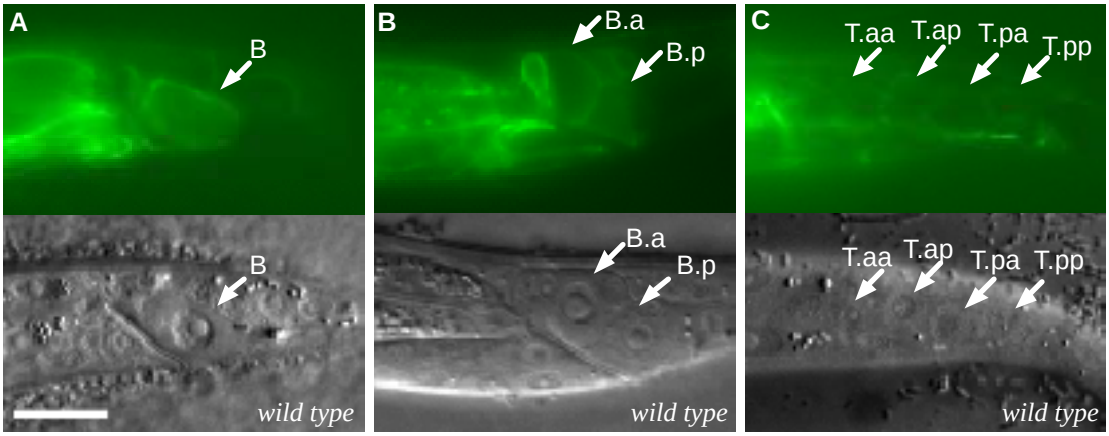


Figure 2 POP-1 is involved in the control of B cell polarity and its asymmetric distribution to the B.a and B.p cell nuclei is controlled by *lin-44* and *lin-17*.

(A) The nuclear size of B.a can be equal to B.p in *pop-1* mutant males and (B) in *lin-44(n1792) pop-1(q645)*. Panels (C-E) show fluorescence above and corresponding DIC images below. Asymmetric distribution of GFP::POP-1 to the B.a and B.p nuclei (arrows) is normal in (C) wild-type, lost in (D) *lin-17* and reversed in (E) lin-44 males. Wild-type B cell lineage (F). *pop-1* mutant B cell lineages observed in 3/18 (G) and 6/18 males (H).

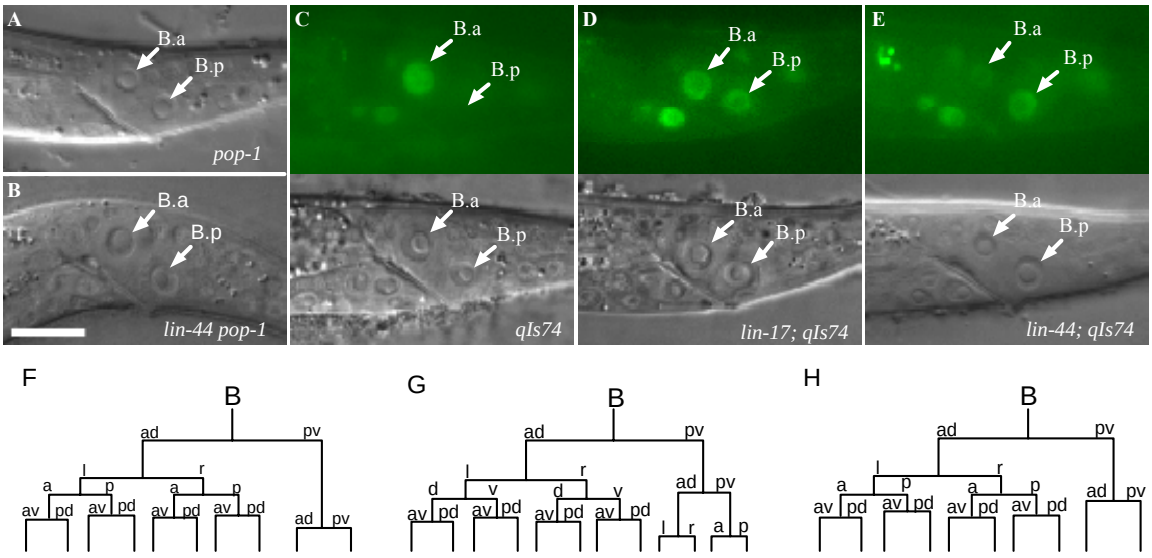


Figure 3 MIG-5 is involved in the control of B cell polarity, functions downstream of LIN-44 and is expressed in the B cell and its descendants.

In *mig-5(ok280)* males (A) and *lin-44(n1792); mig-5(RNAi)* males (B) the size of the B.a nucleus can be equal to the B.p nucleus. (C) mig-5::gfp is expressed in the B cell in a punctate pattern.

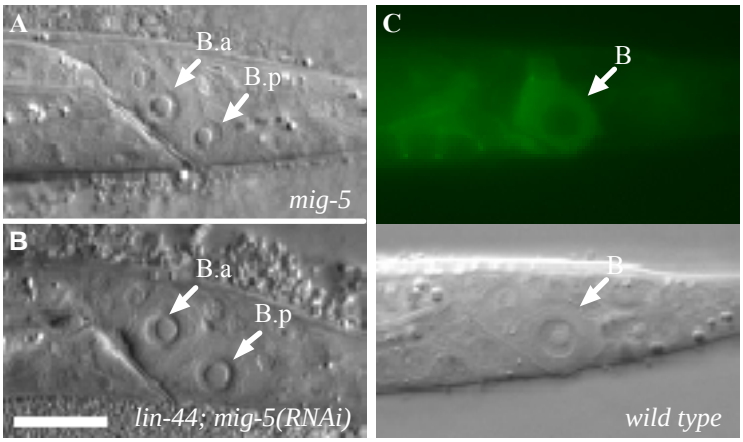


Figure 4 *lit-1* and *wrm-1* play different roles in the regulation of T and B cell polarities.

(A) POP-1 is asymmetrically distributed to the T.a and T.p nuclei in *qIs74* males (arrows). (B) POP-1 was distributed equally to the T.a and T.p nuclei in *qIs74; lit-1(RNAi)* (arrows). (C) POP-1 was distributed equally to the T.a and T.p nuclei in *qIs74; wrm-1(RNAi)* (arrows). POP-1 was distributed asymmetrically to the B.a and B.p nuclei in *qIs74; lit-1(RNAi)* (D) and *qIs74; wrm-1(RNAi)* males (E) and the polarity of the division was normal (arrows).

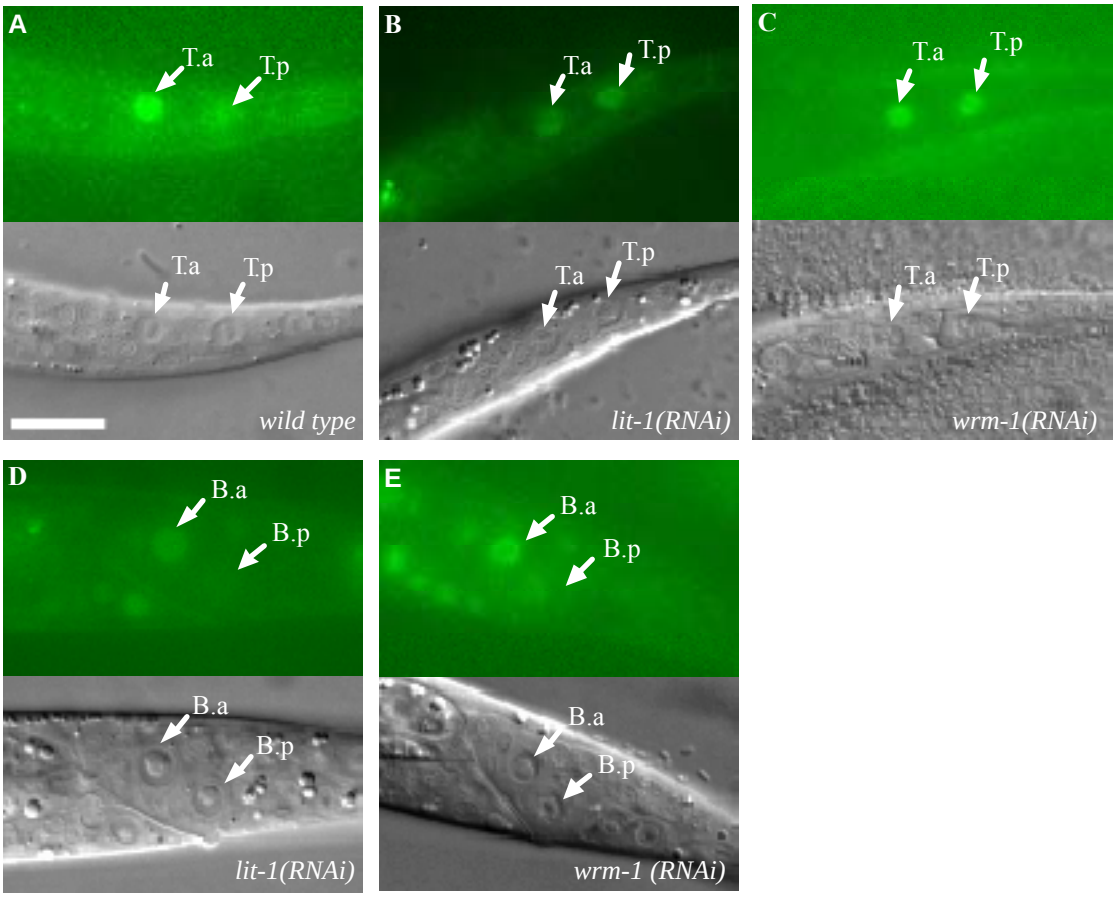


Figure 5 RHO-1 and LET-502 function after LIN-44 in the control of B cell polarity and affect the asymmetric distribution of POP-1 to the daughter nuclei.

Panels (A-D) and (K, L) show the fluorescent image above and corresponding DIC images below. (A, B) The size of B.a is equal to (A) or slightly larger (B) than B.p nuclei in *rho-1(RNAi)* males and GFP::POP-1 is equally distributed to each nucleus. (C, D) *rho-1(RNAi); mhIs9* males, (C) B.a and B.p nuclei or (D) ten descendant nuclei are contained within a common membrane. White line in lower panels indicates B cell membrane traced from *lin-17::gfp* localization in upper panel. (E) The spindle axis of B cell division (white dashed line) is almost parallel to the rectum (white dashed line) in wild-type males; the angle between the spindle axis and the rectum is less than 9° (n=15). (F) The angle between the B cell division spindle and the rectum is 30° in *rho-1(RNAi)* males. (G) Summary of spindle orientation defects in *rho-1(RNAi)* males (n=70) and wild-type worms (n=15). (H) *lin-44(n1792); rho-1(RNAi)* male displayed a loss of B cell polarity. (I) *let-502(RNAi); rrf-3* male displayed cytokinesis and B cell polarity defects at the B cell division. (J) The B cell defect of *lin-44 let-502(RNAi)* male is similar to *let-502(RNAi)* alone. (K) *cyk-4(RNAi)* male in which cytokinesis has failed at the B cell division, yet the B.a nucleus is larger than the B.p nucleus. (L) *mlc-4(zygotic RNAi)* male displayed an symmetric distribution of GFP::POP-1 to the B.a and B.p nuclei and B cell polarity defect.

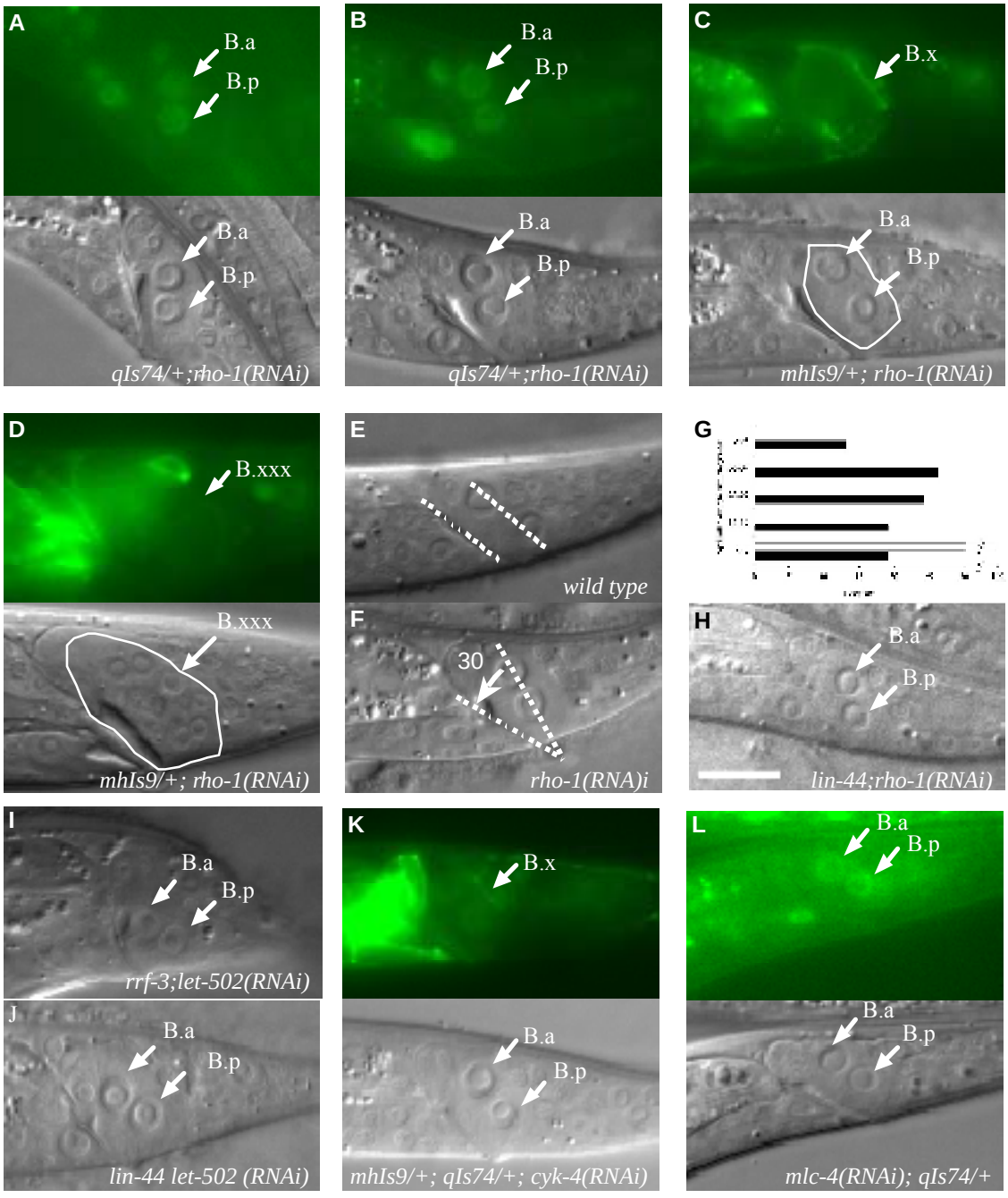
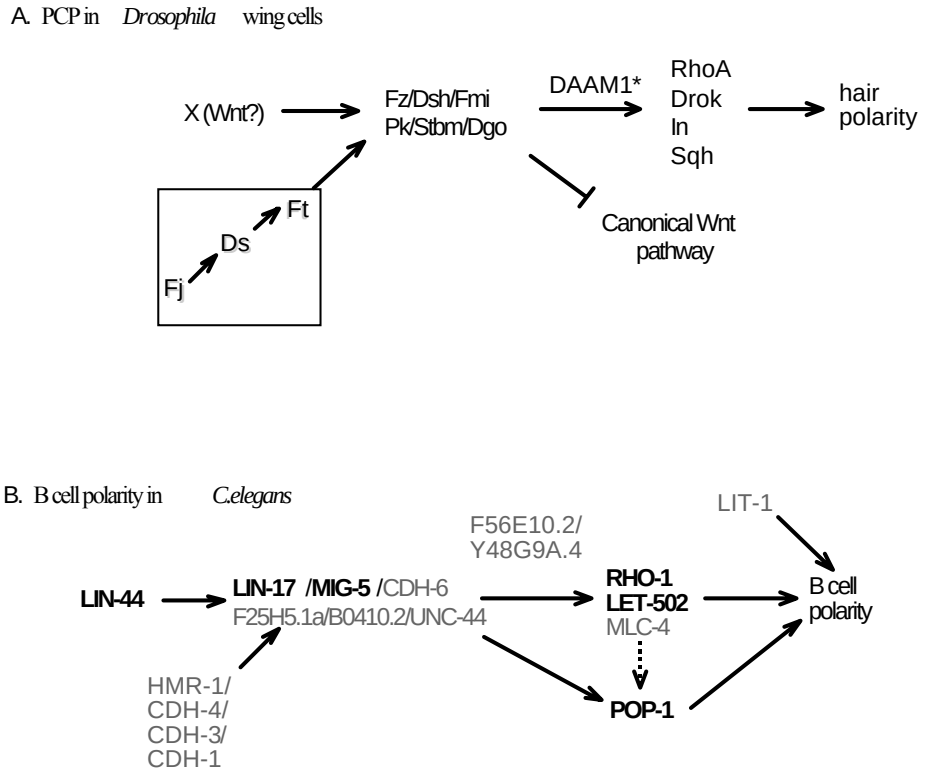

A
B.a
B.p
B.a
B.p
qIs74/+;rho-1(RNAi)
B
B.a
B.p
B.a
B.p
qIs74/+;rho-1(RNAi)
C
B.x
B.a
B.p
mhIs9/+; rho-1(RNAi)
D
B.xxx
B.xxx
mhIs9/+; rho-1(RNAi)
E
wild type
F
30
rho-1(RNA)i
G
H
B.a
B.p
lin-44;rho-1(RNAi)
I
B.a
B.p
rrf-3;let-502(RNAi)
J
B.a
B.p
lin-44 let-502 (RNAi)
K
B.x
B.a
B.p
mhIs9/+; qIs74/+; cyk-4(RNAi)
L
B.a
B.p
B.a
B.p
mlc-4(RNAi); qIs74/+

Figure 6 A PCP-like pathway might regulate B cell polarity.

(A). PCP pathway that regulates wing hair polarity modified from Tree et al. (Tree et al., 2002). *The function of *Drosophila* DAAM1 is unclear. (B). A PCP-like pathway might regulate B cell asymmetric division in *C. elegans*. Black: components that play a major role in the control of B cell polarity; Gray: components that play a minor role. Pathway arrangement is based on the *Drosophila* PCP pathway shown in A. Except for *cdh-3*, *cdh-4* and *hmr-1*, each gene functions after *lin-44*. The functional order among the genes downstream of *lin-44* was not able to be determined as they have a similar phenotype. However *lin-44*, *lin-17*, *mig-5*, *rho-1* and *mlc-4* affected POP-1 asymmetric localization, thus might function upstream of *pop-1* (dashed line).



Chapter 4
Asymmetric localizations of LIN-17/Fz and MIG-5/Dsh are involved in the asymmetric B cell division

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Summary

The polarities of several cells that divide asymmetrically during *C. elegans* development are controlled by Wnt signaling. LIN-44/Wnt and LIN-17/Fz control the polarities of cells in the tail of developing *C. elegans* larvae, including the male-specific blast cell, B, which divides asymmetrically to generate a larger anterior daughter and a smaller posterior daughter. In our previous work, we determined that a non-canonical Wnt pathway, which was different from other Wnt pathways in *C. elegans*, but similar to planar cell polarity (PCP) pathways, appeared to regulate B cell polarity. We are interested in the molecular mechanisms that regulate B cell asymmetric cell division. In *Drosophila*, asymmetric localization of the six core PCP proteins is required for PCP signaling. To determine whether LIN-17/Fz and MIG-5/Dsh are asymmetrically localized during the B cell division, we constructed functional *lin-17::gfp* and *mig-5::gfp* fusion constructs (Chapter 3). We determined that LIN-17::GFP was asymmetrically localized to the B and B.p cell cortex and was asymmetrically distributed to the daughters during division. Asymmetric distribution of LIN-17::GFP correlated with B cell polarity. The seven-transmembrane domain and KTXXXW motif, but not the CRD domain, were required for LIN-17 asymmetric distribution, although each of these domains was required for LIN-17 to rescue the *lin-17* mutants. MIG-5::GFP was also asymmetrically localized to the B cell prior to and after B cell division in a LIN-17-dependent manner. In addition, MIG-5 appeared to be asymmetrically distributed to the B cell daughters, with the B.a cell having a higher level of MIG-5 than the B.p cell. We examined the functions of the MIG-5 domains. The DEP domain was required for MIG-5 membrane association,

while the PDZ and DIX domains were responsible for asymmetric distribution of MIG-5 to the B daughters. Functionally, the DEP and PDZ domains were required to rescue B cell polarity defects of *mig-5* males, while the DIX domain was not that important. In summary, a novel PCP-like pathway, in which LIN-17 and MIG-5 are asymmetrically localized, is involved in the regulation of B cell polarity.

Key words: LIN-17, MIG-5, asymmetric localization, CRD domain, seven-transmembrane domains.

Introduction

Wnt signaling pathways regulate diverse developmental processes (Cadigan and Nusse, 1997; Nelson and Nusse, 2004; Veeman, Axelrod and Moon, 2003; Reya and Clevers, 2005). In the canonical Wnt pathway, signaling is initiated by the interaction of the secreted Wnt glycoprotein and extracellular domains of both the Frizzled/Fz receptor (Bhanot and Brink, et al, 1996) and Low-density lipoprotein receptor-related protein (LRP) 5 and 6/LRP5 and LRP6 in mammals or Arrow in *Drosophila* (Cong, Schweizer and Varmus, 2004a; Mao and Wang, et al, 2001; Pinson and Brennan, et al, 2000; Tamai and Semenov, et al, 2000; Wehrli and Dougan, et al, 2000). Ryk, a receptor protein tyrosine kinase (Halford and Stacker, 2001), was shown to be another Wnt co-receptor and is also involved in the interaction between Wnt and Fz, but how Ryk physically interacts with Wnt and other receptors is still not clear (He, 2004; Inoue and Oz, et al, 2004; Lu and Yamamoto, et al, 2004). After signal initiation, Dishevelled/Dsh is phosphorylated, possibly in a LRP5 and LRP6 independent manner (Gonzalez-Sancho and Brennan, et al, 2004), and is translocated to the membrane where it binds to Fz

(Rothbacher and Laurent, et al, 2000). Upon signal initiation, LRP6 is also phosphorylated, providing a docking site for Axin. Axin then binds to phosphorylated Dsh and LRP6, which causes the separation of Axin from the β-catenin destruction complex and disruption of that complex (He and Semenov, et al, 2004; Tamai and Zeng, et al, 2004). β-catenin translocates to nucleus, as its level elevated due to the disruption of the destruction, and activates the expression of target gene.

In the non-canonical planar cell polarity (PCP) pathway, β-catenin is not involved. The PCP pathway is conserved from *Drosophila* to vertebrates and regulates orientation of body surface hairs and cell migrations (Klein and Mlodzik, 2005; Veeman, Axelrod and Moon, 2003). Unlike mammals, in which Wnt ligands are involved in the PCP pathway to regulate cell movements during the gastrulation (Heisenberg and Tada, et al, 2000; Tada, Concha and Heisenberg, 2002; Weidinger and Moon, 2003), a Wnt ligand for the PCP pathway in *Drosophila* has not been identified. Instead the proto-cadherins Dachsous (Ds), four jointed (fj) and Fat might cooperate to the regulation of Fz signaling to control polarity in the eye and the wing discs (Ma and Yang, et al, 2003; Rawls, Guinto and Wolff, 2002; Simon, 2004; Yang, Axelrod and Simon, 2002). When PCP signaling is initiated, the uniformly localized PCP core proteins are transported to the proximal or distal cortex of an individual cell. Fz and Dsh accumulate at the distal cortex of the cell, and Stbm and Pk accumulate at the proximal cortex, while Fmi and Dgo accumulate at both ends (Adler, 2002; Mlodzik, 2002; Strutt, 2003). Of the six PCP core genes, Fz and Dsh are the major components, and the other four are required for the correct asymmetric localization and activation of Fz and Dsh (Das and Jenny, et al, 2004; Jenny and Darken, et al, 2003; Rawls and Wolff, 2003; Tree and Shulman, et al, 2002;

Tree, Ma and Axelrod, 2002). In *C. elegans*, asymmetric localization of MOM-5/Dsh and DSH-2 have been reported during embryogenesis (Hawkins and Ellis, et al, 2005; Park, Tenlen and Priess, 2004a), which might indicate that a PCP-like pathway is also conserved in *C. elegans*. Progress on the study of the PCP pathway has been made (Klein and Mlodzik, 2005), but the molecular mechanisms of how each core protein is asymmetrically localized, especially Fz and Dsh, during PCP signaling transduction has not been completely worked out.

Structural and functional analysis of Fz is essential to understand the mechanism of how Wnt ligands or proto-cadherins signal through Fz receptors to activate the cytoplasmic protein Dsh. Such analysis will lead to an understanding of how Fz and Dsh are asymmetrically localized within the epithelial cell sheet. Each Fz receptor family member has an extracellular cysteine-rich domain (CRD), seven putative transmembrane domains with three extracellular and three intracellular loops and a carboxyl cytoplasmic domain (Bhanot and Brink, et al, 1996). The CRD usually has 10 cysteine residues, and can physically interact with Wnt ligand in cell culture assays (Hsieh and Rattner, et al, 1999), which was also confirmed by mutational analysis (Dann and Hsieh, et al, 2001). The affinity between CRD and Wingless determines Fz involvement in the canonical or PCP pathway (Rulifson, Wu and Nusse, 2000). Over-expression of the CRD causes it to dimerize which is sufficient to activate Wnt/β-catenin signaling (Carron and Pascal, et al, 2003). However, an in vivo test shows that the CRDs of DFz and DFz2 are not essential for Wingless transduction (Chen and Strapps, et al, 2004), which indicates that the seven-transmembrane domain might be important for signal transduction. It is also possible that the N-terminus of the CRD deleted Fz and Fz2 still possessed the ability to capture Wnt

to initiate signaling (Povelones and Nusse, 2005). Mutational analysis indicated that several residues in the Fz loops also affected the Wnt/β-catenin signaling initiation (Cong, Schweizer and Varmus, 2004b). The length and similarity of the cytoplasmic domains among Fz members varies, but there is a conserved motif Lys-Thr-X-X-X-Typ (KTXXXW) located two amino acids after the seventh transmembrane domain in most Fz receptors (Umbhauer and Djiane, et al, 2000). The KTXXXW motif is required for canonical signal transduction and also membrane localization and phosphorylation of Disheveled (Dsh) (Umbhauer and Djiane, et al, 2000). Dsh contains three conserved domains: PDZ, DIX and DEP. The PDZ domain was shown to bind the KTXXXW motif directly (Wong and Bourdelas, et al, 2003), however, the PDZ domain was not required for Dsh membrane localization, instead the DEP domain was required (Rothbacher and Laurent, et al, 2000). The KTXXXW motif might interact with Dsh to regulate the canonical Wnt pathway (Boutros and Mlodzik, 1999; Li and Yuan, et al, 1999) and function in PCP to recruit Dsh to the plasma membrane.

We showed that LIN-44, LIN-17, MIG-5, RHO-1, LET-502 and POP-1 played large roles in regulation of the B cell polarity, while other PCP homologs played minor roles. This indicated that a PCP-like pathway regulated B cell polarity (Chapter 3). The insolubility of Wnt ligands limits the utility of biochemical assays to study the interaction between Wnt and Fz in mammals. Therefore, we have taken advantage of the powerful genetic and molecular tools in *C. elegans* to study the functions of LIN-17/Fz and the interaction between LIN-44/Wnt and LIN-17/Fz in the B cell. Interestingly, we found that the functions of the CRD domain in signal transduction varied. The CRD domain was not responsible for the asymmetric distribution of Fz. Instead, the seven-transmembrane

domain and the KTXXXW motif of LIN-17 were required for its function and asymmetric distribution. Functions of different domains of MIG-5 were conserved, as the DEP domain was required for MIG-5 membrane localization and the PDZ and DEP domains were required to regulate B cell polarity.

Materials and methods:

General methods and strains

Nematodes were cultured and manipulated by standard techniques (Brenner, 1974). N2 was used as the wild-type strain. The following mutations and transgenic lines were used:

Linkage Group I (LGI): *lin-17(n671)* and *lin-44(n1792)*; LGII: *mig-5(ok280), mhIs009* (Chapter 3); LGIII: *unc-119(e2498)*; LGV: *him-5(e1490)*.

Strains were obtained from the *C. elegans* Genetics Center (University of Minnesota), or from *C. elegans* Gene Knockout Consortium.

The transgenic lines and their corresponding constructs are listed in the table 1.

***mig-5* expression constructs**

A functional *mig-5::gfp* construct, with 5520bp upstream regulatory sequence and 1035 bp downstream regulatory, was made (Chapter 3). In order to analyze the MIG-5 promoter, constructs with five different length of promoter sequences were generated: *4.6KBPromig-5::gfp* has a 4.6 kb promoter, *3.6KBPromig-5::gfp* has a 3.6 kb promoter, *2.6KBPromig-5::gfp* has a 2.6 kb promoter, *1.9KBPromig-5::gfp* has a 1.9 kb promoter

and *1.2KBPromig-5::gfp* has a 1.2 kb promoter. The construction of *1.2KBPromig-5::gfp* was described previously (Chapter 3).

All the other four promoters were amplified by PCR using *mig-5::gfp* as template. The antisense primer was shared by four constructs and the sequence was: TCACCGGTAAAAGAACTACTGGACAAGGC and all the four sense primers were tagged with a *Spe*I site and the sequences for the specific construct were *4.65KBPromig-5::gfp*: ATACTAGTGACCGACGTCTAGAAGAGCAGAGTGTCTAGAAG, *3.6KBPromig-5::gfp:* ATACTAGTCTCGAATAGTTTGGCGAAGGGCAATGTAGC, *2.6KBPromig-5::gfp*: ATACTAGTCGCGATTTCACATCACTGGGCGGCGATGAG, *1.9KBPromig-5::gfp:* ATACTAGTGCTTGCCAAAACCTTCGGAATGATGAGAAGC.

The ability to rescue lethality of *mig-5* mutants of each construct was investigated by injecting each construct at concentration of 5 ng/µl, with the co-injection marker *rol-6* (+) at a concentration of 100 ng/µl, into *mig-5(ok280); him-5(e1490)*.

In order to investigate the function of each of the MIG-5 domains, we made three constructs with different domain deletion. *ΔDEPmig-5::gfp* encodes a truncated MIG-5 protein, in which GFP was fused to the carboxyl terminus of the first 357 amino acids of MIG-5, deleting the DEP domain. Specifically, we used *3.6KBPromig-5::gfp* (MIG-5::GFP) to amplify a fragment that excluded the MIG-5 DEP domain. The primers used were: MIG-5DDPL: CGGATCGGCTGCGCAAATCCCAGAATGG and MIG-5DDPR: ATGCTAGCGTTTGCTCCTGTGGATCCAAACCCAGAGC, which was tagged with a *Nhe*I site. This fragment was cloned into pCR2.1 TOPO (Invitrogen), subsequently digested by *Nhe*I and *Hpa*I and ligated to *Nhe*I-*Hpa*I digested *3.6KBPromig-5::gfp*.

ΔPDZmig-5::gfp encodes a truncated MIG-5 protein, in which the PDZ domain, consisting of amino acid residues 219 to 318, was deleted and GFP was fused to the carboxyl terminus of the truncated MIG-5 protein. Specifically, primers were designed to amplify a fragment that excluded the PDZ domain by using *3.6KBPromig-5::gfp* as template. The *Nhe*I-*Hpa*I fragment in pCR2.1 TOPO vector was used to replace the NheI-HpaI fragment in *3.6KBPromig-5::gfp*. The primers used were: MIG-5PDPL: ATGTTAACCCACTCGCGTCCATGGCGTCTGAAACGATG, which was tagged with a *Hpa*I site and MIG-5PDPR: GTTTTCCGTATGTTGCATCACCTTCACCCTC.

ΔDIXmig-5::gfp encodes a truncated MIG-5 protein, in which the DIX domain, consisting of amino acid residues from 11 to 101 of MIG-5, was deleted and the GFP was fused to the carboxyl terminus of the truncated MIG-5 protein. Specifically, we designed primers to amplify a fragment that excluded the DIX domain using *3.6KBPromig-5::gfp* as template. The *Pst*I and *Hpa*I fragment from the pCR2.1 TOPO vector was used to replace the *Pst*I and *Hpa*I fragment in *3.6KBPromig-5::gfp*. The primers used were: MIG-5DIXDP5': ACTGCAGCGGAACAATGACGCGACCACAGAGAACTGCTC, which was tagged with a *Pst*I site and MIGMIG-5DIXDP3': TCCAGAATTCGAGGGAGGCCGCTGTTA.

LIN-17 expression constructs:

In order to analyze the functions of CRD, four different constructs that encode different lengths of the cystine rich domain (CRD) of LIN-17 were made: *CRDΔ19-204lin-17::gfp*, *CRDΔ40-204lin-17::gfp*, *CRDΔ40-174lin-17::gfp* and *CRDΔ50-174lin-17::gfp*.

lin-17::gfp has the full length of CRD domain (Chapter 3). The following is the amino acid sequence of the CRD with the residue numbers indicated. (The signal peptide is bold and the cys residues are bold and underlined.)

19 40 50

MMHSLGIILLFIPLATGSIFDQAVKGK**C**IPIDIEL**C**KDLPYNYTYFPNTILHNDQH TLQTHTEHFKPLMKTK**C**HPHIHFFI**C**SVFAPM**C**PIGMPQAVTS**C**KSV**C**EQVKAD **C**FSILEEFGI

174

GWPEPLN**C**AQFPDPPEL**C**MKPTEDEITGGFSAPRLPTKGSSSSSSKPTG**C**PSDLVD VDPH

204

DPKSH**C**AFA**C**QSNVMFSTDNKRMVR

The sequences of the four truncated CRDs were:

CRDΔ19-204LIN-17::GFP: **MMHSLGIILLFIPLATGS**TR-Δ19-204R
CRDΔ40-204LIN-17::GFP:**MMHSLGIILLFIPLATGS**IFDQAVKGK**C**IPIDIEL**C**KDLTR-Δ40-204R
CRDΔ40-174LIN-17::GFP:**MMHSLGIILLFIPLATGS**IFDQAVKGK**C**IPIDIEL**C**KDLTR-Δ40-174VDVDPH DPKSH**C**AFA**C**QSNVMFSTDNKRMVR
CRDΔ50-174LIN-17::GFP:**MMHSLGIILLFIPLATGS**IFDQAVKGK**C**IPIDIEL**C**KDLPYNYTYFPNTTR-Δ50-174VDVDPHDPKSH**C**AFA**C**QSNVMFSTDNKRMVR.

Specifically, *CRDΔ19-204lin-17::gfp* contains three fragments: MluI-SphI fragment (1,725 bp), which was amplified by PCR using *lin-17::gfp* as template with the sense primer tagged with a *Mlu*I site, and *Sph*I-*EcoR*V fragment, which came from LIN-17::GFP and *EcoR*V-*Mlu*I fragment (6,964 bp), which was amplified using *lin-17::gfp* as template with the antisense primer tagged with a *Mlu*I site. The primers for *Mlu*I-*Sph*I fragment were LIN-17CRDP15':

AACGCGTCGTTCATGGTCAATTTGGTTTGCCGCCGC and

LIN-17CRDP13': CGTGGCCAGCGACGACAATCCCCAGGC.

Primers for *EcoR*V-*Mlu*I were LIN-17CRDP25': GGCTGAAATCACTCACAACGATGGATACGC and LIN-17CRDP23': AACGCGTGGCGAGTGGAATAAATAGTAGAATGATGCC.

CRDΔ40-204lin-17::gfp is similar to *CRDΔ19-204lin-17::gfp*, except the *EcoR*V-*Mlu*I fragment (7443bp) is longer. Primers for *EcoR*V-*Mlu*I are LIN-17CRDP25': GGCTGAAATCACTCACAACGATGGATACGC and LIN-17CRDP23'2: ACGCGTCAGGTCTTTGCATAGCTCAATGTCGATCGG.

CRDΔ40-174lin-17::gfp is similar to *CRDΔ40-204lin-17::gfp*, except the *Mlu*I-*Sph*I fragment (2848bp) is longer. The primers for *Mlu*I-*Sph*I fragment were LIN-17CRDP15'2: ACGCGTGTCGATGTGGATCCACACGATCCCAAGTCG and LIN-17CRDP13': CGTGGCCAGCGACGACAATCCCCAGGC.

CRDΔ50-174lin-17::gfp is similar to *CRDΔ40-174lin-17::gfp*, except the *EcoR*V-*Mlu*I fragment (7473bp) is longer. Primers for *EcoR*V-*Mlu*I were LIN-17CRDP25': GGCTGAAATCACTCACAACGATGGATACGC and LIN-7CRDP233: ACGCGTGGTATTCGGGAAATATGTGTAGTTGTAGGG.

In order to investigate the function of the KTXXXW motif, we performed site-directed mutagenesis (Stratagene) to change K497 to M or R, T498 to A or S, W502 to G singly or in various combinations to generate constructs K/M*lin-17::gfp* or K/R*lin-17::gfp*, T/A*lin-17::gfp*, T/S*lin-17::gfp*, W/G*lin-17::gfp* or KTW/MAG*lin-17::gfp*. H499 was changed to A to generate H/A*lin-17::gfp* as a control. A/B means residue A is replaced by B.

The primers for these mutations were:

LIN-17K/ML: gatgtgggtgttgtcggcgatgaccgtccacgcctgg,

LIN-17K/MR: ccaggcgtggacggtcatcgccgacaacacccacatc,

LIN-17T/AL: gtgggtgttgtcggcgaaggccgtccacgcctggaag,

LIN-17T/AR: cttccaggcgtggacggccttcgccgacaacacccac,

LIN-17W/GL: cggcgaagaccgtccacgccgggaagaacttcatcttctg,

LIN-17W/GR: cagaagatgaagttcttcccggcgtggacggtcttcgccg,

LIN-17KT/MAL: ctgatgtgggtgttgtcggcgatggccgtccacgcctggaagaac,

LIN-17KT/MAR: gttcttccaggcgtggacggccatcgccgacaacacccacatcag,

LIN-17KTW/MAGL: gggtgttgtcggcgatggccgtccacgccgggaagaacttcatcttctg,

LIN-17KTW/MAGR: cagaagatgaagttcttcccggcgtggacggccatcgccgacaacaccc,

LIN-17KW/MGL: gatgtgggtgttgtcggcgatgaccgtccacgccggg,

LIN-17KW/MGR: cccggcgtggacggtcatcgccgacaacacccacatc,

LIN-17TW/AGL: gtgggtgttgtcggcgaaggccgtccacgccgggaag,

LIN-17TW/AGR: cttcccggcgtggacggccttcgccgacaacacccac,

LIN-17K/RL: gatgtgggtgttgtcggcgaGgaccgtccacgcctgg,

LIN-17K/RR: ccaggcgtggacggtcCtcgccgacaacacccacatc,

LIN-17T/SL: gtgggtgttgtcggcgaagTccgtccacgcctggaag,

LIN-17T/SR: cttccaggcgtggacggActtcgccgacaacacccac,

LIN-17H/AL: gttgtcggcgaagaccgtcGCcgcctggaagaacttcatc,

LIN-17H/AR: gatgaagttcttccaggcggcgacggtcttcgccgacaac.

In order to investigate the function of the CRD and the seven-transmembrane domain of LIN-17, we made the construct *1TMDlin-17::gfp*, in which the transmembrane domain of DAF-16 replaced the seven-transmembrane domain of LIN-17. *1TMDlin-17::gfp* contains four fragments: *Eco*RV-*Nhe*I (7,664 bp), *Nhe*I-*Mlu*1 (4,072 bp), *Mlu*I-*Pst*I (115

bp) and *Pst*I-*EcoR*V (4,872 bp). The *EcoR*V-*Nhe*I (7,664 bp) fragment came from *lin-17::gfp*. The *Nhe*I-*Mlu*1 (4,072 bp) was PCR amplified using *lin-17::gfp* as template and the primers were 1TMDLIN-17::GFPL2: GGGTGATTTGCATAGAGGAATGG and 1TMDLIN-17::GFPR2: ATACGCGTACGTACCATACGCTGTAATTAATAGTAATGATCGC with the antisense primer tagged with a *Mlu*I site. The *Mlu*I-*Pst*I (115 bp) fragment came from a synthesized single strand DNA fragment. The sequence of the synthesized fragment was CTGACGCGTTCATCCACGTGGCTGATTCTTACTATCCTCGCCCTGCTCACGTT CATCGTGCTCCTGGGCATTGCAATTTTCCTCACCAGAAAATCATGGGAGGCG AAATTCGACCTGCAGTCG (123 bp), which contained a *Mlu*I site at 5' and a *Pst*I site at 3'. The single strand DNA was used as template to generate double stranded DNA by PCR using primers DAF-1TML: CTGACGCGTTCATCCACGTGGC and DAF-1TMR: CGACTGCAGGTCGAATTTCGCC. A 4,872 bp *Pst*I-*EcoR*V fragment was PCR amplified using *lin-17::gfp* as template and the primers are 1TMDLIN-17::GFPL: TCGCTGCAGTCGGCGAAGACCGTCCACGCCTGGAAG and 1TMDLIN-17::GFPR: CCTGAGTGAGACATGGATTGAATGG with the sense primer tagged with *Pst*I site. Finally, the four fragments were ligated together to generate *1TMD lin-17::gfp*.

In order to examine the function of the carboxyl terminal domain (CTD) of LIN-17, several constructs with different lengths of CTDs encoding sequences were made. *KTWlin-17::gfp* encodes a GFP fused LIN-17 protein with the deletion of the CTD except 11 residues right after the seventh transmembrane domain. Specifically, the *Sph*I-*Not*I fragment (694 bp) in *lin-17::gfp* was replaced by a new *Sph*I-*Not*I fragment (532 bp) amplified from *lin-17::gfp* to generate *KTWlin-17::gfp*. The primers were LIN-

17CDNA5': CAGTACAGCGGCTGCCCTTTGGTGGTTAAT and LIN-17CTD3': GCGGCCGCCGTTCTTCCAGGCGTGGACGGTCTTCGCCGAC with the sense primer tagged with a *Not*I site.

ΔCTDlin-17::gfp is similar to *KTWlin-17::gfp* except for the additional deletion of residues KTVHAWKNAA. The construct was made similar to *KTWlin-17::gfp* and both constructs used the same sense primer, while the antisense primer of *ΔCTDlin-17::gfp* is LIN-17CTD3'3: GCGGCCGCTCGCCGACAACACCCACATCAGACACGAGAATC. *ΔKTWlin-17::gfp* encodes a mutated LIN-17::GFP with the KTXHAW replaced by SGR residues. The primers were KTWLIN-17::GFPL: TATGCGGCCGCAAGAACTTCATCTTCTGTGGAATGTGCTCG and KTWLIN-17::GFPR: CATATGGCGGCCGCCGACGACCTTACT with the sense primer tagged with a *Not*I site.

The alignment of CTDs in these truncated LIN-17::GFP was shown below.

KTWLIN-17::GFP: SAKTVHAWKNAA::GFP

ΔCTDLIN-17::GFP: SASGR::GFP

ΔKTWLIN-17::GFP:

ASGRKNFIFCGMCSSAPVKNPIEPSTRPLLEPPTAPPPQPPVYMQMA

TNPQNSWRPSKVV::GFP

LIN-17::GFP:

SAKTVHAWKNFIFCGMCSSAPVKNPIEPSTRPLLEPPTAPPPQPPVYMQM

ATNPQNSWRPSKVV::GFP

Functional analysis of constructs

All the *lin-17::gfp* and *mig-5::gfp* constructs described above were microinjected at a concentration of 10 ng/µl and 5 ng/µl respectively, with the co-injection marker pPDMM0166 [*unc-119* (+)] (Maduro and Pilgrim, 1995) at a concentration of 40 ng/µl, into *unc-119(e2498); him-5(e1490)* or *mig-5(ok280); unc-119(e2498); him-5(e1490)* hermaphrodites.

Microscopy and cell lineage analysis

Living animals were observed using Nomarski optics or a zeiss laser scanning confocal microscope; cell nomenclature and cell lineage analysis were as previously described (Sulston and Horvitz, 1977). N.x. refers to both daughters of cell N. Fates of the T and B cell descendants were determined by nuclear morphologies and size; orientation to the body axis (Herman and Horvitz, 1994) was used as an indicator of T and B cell polarities, as previously described (Herman et al., 1995). Phasmid dye-filling was also used as an indicator of T cell polarity (Herman and Horvitz, 1994). Spatial and time course expression pattern of LIN-17 were examined by using the Zeiss laser scanning confocal microscope.

Results

LIN-17/Fz is asymmetrically localized prior to and after the B cell division

In the pupal wing of *Drosophila*, the asymmetric localization of Fz and Dsh to the distal cell cortex is essential to initiate wing hair formation (Strutt, 2001). In *Drosophila* eye development, asymmetric localization of Frizzled activity correlates with the bias of Notch activity between R3/R4 with the cell having higher Fz receptor activity taking on

the R3 cell fate (Strutt and Johnson, et al, 2002). As B cell polarity might be controlled by a PCP-like pathway (Chapter 3), we asked whether LIN-17/Fz and MIG-5/Dsh were also asymmetrically localized to the B cell and its daughters. There are three Fz receptors in *C. elegans*. LIN-17/Fz is involved in the regulation of cell polarities, cell migration and cell fate specification (Herman, 2002). The *lin-17(n671)* mutation terminates the coding sequence of the receptor before the seventh transmembrane domain (Sawa, Lobel and Horvitz, 1996). Although this allele truncates LIN-17/Fz 347 amino acids downstream of the initiator ATG, it functions as a genetic null, as 89% of *lin-17(n671)* males displayed B cell polarity defect (Herman and Horvitz, 1994), which we confirmed. We made an expression construct in which the GFP coding sequence was inserted just before the stop codon of the *lin-17(n671)* mutation, and six out of seven transgenic lines did not display detectable GFP expression, while only one out of seven lines displayed weak GFP expression in the cytoplasm and nucleus, which could not rescue *lin-17(n671)* (data not shown). So, we used *lin-17(n671)* as the genetic background to examine the rescuing ability of all the *lin-17::gfp* constructs.

LIN-17::GFP was localized to the B cell membrane and rescued *lin-17*, thus it was functional (Chapter 3). As B.a and B.p cell take different cell fates, we ask whether there is different distribution of LIN-17 to the two daughters during division and whether the asymmetric distribution of LIN-17 contributes to the different cell fates. We investigated the expression pattern of LIN-17::GFP during the B cell division. Before division, LIN-17::GFP accumulated in the anterior cortex and cytoplasm of the B cell in 81% of the *lin-17::gfp; lin-17; unc-119* males (n=31) (Fig. 1A) and the posterior membrane and cytoplasm displayed a lower level of GFP. After division, LIN-17::GFP was not

asymmetrically distributed to the two daughters, instead, was asymmetrically localized to the B.p cell in 76% of males, with the posterior membrane and cytoplasm of B.p cell displaying a lower level of LIN-17::GFP than the anterior of B.p (n=33) (Fig. 1B). LIN-17::GFP was also asymmetrically localized to the posterior cortex of the T cell prior to division in 45% of animals (n=34), with the posterior cortex showing a higher level of GFP than the anterior (Fig 1C). The asymmetric localization of LIN-17::GFP to the anterior cortex and cytoplasm of B and B.p cells was confirmed by a Z series of sections of confocal fluorescence images (Fig. 1D and data not shown).

Asymmetric localization of LIN-17/Fz is MIG-5/Dsh and LIN-44/Wnt dependent

In the *Drosophila* pupal wing epithelial cells, the six PCP core proteins are asymmetrically localized within individual cell in a codependent manner (Strutt, 2003; Strutt, 2002). In the pathway that controls B cell polarity, homologs of Stbm, Pk, Fmi and Dgo played minor roles, while *lin-17/Fz* and *mig-5/Dsh* played major roles (Chapter 3). We examined the localization of LIN-17::GFP in *mig-5*, and as expected, LIN-17::GFP asymmetric localization was lost in 81% of *mig-5* males (n=19) before cell division (Fig. 2A and B) and in 80% of *mig-5* males (n=31) after division (Fig 2D and E). In contrast to the pathways that control the *Drosophila* wing hair and eye polarities, a Wnt ligand, LIN-44, is involved in the control of B cell asymmetric division (Herman and Horvitz, 1994; Herman and Vassilieva, et al, 1995). Surprisingly, LIN-17::GFP asymmetric localization was affected in *lin-44* mutants. 73% of *lin-17::gfp; lin-17lin-44; unc-119* males displayed symmetric localization of LIN-17::GFP before the B cell division (Fig. 2A and C). After division, 19% of males displayed normal asymmetric localization, and 23% displayed

reversed asymmetric distribution (n=52) with the B.p cell membrane showing a higher level of LIN-17::GFP than B.a (Fig. 2D, F) and the other 58% displayed symmetric localization and distribution (Fig. 2D,G). In order to examine the correlation between B cell asymmetric division and LIN-17::GFP asymmetric localization, we examined the expression pattern of LIN-17::GFP during the B cell divisions in 16 *lin-17::gfp; lin-17; unc-119* males and six *lin-17::gfp; lin-17 lin-44; unc-119* males. Interestingly, we did not find any difference between the two genotypes until five hours after hatching, as there was no LIN-17::GFP expression in the posterior membrane of the B cell in either genotypes (Fig. 2H1 and data not shown), but there was accumulation of GFP in the anterior cytoplasm and membrane of B cell. Although there was strong GFP expression in the anterior membrane of the B cell, neighboring cells also possessed strong LIN-17::GFP expression, so we could not determine whether LIN-17::GFP was asymmetrically localized to the anterior membrane of B cell or there was no expression in the B cell membrane at that stage (Fig. 2H1). After five hours of post embryonic development, in all the *lin-17::gfp; lin-17* (n=16) and two of six *lin-17::gfp; lin-17 lin-44* males, LIN-17::GFP expression extended from the anterior B cell membrane to the posterior membrane slowly, but there was a clear gap with no GFP expression to the posterior membrane of B cell and lower level of GFP in the posterior cytoplasm than the anterior (Fig. 2H2). As these worms grew, this gap shrank and when the B cell was ready to divide, there was no gap, but the posterior membrane of the B cell still showed weaker LIN-17::GFP than the other parts of the B cell membranes (Fig 2H3). During the B cell division, LIN-17::GFP still displayed asymmetric localization with the posterior membrane weaker than anterior, and right after membrane separation, LIN-17::GFP was

asymmetrically distributed to B daughter cells, with B.a cell membrane having a higher level of LIN-17::GFP than B.p (data not shown), but there was no asymmetric localization in B.a or B.p cell membrane. In less than twenty minutes, LIN-17::GFP accumulates to the anterior membrane and cytoplasm of the B.p cell, while the posterior portion decreased in the B.p cell (Fig. 2H4), which results in the asymmetric localization of LIN-17::GFP to B.p cell, while obvious expression pattern change in B.a cell was not observed. The asymmetric localization of LIN-17::GFP in the B.p cell was maintained until B.a cell is ready to divide. Expression pattern of LIN-17::GFP in *lin-44* males is different. In four of six *lin-17::gfp; lin-17 lin-44* males, after 5 hours of post embryonic development, LIN-17::GFP was uniformly expressed to the posterior membrane of the B cell weakly. As the worms grew, LIN-17::GFP expression became stronger, but there was no asymmetric localization to the B cell membrane. During and after division, clear asymmetric distribution to B daughter cells was not observed, even when the polarity was reversed (n=4). In summary, prior to division, LIN-17::GFP was initially asymmetrically localized such that the posterior membrane of B cell had a lower level of LIN-17::GFP than anterior membrane. Immediately after division, LIN-17::GFP was uniformly distributed within the daughter cell membranes, but the B.a cell membrane had a higher level than did the B.p membrane. However, the weaker but uniform expression of LIN-17::GFP in B.p cell membrane soon became asymmetric as the posterior B.p membrane had a lower level. The asymmetric localization of LIN-17::GFP in B.p cell is not constant and when the B.a cell is ready to divide, LIN-17::GFP displayed symmetric distribution to B.a and B.p cells. The asymmetric localization of LIN-17::GFP prior to the B cell division might be required for LIN-17::GFP asymmetric distribution to the B daughters,

which correlated with B cell polarity. The loss or reversed asymmetric distribution of LIN-17::GFP in *lin-44* males may indicate that LIN-44 binding to LIN-17/Fz is required for the asymmetric distribution.

Cysteine rich domain (CRD) of LIN-17/Fz plays a role in signaling transduction but not in LIN-17::GFP asymmetric distribution.

Like the other members of the Fz family, LIN-17 has a CRD, but while most of other Fz members have ten cysteine residues, LIN-17 has 13. How the disulphide bonds are formed among these cysteine residues and how the CRD of LIN-17 is folded are unknown. The CRD domain physically interacts with Wnt proteins in cell culture assays (Hsieh and Rattner, et al, 1999) and the affinity of the Wnt-CRD interaction may correlate with the signal strength (Rulifson, Wu and Nusse, 2000; Wu and Nusse, 2002). It seems that the CRD is essential for signal transduction (Cong, Schweizer and Varmus, 2004; He and Semenov, et al, 2004; Strapps and Tomlinson, 2001). However CRD-deleted DFz could rescue *Fz* mutant flies, which indicated that the CRD is not absolutely required for signal transduction (Chen and Strapps, et al, 2004). Although several soluble Wnts are now available (Willert and Brown, et al, 2003), it is still difficult to produce other soluble Wnts, which has limited studies of the interaction between specific Wnts and Fzs. In *C. elegans*, powerful genetic and molecular tools enable us to investigate the interaction between LIN-44 and LIN-17. We are interested in whether the CRD of LIN-17 is required for signal transduction and asymmetric localization of LIN-17. Since LIN-44/WNT was required for LIN-17::GFP asymmetric localization, we hypothesized that the CRD of LIN-17 was required to interact with LIN-44 to transduce signals and to

cause the asymmetric localization of LIN-17::GFP. We made four constructs with different extents of CRD deletions (Table 2 and Fig. 3). Although all four truncated proteins contained the signal peptide at amino terminus, all displayed partial cytoplasm localization. We divided the extent of membrane localization into four categories: type I, in which the membrane localization is similar to wild-type, but there was partial localization to the cytoplasm (Fig 3A); type II, in which the membrane outlined by LIN-17::GFP can be roughly seen (Fig 3B); and type III, in which the majority of LIN-17::GFP is localized to the cytoplasm and the plasma membrane could not clearly be seen (Fig 3C) and type IV, in which LIN-17::GFP expression in B cell is weak and could not be seen. Worms that displayed type I, II, III membrane localization were considered as having membrane localization, while worms that display type IV membrane localization were considered not to have membrane localization.

The majority of CRDΔ40-174LIN-17::GFP and CRDΔ50-174LIN-17::GFP membrane localizations were type I (Fig 3A) and small percentage of worms display type II (Fig 3C), while the majority of CRDΔ19-204LIN-17::GFP and CRDΔ40-204LIN-17::GFP membrane localizations were type II (Fig 3B) and small percentage of worms displayed type III (Fig 3C). Comparing the function of the four proteins, CRDΔ19-204LIN-17::GFP, with no cysteine residues in the extracellular domain (ED) and CRDΔ40-204LIN-17::GFP, with two cysteine residues in the ED, could not rescue the T or B cell polarity defects of *lin-17* mutants, while CRDΔ40-174LIN-17::GFP, with four cysteine residues in the ED, could partially rescue *lin-17*. When we counted the percentage of normal polarity worms out of the total number of membrane localized worms (rescue ability) (Table 2), CRDΔ40-174LIN-17::GFP almost fully rescued *lin-17* B cell defect,

which suggests that the ED of CRDΔ40-174LIN-17::GFP can interact with LIN-44/Wnt to trigger signaling. CRDΔ50-174LIN-17::GFP, which also has four cysteines but a longer ED, surprisingly did not rescue *lin-17* as well as CRDΔ40-174LIN-17::GFP (Table 2), which suggests that the three dimensional structure of ED might determines the interaction between the ED of LIN-17 and LIN-44 and the activation of Wnt signaling.

Unexpectedly, proteins CRDΔ19-204LIN-17::GFP and CRDΔ40-204LIN-17::GFP, which could not rescue *lin-17*, and CRDΔ40-174LIN-17::GFP and CRDΔ50-174LIN-17::GFP, which could at least partially rescue *lin-17*, all displayed asymmetric distribution to B.a and B.p cell membrane and cytoplasm (Fig. 3A, B and D and Table 3). Normal asymmetric distribution of GFP to the B daughters with the B.a cell displaying a higher level than B.p, correlated with normal polarity with B.a size being larger than B.p (Fig 3A and Table 3), while reversed distribution of LIN-17::GFP with the B.p cell having a higher level of LIN-17::GFP than did the B.a cell correlated with reversed polarity with B.p being larger than B.a (Fig. 3B, D). In most of the transgenic lines, the percentage of asymmetric and reversed localization or distribution in worms to the total number of worms displaying membrane localization is high (Table 3), which indicates that the CRD is not required for the asymmetric distribution of LIN-17.

The LIN-17 Seven-transmembrane domain is required for asymmetric distribution and function

The interaction between the Wnt ligand and the CRD of Fz is widely recognized to be important for signal transduction (Dann and Hsieh, et al, 2001), while the role of the interaction between Wnt and the three extracellular Fz loops is not so clear, although it

appears that several residues may be important for signal transduction (Cong, Schweizer and Varmus, 2004). Since LIN-44 is required for LIN-17 asymmetric localization in B cell and its daughters, and we already showed that the deletion of CRD domain did not affect LIN-17 asymmetric distribution, we hypothesized that LIN-44 might interact with the extracellular loops of LIN-17 to regulate LIN-17 asymmetric localization and signal transduction. We replaced the seven-transmembrane domains with the single transmembrane domain of DAF-16 (Inoue and Oz, et al, 2004) to generate *1TMDlin-17::gfp*. 1TMDLIN-17::GFP was well localized to the B cell membrane, but failed to rescue B cell polarity defect of *lin-17* males, as only 10% of the worms displayed normal polarity (n=77) (Fig 3E and Table 2). 1TMDLIN-17::GFP symmetrically localized to B daughter cells in 89% (n=71) of *lin-17* males. The failure of 1TMDLIN-17::GFP to rescue *lin-17* mutants and its symmetric distribution to the B daughter cells indicates that the seven-transmembrane domain and the six intracellular or extracellular loops of LIN-17 are important for the function and also asymmetric distribution of LIN-17.

The conserved KTXXXW motif is required for LIN-17 function.

The KTXXXW motif, located immediately after the seven-transmembrane domain, is conserved in different Frizzled receptors (Umbhauer and Djiane, et al, 2000) and interacts with the PDZ domain of Dsh (Wong and Bourdelas, et al, 2003). This motif is also conserved in LIN-17. As MIG-5/Dsh is required for LIN-17 asymmetric localization, we asked whether the KTXXXW motif was also important for the asymmetric localization of LIN-17::GFP. To test whether the function of this motif is conserved in *C. elegans*, we deleted the KTXXXW motif or individually mutated the residues of K497 to M or R,

T498 to A or S, H500 to A and W502 to G and checked the ability of mutated proteins to rescue *lin-17* mutants. When the carboxyl terminal domain (CTD) including the KTXXXW motif of LIN-17 was deleted, the truncated protein ΔCTDLIN-17::GFP was not localized to the B cell membrane, although it was localized to the membranes of some other cells (Table 3 and data not shown), and it failed to rescue *lin-17* mutants. Mutations of each of the three conservative residues in the KTXXXW motif affected LIN-17::GFP membrane localization as visualized by GFP (Fig. 4 and Table 3) and the function of LIN-17 (Table 2 and 3). Specifically, when K was changed to M, 41 out of 76 worms displayed type II or III membrane localization (Fig. 4A and data not shown) and only one out of these 41 displayed normal polarity, which indicated that the K497 residue was important for LIN-17 membrane localization and also function in the control of B and T cell polarities (Table 2 and 3). When T498 was changed to A, 60 out of 74 animals displayed type I or II membrane localization of T/ALIN-17::GFP to the B cell membrane (Fig. 4B and data not shown) and 36 out of these 60 displayed normal polarity, which indicates that the T498 residue plays some role in membrane localization and function, but its role is not as large as K497. When W502 was replaced by G, membrane localization and the ability to rescue B or T cell polarity defect were severely affected and worms displayed type II or III membrane localization (Fig. 4C and data not shown) (Table 2 and 3). Overall, K497 and W502 were important for membrane localization and function of LIN-17::GFP, while T498 was not that important. We deleted the KTXXXW motif, (which introduced SGR for technical reasons), to generate ΔKTXLIN-17::GFP. ΔKTWLIN-17::GFP was localized to the B cell membrane similar to LIN-17::GFP and displayed asymmetric localization pattern prior to and after division (Fig. 4, E-H).

However, while ΔKTWLIN-17::GFP rescued T cell polarity, it did not rescue B cell polarity (Table 2). In order to exclude the possibility that the mutant failed to localize in the membrane of the T and B cells, we examined ΔKTWLIN-17::GFP expression pattern in T and B cells. We found that ΔKTWLIN-17::GFP was localized to T and B cells membrane as well as LIN-17::GFP was, and displayed asymmetric distribution to B cell prior to and after B cell division (Fig. 4D and 4E). However, asymmetric localization of ΔKTWLIN-17::GFP did not correlate to the B cell polarity, only 17 of 38 animals, that displayed asymmetric localization to the B daughters, displayed normal B cell polarity (Fig. 4F). One male, whose B cell polarity was reversed, displayed normal asymmetric localization (Fig. 4G).

The KTXXXW motif binds to the PDZ domain of Dsh (Wong and Bourdelas, et al, 2003) and the interaction of the KTXXXW motif with Dsh is required for activation of the canonical Wnt pathway and also for the phosphorylation and membrane association of Dsh (Umbhauer and Djiane, et al, 2000). The rescue of T cell polarity but not B cell polarity by ΔKTWLIN-17::GFP indicates that the KTXXXW motif is important in the signal transduction in the B cell and also indicates that the pathways that control B cell and T cell polarity are different, which is consistent with the observation that MIG-5 was only expressed in the B cell and was not involved in the regulation of T cell polarity.

MIG-5::GFP was asymmetrically localized to the B cell prior to and after cell division dependent upon *lin-17*

The *gfp* sequence was inserted just before the *mig-5* stop codon to generate *mig-5::gfp*. This construct rescued *mig-5*, so it is functional. MIG-5::GFP was expressed in the B cell

and its daughters (Chapter 3). Prior to the B cell division, MIG-5::GFP accumulated asymmetrically as puncta at the anterior cortex or anterior B cell membrane in nine out of 13 males (Fig. 5A). This is similar to the unipolar membrane association of Dsh during PCP signal transduction (Axelrod, 2001). After the B cell division, MIG-5::GFP was also asymmetrically localized to B daughters. Except for 17% of males, which displayed puncta all around the B.a and B.p cell cytoplasm (n=46), the displayed MIG-5::GFP accumulation or puncta at posterior cortex of B.a cell (Fig. 5B). 80% of the males that displayed puncta at posterior cortex of B.a displayed MIG-5::GFP puncta at ventral posterior cortex of B.p cell (Fig. 5B). A low percentage of worms displayed puncta at anterior cortex of B.a cell or puncta all round the B.p cell membrane (21%, n=46) (data not shown). MIG-5::GFP did not associate with the plasma membrane in *lin-17* males (n=13) (Fig. 5C), although two displayed puncta all around cytoplasm (data not shown). In addition, in *lin-17* males, the level of the MIG-5::GFP in B.a cell was higher than that in B.p (n=11) (Fig. 5C). Surprisingly, MIG-5::GFP was also asymmetrically localized to the B cell and its daughters in *lin-44* males, but was in a pattern different from that observed in the wild-type background. Prior to the B cell division, in 16 out of 28 animals, MIG-5::GFP accumulated at both anterior and posterior cortex of the B cell with weak puncta associating with the membrane (data not shown), and in four out of 28 worms, there were puncta only at anterior cortex of the B cell, while the remaining eight animals displayed puncta all round the B cell (data not shown). After division, in 34 out of 54 males, there were puncta at anterior cortex of both B.a and B.p cells (Fig. 5D) and 10 of these 34 animals also displayed puncta at posterior ventral cortex of B.p cell. Eight out of 54 males displayed puncta throughout the B.a and B.p cytoplasm. So, it seemed

that in the absence of LIN-44/Wnt ligand, MIG-5 was asymmetrically localized to the B cell or B daughter membranes, but in a reverse manner. This indicates that without LIN-44, another signal may recruit MIG-5::GFP to membrane.

The DIX, PDZ and DEP domains play different roles in MIG-5 asymmetric distribution and function.

Disheveled adaptor proteins function at the branch point of the canonical and PCP pathways. Dsh has three conserved domains: DIX, PDZ and DEP (Boutros and Mlodzik, 1999; Capelluto and Kutateladze, et al, 2002; Li and Yuan, et al, 1999). The PDZ and DEP, but not the DIX, are required for the PCP pathway, while the DIX and PDZ, but not the DEP, are required for the canonical Wnt pathway (Axelrod and Miller, et al, 1998; Boutros and Paricio, et al, 1998; Boutros and Mlodzik, 1999). We have already shown that MIG-5 was involved in the regulation of B cell polarity (Chapter 3) and that the subcellular localization of MIG-5::GFP was regulated by LIN-17 (Fig. 5). In order to examine whether a PCP-like pathway functions to regulate B cell polarity, we investigated the functions of each MIG-5 domain in the regulation of B cell polarity. We made three constructs that expressed truncated MIG-5::GFP proteins: ΔDEPMIG-5::GFP, ΔDIXMIG-5::GFP and ΔPDZMIG-5::GFP. ΔDEPMIG-5::GFP was not localized to membrane and did not display a punctate expression pattern (n=13) (Fig. 6B). This suggested that ΔDEPMIG-5::GFP did not bind to the Fz receptor in the membrane and lipid vesicles, although it contained the DIX domain, which has been shown to be responsible for binding to vesicular membrane (Capelluto and Kutateladze, et al, 2002). ΔDEPMIG-5::GFP also displayed different expression levels in the B.a and B.p cells,

with B.a having a higher level than that of B.p (n=13) (Fig. 6B), which implied that DEP domain was not responsible for the asymmetric distribution of MIG-5 to the B.a and B.p cells. ΔDIXMIG-5::GFP was localized to the membrane in a form of puncta in 16 out of 18 animals (Fig. 6C), and one out of 18 animals had puncta in the cytoplasm. ΔPDZMIG-5::GFP displayed membrane association in 24 out of 26 animals (Fig. 6D), and in one, ΔDIXMIG-5::GFP accumulated in the nucleus (Fig. 6E). In the 24 worms in which ΔDIXMIG-5::GFP associated with membrane, four displayed a puncta expression pattern in the posterior cortex of B daughters and the other 20 displayed uniform expression of ΔDIXMIG-5::GFP to the cell membranes (Fig. 6D). The three truncated proteins had different abilities to rescue *mig-5* B cell polarity. MIG-5::GFP can rescue *mig-5* B cell polarity defect, as 56% of *mig-5* males displayed B cell polarity defect (n=58), whereas only 16% of *mig-5* males that expressed MIG-5::GFP displayed abnormal B cell polarity (n=44). ΔDEPMIG-5::GFP or ΔPDZMIG-5::GFP failed to rescue the *mig-5* B cell polarity defect: as *mig-5* worms that expressed ΔDEPMIG-5::GFP or ΔPDZMIG-5::GFP showed a 58% (n=41) or 60% (n=31) B cell polarity defect, respectively. ΔDIXMIG-5::GFP partially rescued the *mig-5* B cell polarity defect as *mig-5* worms that expressed ΔDIXMIG-5::GFP displayed a 40% (n=38) B cell polarity defect. Although LIN-17 was required for MIG-5 membrane association, LIN-17 was not involved in asymmetric distribution of MIG-5 to the B.a and B.p cells (Fig. 5C) (n=13). In wild-type background, MIG-5::GFP was expressed in a punctate pattern (Fig. 5B and C), so we could not determine the asymmetric distribution of MIG-5::GFP to the B.a and B.p cells. Since ΔDEPMIG-5::GFP was not localized to the membrane and was expressed at a higher level in the B.a cell than in the B.p cell, the DEP domain did not

appear to be involved in the MIG-5::GFP asymmetric distribution to the B.a and B.p cells. In order to investigate which domain of MIG-5 was required for MIG-5::GFP asymmetric distribution to B.a and B.p cells, ΔDIXMIG-5::GFP and ΔPDZMIG-5::GFP were expressed in the *lin-17* mutants. We found that ΔPDZMIG-5::GFP (n=6) and ΔDIXMIG-5::GFP (n=7) displayed similar expression levels in the B daughters in *lin-17* males (data not shown), which indicated that both DIX and PDZ might be important for MIG-5::GFP asymmetric distribution to the B cell daughters.

Discussion

We have shown that LIN-17::GFP and MIG-5::GFP were asymmetrically localized to the cell cortex of the B cell and its daughters. We provided evidence that the LIN-17 seven-transmembrane domains and the KTXXXW motif were required for LIN-17 asymmetric distribution to B daughters, and the CRD, KTXXXW motif and seven-transmembrane domain of LIN-17 were required for its function. The MIG-5/Dsh DEP and PDZ domains played larger roles than did the DIX domain in rescuing B cell polarity. The DEP domain was required for MIG-5 membrane localization, while the PDZ and DIX domains were required for asymmetric distribution of MIG-5 to the B daughter cells. This work showed that the functions of different domains of LIN-17/Fz and MIG-5/Dsh were conserved and the mechanism of a PCP-like pathway that regulates B cell polarity may be also conserved.

Asymmetric distribution of LIN-17 correlated with B cell polarity, but was not sufficient for B cell polarity

Prior to the B cell division, asymmetric localization of LIN-17::GFP to the B cell was observed and the posterior membrane of B cell displayed a lower level of LIN-17::GFP than the anterior membrane in a LIN-44 and MIG-5 dependent manner. β-arrestin 2 is involved in the Fz4 endocytosis (Chen, Nusse, Lefkowitz 2004), however disruption of the *C. elegans* β-arrestin 2 homolog, *arr-1*, did not affect LIN-17::GFP localization (unpublished data), suggesting that if endocytosis is involved in, it might be ARR-1 independent. Thus asymmetric localization of LIN-17::GFP might be due to endocytosis in an ARR-1 independent manner (Fukuto, Ferkey et al 2004). During the B cell division, LIN-17::GFP is asymmetrically distributed to B.a and B.p cells with the B.p cell having a lower level than the B.a cell. The asymmetric distribution of LIN-17::GFP appear to result from its asymmetric localization before division, which might indicate that asymmetric localization of LIN-17::GFP prior to cell division is required for its asymmetric distribution. After LIN-17::GFP became asymmetrically localized within the B.p membrane, the asymmetrical distribution between B.a and B.p is no longer obvious. Several lines of evidence indicated that asymmetric distribution of LIN-17 correlated with B cell polarity. First, LIN-17::GFP rescued the B cell polarity defect of *lin-17* males and displayed normal asymmetric distribution to B daughter cells during B cell division. In *lin-44* males, distribution of LIN-17::GFP also correlated with B cell polarity, as all the worms that displayed reversed distribution of LIN-17::GFP with the B.p cell membrane having a higher level than B.a, displayed reversed polarity. LIN-17::GFP was also strongly expressed in the F and K cells, which were located to the anterior and dorsal sides of the B cell or B.a cell, which made it difficult to observe the reversed distribution

of LIN-17::GFP in the B and B.a cells in *lin-44* males. Reverse distribution of LIN-17::GFP in *lin-44* males is likely under estimated for this reason.

Second, CRDΔ19-204LIN-17::GFP, CRDΔ40-204LIN-17::GFP, CRDΔ40-174LIN-17::GFP and CRDΔ50-174LIN-17::GFP all displayed asymmetric distribution and asymmetric distribution of these proteins correlated with cell polarity to some extent (Fig. 3 and Table 3). As expression of these proteins in the surrounding cells was weaker, we could more easily see the LIN-17::GFP asymmetric distribution to the B cell and its daughters. The distribution pattern of these proteins correlated with polarity, as in worms with normal B cell polarity, B.a cell had a higher level of GFP in the membrane and cytoplasm than did the B.p cell (Fig. 3A). While in worms with reversed B cell polarity, the B.p cell had a higher level of GFP than did the B.a cell (Fig. 3B and D). Truncated proteins with the ability to rescue *lin-17* (CRDΔ40-174LIN-17::GFP and CRDΔ50-174LIN-17::GFP) or without the ability to rescue *lin-17* (CRDΔ19-204LIN-17::GFP and CRDΔ40-204LIN-17::GFP) all displayed asymmetric distribution to the B cell daughters, which indicates that the CRD domain is not required for asymmetric distribution. That LIN-44 affected LIN-17::GFP asymmetric distribution and the CRD domain was not involved, suggested that LIN-44 might affect LIN-17::GFP asymmetric distribution through the seven-transmembrane domain. This assumption was confirmed by 1TMDLIN-17::GFP, in which the seven-transmembrane domain of LIN-17 was replaced by the transmembrane domain of DAF-16 (Table 2 and 3).

Third, 1TMDLIN-17::GFP failed to rescue T and B cell polarity and displayed symmetric distribution and localization to the B daughter cells, with the two daughter cells having similar level of GFP in the membrane and cytoplasm. The results imply that

the seven-transmembrane domain is required for the function and asymmetric distribution and localization of LIN-17. LIN-44 might also affect LIN-17 asymmetric localization via the seven-transmembrane domain, although more work needs to be done to demonstrate a physical interaction between LIN-44/Wnt and the LIN-17 seven-transmembrane domain.

Fourth, residues K497 and W502 in KTXXXW motif were important for function and also asymmetric distribution. K497, T498 and W502, especially K497 and W502, in the KTXXXW motif were important for LIN-17 membrane localization (Fig. 4 and Table 2 and 3). This was different from results observed with Xfz3 (Umbhauer and Djiane, et al, 2000), in which the KTXXXW motif did not function in the membrane localization of Xfz3 in *Xenopus*. Functionally, K497 and W502 were also important for LIN-17, since even when the mutated proteins were localized to the B cell membrane, they still failed to rescue B cell polarity of *lin-17* males. Surprisingly, the majority of membrane localized mutant proteins neither displayed asymmetric distribution to the B.a and B.p cells nor rescued B cell polarity, indicating that the K and W residues might be also important for LIN-17 asymmetric distribution. This might be consistent with the observation that MIG-5 was also required for LIN-17 asymmetric localization and distribution, as the KTXXXW motif was shown to interact with PDZ domain of Dsh (Wong and Bourdelas, et al, 2003). However, T/ALIN-17::GFP could partially rescue B cell polarity and was asymmetrically distributed to the B cell daughters that had normal B cell polarity and displayed normal asymmetric localization of LIN-17::GFP.

Fifth, although many lines of evidence indicate that asymmetric distribution of LIN-17::GFP correlates with normal B cell polarity, there was one exception: ΔKTWLIN-17::GFP (Table 3). ΔKTWLIN-17::GFP was localized to the B cell membrane as well as

the full length LIN-17::GFP (Fig. 5) and displayed asymmetric localization prior to cell division and asymmetric distribution after division, but failed to rescue *lin-17*. When B cell polarity was lost, GFP was localized asymmetrically, and when B cell polarity was reversed, GFP was also asymmetrically localized, which was never seen in *lin-17::gfp; lin-17* or *lin-17::gfp; lin-17 lin-44* males. This result indicates that the asymmetric distribution of LIN-17 might be required for B cell polarity, but not sufficient to rescue B cell polarity of *lin-17* males.

A noncanonical Wnt pathway regulates B cell polarity

Wnt pathways are involved in the regulation of cell specification, cell migration and cell polarity during *C. elegans* development. In *C. elegans*, POP-1 is the only Tcf (Lin, Thompson and Priess, 1995; Lo and Gay, et al, 2004) (Reviewed by (Herman and Wu, 2004)), while there are four β-catenin homologs (Kidd and Miskowski, et al, 2005; Natarajan, Witwer and Eisenmann, 2001). The sequence of the four *C. elegans* β-catenins are widely divergent among themselves and to the vertebrate β-catenin and *Drosophila* Armadillo (Natarajan,Witwer and Eisenmann, 2001). The functions of the four *C. elegans* β-catenin are also divergent, as HMP-2 is involved in cell adhesion, WRM-1 functions with LIT-1 to regulate POP-1 sub-cellular localization (Rocheleau and Yasuda, et al, 1999). Both BAR-1 and SYS-1 interact with POP-1 directly to regulate POP-1 function (Kidd and Miskowski, et al, 2005; Rocheleau and Yasuda, et al, 1999). BAR-1 can respond to Wnt signaling and bind to POP-1 to switch POP-1 from a repressor to an activator (Korswagen, Herman and Clevers, 2000), so pathways that possess BAR-1 are considered to be canonical Wnt pathways. Wnt pathways are divergent in *C. elegans* compared to Wnt pathways in vertebrates and *Drosophila* and can be roughly divided to

canonical and non-canonical pathways (Reviewed by (Herman, 2002; Korswagen, 2002). Nocanonical Wnt pathways are involved in the endoderm cell specification, spindle orientation, Z1, Z4, P, T, seam cells and B cell asymmetric division (Bei and Hogan, et al, 2002; Deshpande and Inoue, et al, 2005; Gleason, Korswagen and Eisenmann, 2002; Herman, 2001; Siegfried and Kidd, et al, 2004; Thorpe and Schlesinger, et al, 1997) and the canonical Wnt pathway controls Q cell migration (Korswagen and Coudreuse, et al, 2002; Maloof and Whangbo, et al, 1999). Recent reports of the asymmetric localization of DSH-2 and MOM-5 during *C. elegans* embryogenesis (Hawkins and Ellis, et al, 2005; Park,Tenlen and Priess, 2004b) and a PCP-like pathway in the regulation of B cell polarity (Chapter 3) suggest that the PCP pathway is also conserved in *C. elegans*. In our previous report, we showed that BAR-1, SYS-1, WRM-1 and HMP-2 were not involved in the control of B cell polarity, while RHO-1 and ROCK are involved, which strongly indicates a PCP-like pathway might regulate B cell polarity (Chapter 3). This report provides further evidence that a PCP-like pathway is conserved in *C. elegans* and regulates B cell polarity. First, we showed that LIN-17 can recruit MIG-5 to the membrane prior to and after the B cell division, which is similar to the PCP pathway in *Drosophila*. Both DFz and DFz2 are receptors for the canonical Wnt pathway, but only DFz can function as a PCP signaling receptor and only DFz can recruit Dsh to the membrane in *Drosophila* (Axelrod and Miller, et al, 1998). Second, we showed that the domains of MIG-5 were conserved. The DEP was required for membrane association but not asymmetric distribution to B daughters. The two domains of MIG-5: DEP and PDZ, which were required for PCP (Boutros and Mlodzik, 1999), were also required to rescue B cell polarity, while the DIX was not that important, which indicates that a PCP-like

pathway regulates B cell polarity. Third, the asymmetric localization pattern of LIN-17/Fz and MIG-5/Dsh was similar to Fz and Dsh in *Drosophila*. LIN-17/Fz and MIG-5/Dsh accumulated to the anterior cortex of B cell, opposite to the LIN-44/Wnt source, which is similar to the asymmetric localization patterns of Fz and Dsh to the distal cortex of an individual cell during the *Drosophila* wing hair formation, in which the signal comes from the proximal (Strutt, 2001). Fourth, the KTXXXW motif function was conserved in LIN-17. The KTXXXW motif was shown to be required for the activation of β-catenin and required for Dsh membrane association, as XFz3 with any point mutation of the three residues failed to recruit Xdsh to cell membrane (Umbhauer and Djiane, et al, 2000), which indicates that the KTXXXW motif is also important to PCP signaling, as membrane association of Dsh is required for the PCP signaling (Axelrod and Miller, et al, 1998; Strutt, 2001). In our work, we have shown that the KTXXXW motif was not only important for LIN-17 function, but for membrane localization of LIN-17, as mutation of any of the three affected LIN-17 membrane localization and its function. Overall, a PCP-like pathway might function to regulate B cell polarity.

Model of LIN-17 and MIG-5 in the regulation of B cell polarity

We propose the following model to describe how LIN-44 and LIN-17 regulate B cell polarity (Fig. 7). LIN-44/Wnt ligand, which is expressed in the tail hypodermal cells (Herman and Vassilieva, et al, 1995), travels to the B cell membrane and binds to the CRD and extracellular loops of LIN-17. The interaction of LIN-44 and LIN-17 will recruit MIG-5 to the B cell plasma membrane, possibly with other proteins, such as homologs of PCP core proteins, to form a complex. This complex will be endocytosed or

transported to anterior cortex of B cell and accumulate to the anterior cortex and membrane. Due to the asymmetric localization of this complex, some cell fate determinants, such as VAB-3 (Chamberlin and Sternberg, 1995), will accumulate to anterior part of B cell. The asymmetric localization of LIN-17, MIG-5 and other cell fate determinants will cause the two daughters to acquire different cell fate determinants and take different cell fates. After division, the daughter cell that is closer to the signal will take B.p cell fate and the other daughter will take the B.a cell fate. In *lin-44* males, although we could not reliably detect the reversed asymmetric localization of LIN-17::GFP to B cell prior to and during B cell division in real time due to the strong expression of LIN-17::GFP in the surrounding F and K cells, we showed that some percentage worms with reversed polarity displayed reversed distribution after division, with B.p cell membrane having a higher level of GFP than B.a (Fig. 2F). The correlation of reversed distribution of LIN-17::GFP with reversed polarity is consistent with the model we described above. In *lin-44* males, the posterior daughter possesses higher LIN-17::GFP and MIG-5::GFP, which will recruit B.a cell fate determinants to the posterior part of B cell and after division, the posterior daughter will take B.a cell fate, while the anterior daughter will take the B.p cell fate. How is LIN-17::GFP localization to B daughters reversed in *lin-44* males? We could not exclude the possibility of another signal, but we also think it is highly possible that LIN-17 can auto-activate via LIN-17 dimerization. LIN-17::GFP is expressed strongly in the F cell, which contacts B cell or B.a cell and this strong expression of LIN-17::GFP in the F cell might cause LIN-17 to be auto-activated to initiate the signaling in B cell, which is similar to the Fz auto-activation by dimerization (Carron and Pascal, et al, 2003). This may cause the daughter cell that is

closer to F cell to take the B.p cell fate and the other daughter will take the B.a cell fate in *lin-44* males. When LIN-44 is present, this LIN-44/Wnt signal will overcome the signal initiated in the F cell, and cause the anterior daughter to take the B.a cell fate and the posterior daughter to take the B.p cell fate.

This model explains how Wnt signaling regulates asymmetric B cell division, however more work need to be done to test this model. Although we tested several domains and motifs that are required for LIN-17::GFP asymmetric localization and distribution in a LIN-44 and MIG-5 but not ARR-1 dependent process, the process might be more complicated than we know. Questions remain, for example, what other components that are involved in this process, how asymmetric distribution of LIN-17 couples with the asymmetric distribution of cell fate determinants, how does the other signal, a new ligand or LIN-17 auto-activation in the F cell, regulates B cell division in *lin-44*, does LIN-44 function as a morphogen in the regulation of T and B cell polarity? These questions will be exciting to investigate.

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Table 1 Transgenic lines and their corresponding constructs:

Genotype	KS names for the strains
mhIs9;lin-17;unc-119;him-5	KS411
mhIs9;lin-17lin-44;unc-119;him-5	KS412
mhIs9;mig-5;unc-119;him-5	KS541
mhEx155a,b;{CRDΔ19-204lin-17::gfp};lin-17;unc-119;him-5	KS520, KS521
mhEx151a,b;{CRDΔ40-204 lin-17::gfp};lin-17;unc-119;him-5	KS512, KS513
mhEx150a,b;{CRDΔ40-174 lin-17::gfp};lin-17;unc-119;him-5	KS510, KS511
mhEx153a,b;{CRDΔ40-174 lin-17::gfp};lin-17lin-44;unc-119;him-5	KS516, KS517
mhEx149a,b;{CRDΔ50-174 lin-17::gfp};lin-17;unc-119;him-5	KS508, KS509
mhEx157a,b;{1TMD lin-17::gfp};lin-17;unc-119;him-5	KS523, KS524
mhEx156;{ΔCTD lin-17::gfp};lin-17;unc-119;him-5	KS522
mhEx153a,b;{ΔKTW lin-17::gfp};lin-17;unc-119;him-5	KS514, KS515
mhEx159a,b;{K/M lin-17::gfp};lin-17;unc-119;him-5	KS527, KS528
mhEx168a,b;{K/R lin-17::gfp};lin-17;unc-119;him-5	KS539, KS540
mhEx160a,b;{T/A lin-17::gfp};lin-17;unc-119;him-5	KS529, KS530
mhEx158a,b;{W/G lin-17::gfp};lin-17;unc-119;him-5	KS525, KS526
mhEx124;{ΔDEPMIG-5::gfp};mig-5;unc-119;him-5	KS454
mhEx125;{ΔPDZMIG-5::gfp};mig-5;unc-119;him-5	KS455
mhEx126;{ΔDIXMIG-5::gfp};mig-5;unc-119;him-5	KS456
mhEx161;{ΔPDZMIG-5::gfp};lin-17;unc-119;him-5	KS531
mhEx162;{ΔDIXMIG-5::gfp};lin-17;unc-119;him-5	KS532
mhEx164;{mig-5::gfp};lin-17;him-5	KS534
mhEx165;{mig-5::gfp};lin-44;him-5	KS535
mhEx111;{mig-5::gfp}; him-5	KS432
mhEx166;{mig-5::gfp};him-5	KS536
mhEx110a,b,c;{ΔDEPMIG-5::gfp};unc-119;him-5	KS429-KS431
MhEx105b,c;{ΔPDZMIG-5::gfp};unc-119;him-5	KS426, KS427
mhEx108b,c{ΔDIXMIG-5::gfp};unc-119;him-5	KS425, KS428

Table 2 The ability of LIN-17 variants to rescue *lin-17(n671)* varied.

LIN-17 variants[#]	Normal T cell polarity % (M)	Normal B cell polarity (N)	Rescuing ability *(O)
LIN-17(n671)	2% (120)	15% (68)	NA
LIN-17::GFP[@]	75% (96)	88%% (27)	ND
LIN-17::GFP[&]	96% (54)	93% (54)	ND
CRDΔ19-204LIN-17::GFP	1% (204)	12% (34)	16% (19)
CRDΔ40-204 LIN-17::GFP	13% (314)	13% (120)	18% (76)
CRDΔ40-174 LIN-17::GFP	59% (266)	67% (70)	90% (58)
CRDΔ50-174 LIN-17::GFP	21% (182)	31% (73)	34% (56)
1TMDLIN-17::GFP	7% (196)	10% (77)	11% (71)
ΔCTDLIN-17::GFP	6% (100)	5% (26)	7% (16)
ΔKTWLIN-17::GFP	71% (356)	22% (86)	21% (72)
K/M LIN-17::GFP	2% (216)	8% (76)	9% (41)
K/R LIN-17::GFP	30% (206)	62% (61)	72% (36)
H/ALIN-17::GFP	82% (250)	72% (57)	91% (44)
T/A LIN-17::GFP	30% (450)	51% (74)	60% (60)
W/GLIN-17::GFP	6% (214)	10% (77)	12% (57)

LIN-17 variants[#]: the functions of LIN-17 variants were examined by their ability to rescue *lin-17(n671)*. M: the total socket cells examined; N: total worms examined after B cell division; O: number of worms in which GFP was membrane localized (O); Rescue ability*: the percentage of worms with normal polarity out of total number of worms with LIN-17::GFP localized to the membrane; ND: not determined. @: extrachromosomal array; &: Integrated line.

Table 3 Asymmetric distribution of LIN-17::GFP correlates with B cell polarity.

LIN-17 variants	A	B	C	D	E
LIN-17::GFP[@]	10/10	10/12	12/12	12/28	22/52
LIN-17::GFP	25/25	25/33	NA	NA	25/33
LIN-17::GFP[#]	5/5	5/14	NA	NA	5/31
CRDΔ19-204LIN-17::GFP	3/7	3/3	0/2	NA	9/19
CRDΔ40-204 LIN-17::GFP	6/14	6/14	12/20	12/20	34/76
CRDΔ40-174 LIN-17::GFP	42/47	42/43	3/5	3/3	52/58
CRDΔ50-174 LIN-17::GFP	21/23	21/21	13/14	13/13	37/56
1TMDLIN-17::GFP	0/5	0/8	3/8	3/3	13/71
ΔCTDLIN-17::GFP	1/4	1/1	NA	0/4	4/16
ΔKTWLIN-17::GFP	17/38	17/19	1/1	1/3	39/72
K/MLIN-17::GFP	1/5	1/6	3/9	3/3	14/41
K/RLIN-17::GFP	26/27	26/38	NA	0/1	27/36
H/ALIN-17::GFP	30/32	30/35	NA	0/2	32/44
T/ALIN-17::GFP	36/41	36/36	4/9	4/4	50/60
W/GLIN-17::GFP	5/8	5/7	3/8	3/3	16/57

The functions of the LIN-17 variants were examined in *lin-17* background, except @ in *lin-17lin-44* background and [#] in *mig-5* background;
A: Number of normal polarity males with LIN-17::GFP asymmetrically localized to B daughters / Number of males that display asymmetric localization of LIN-17::GFP to B daughters.
B: Number of normal polarity males with LIN-17::GFP asymmetrically localized to B daughters /number of normal polarity males.
C: Number of reversal polarity males with reversed LIN-17::GFP localization to B daughters/ number of males that displayed reversed localization of LIN-17::GFP to B daughters.
D: Number of reversal polarity males with reversed LIN-17::GFP localization to B daughters/ number of reversal polarity worms.
E: asymmetric and reversal localization/total membrane localization.

Figure 1 LIN-17::GFP was asymmetrically localized to the B cell in *lin-17* males prior to and after cell division.

Panels (A-C) show the fluorescent image below and corresponding DIC image above. LIN-17::GFP was asymmetrically localized within the B cell prior to (A) and after division (B). The posterior cortex and cytoplasm (arrow) of the B cell (A) or the B.p cell (B) displayed weaker GFP expression than the anterior of that cell. (C) LIN-17::GFP was asymmetrically localized to the T cell and accumulated to posterior cortex (arrowhead) of T cell. (D1-D5) Fluorescence images of Z series sections with 1 μm per section and pictures from D1 to D5 came from a B cell spatially sectioned from top to bottom respectively. Arrows point to the cortex of B cell that expresses LIN-17::GFP weaker. (D6) is the DIC image of B cell showed in D1-D5.

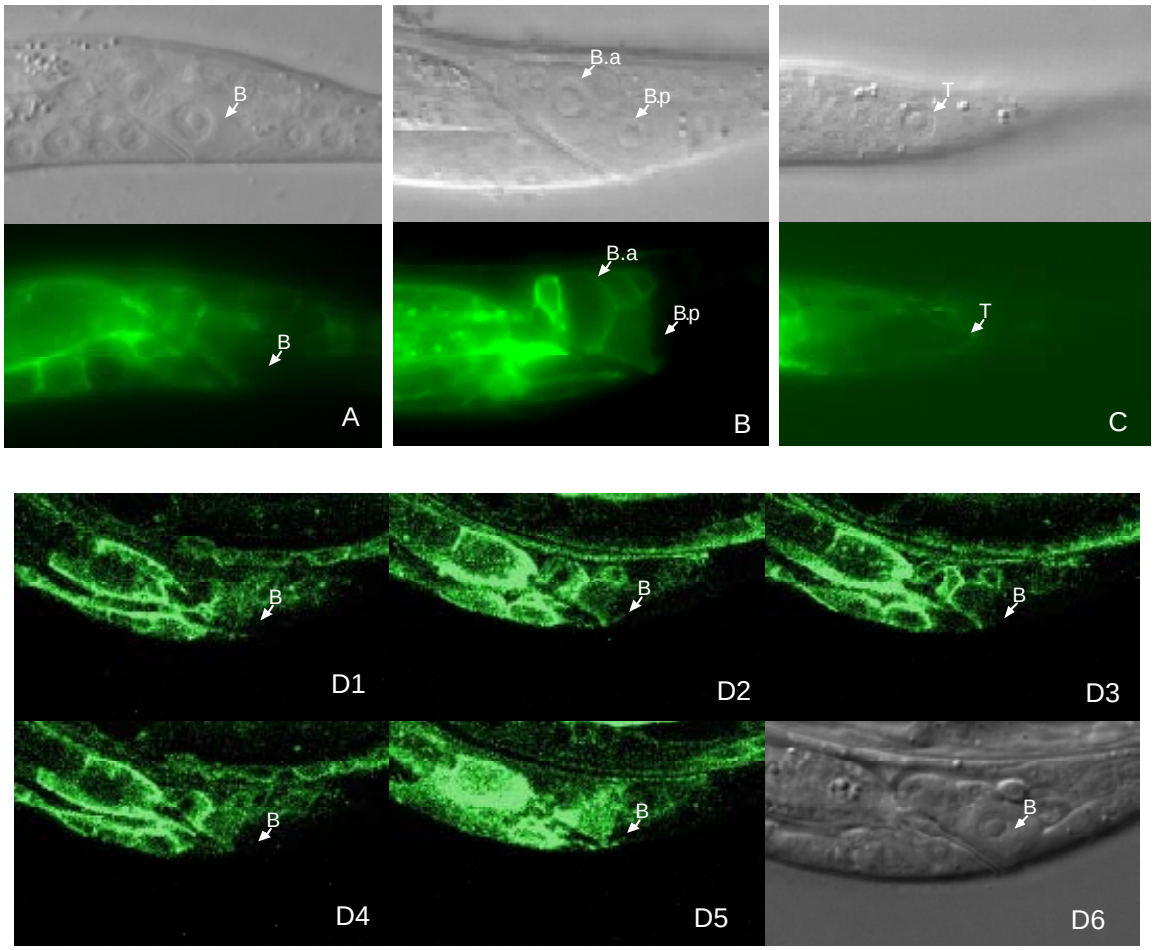


Figure 2 LIN-44 and MIG-5 were required for LIN-17::GFP asymmetric localization.

Panels (A-G) show the fluorescent image below and corresponding DIC image above. LIN-17::GFP was asymmetrically localized to B cell in *lin-17* males (A), while it was symmetrically localized to B cell in *mig-5* males (B) and *lin-17 lin-44* males (C). After the B cell division, LIN-17::GFP was asymmetrically localized to the B.p cell and the posterior membrane (arrow) displayed weaker expression in *lin-17*(D), while the asymmetric localization was lost in *mig-5* (E) and *lin-17 lin-44* mutants (G) or reversed in *lin-17 lin-44* (F) with B.a cell membrane (arrow) having lower level of GFP than B.p. (H1-H4) dynamic expression of LIN-17::GFP at different time points in *lin-17* males: five hours after hatching (H1), before the B cell ready to divide (H2), during B cell division (H3) and right after the B cell division (H4). Arrows point to the cell membrane or cortex that displayed weaker GFP expression.

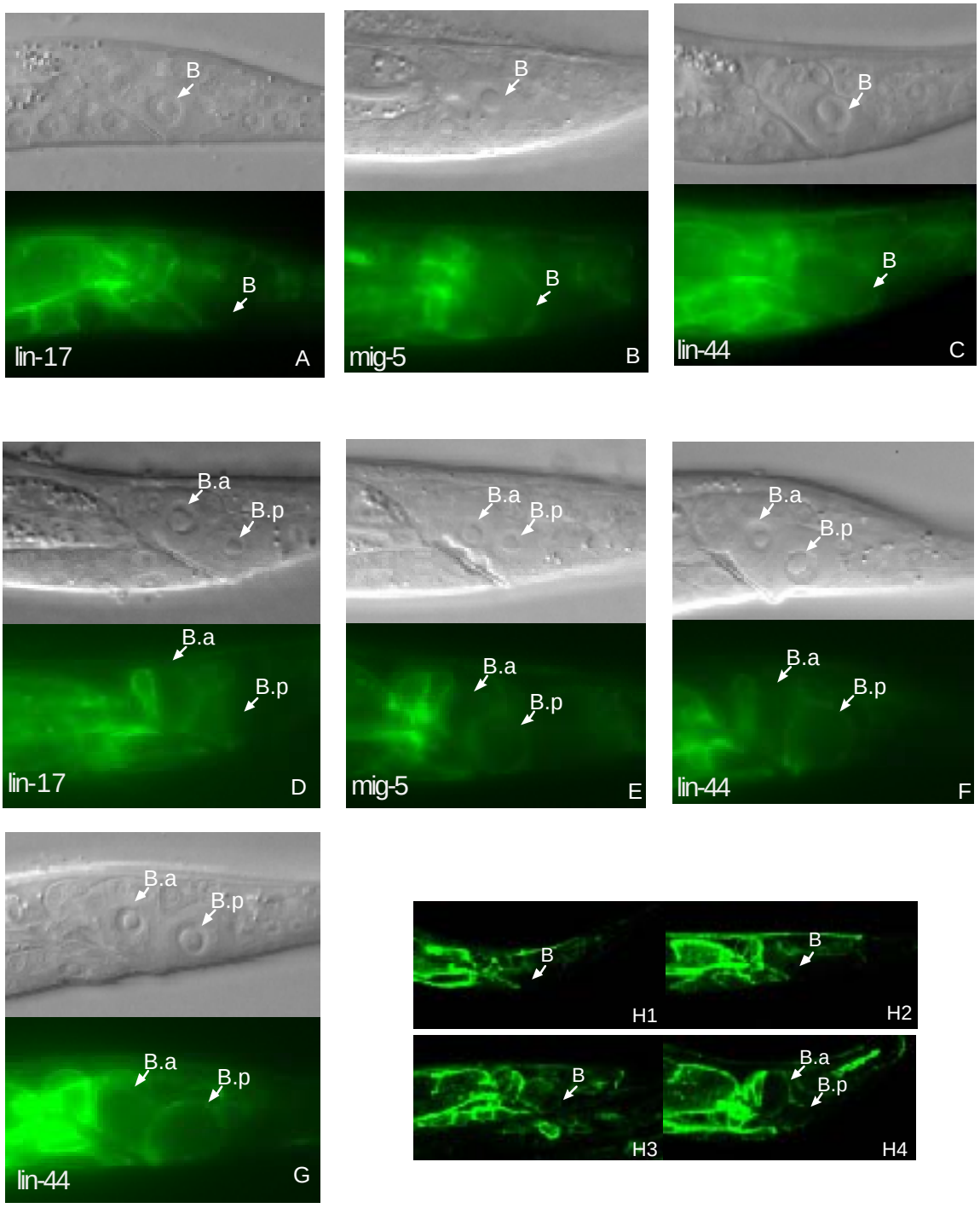

B
B
lin-17
A
B
B
mig-5
B
B
B
lin-44
C
B.a
B.p
B.a
B.p
lin-17
D
B.a
B.p
B.a
B.p
mig-5
E
B.a
B.p
B.a
B.p
lin-44
F
B.a
B.p
B.a
B.p
lin-44
G
B
H1
B
H2
B
H3
B.a
B.p
H4

Figure 3 The CRD was not required for LIN-17 asymmetric localization to the B.a and B.p cells. Above the image is the whole sequence of CRD and the sequence alignment of the four truncated CRDs (The signal peptide is bold and the cys residues were bold and underlined.) (A) CRDΔ40-174LIN-17::GFP displayed Type I membrane localization, in which the membrane localization was similar to LIN-17::GFP that wild-type with partial localization to the cytoplasm. (B) CRDΔ40-204LIN-17::GFP that displayed Type II membrane localization, in which the whole membrane could be roughly seen. (C) CRDΔ40-204LIN-17::GFP that displayed type III membrane localization, in which we could not determine there was a clear membrane localization. (D) CRDΔ40-174LIN-17::GFP that displayed reverse localization to B daughters in *lin-17lin-44.* (E) 1TMDLIN-17::GFP failed to rescue *lin-17* and also displayed a symmetric localization pattern to B daughters.

19 40 50

MMHSLGIILLFIPLATGSIFDQAVKGK**C**IPIDIEL**C**KDLPYNYTYFPNTILHNDQH
TLQTHTEHFKPLMKTK**C**HPHIHFFI**C**SVFAPM**C**PIGMPQAVTS**C**KSV**C**EQVKAD
CFSILEEFGI

174

GWPEPLN**C**AQFPDPPEL**C**MKPTEDEITGGFSAPRLPTKGSSSSSSKPTG**C**PSDLVD
VDPH

204

DPKSH**C**AFA**C**QSNVMFSTDNKRMVR

The sequences of the four truncated CRDs were:
CRDΔ19-204LIN-17::GFP: **MMHSLGIILLFIPLATGS**TR-Δ19-204R
CRDΔ40-204LIN-17::GFP:**MMHSLGIILLFIPLATGS**IFDQAVKGK**C**IPIDIEL**C**KDLTR-Δ40-204R
CRDΔ40-174LIN-17::GFP:**MMHSLGIILLFIPLATGS**IFDQAVKGK**C**IPIDIEL**C**KDLTR-Δ40-174VDVDPH DPKSH**C**AFA**C**QSNVMFSTDNKRMVR
CRDΔ50-174LIN-17::GFP:**MMHSLGIILLFIPLATGS**IFDQAVKGK**C**IPIDIEL**C**KDLPYNYTYFPNTTR-Δ50-174VDVDPHDPKSH**C**AFA**C**QSNVMFSTDNKRMVR.

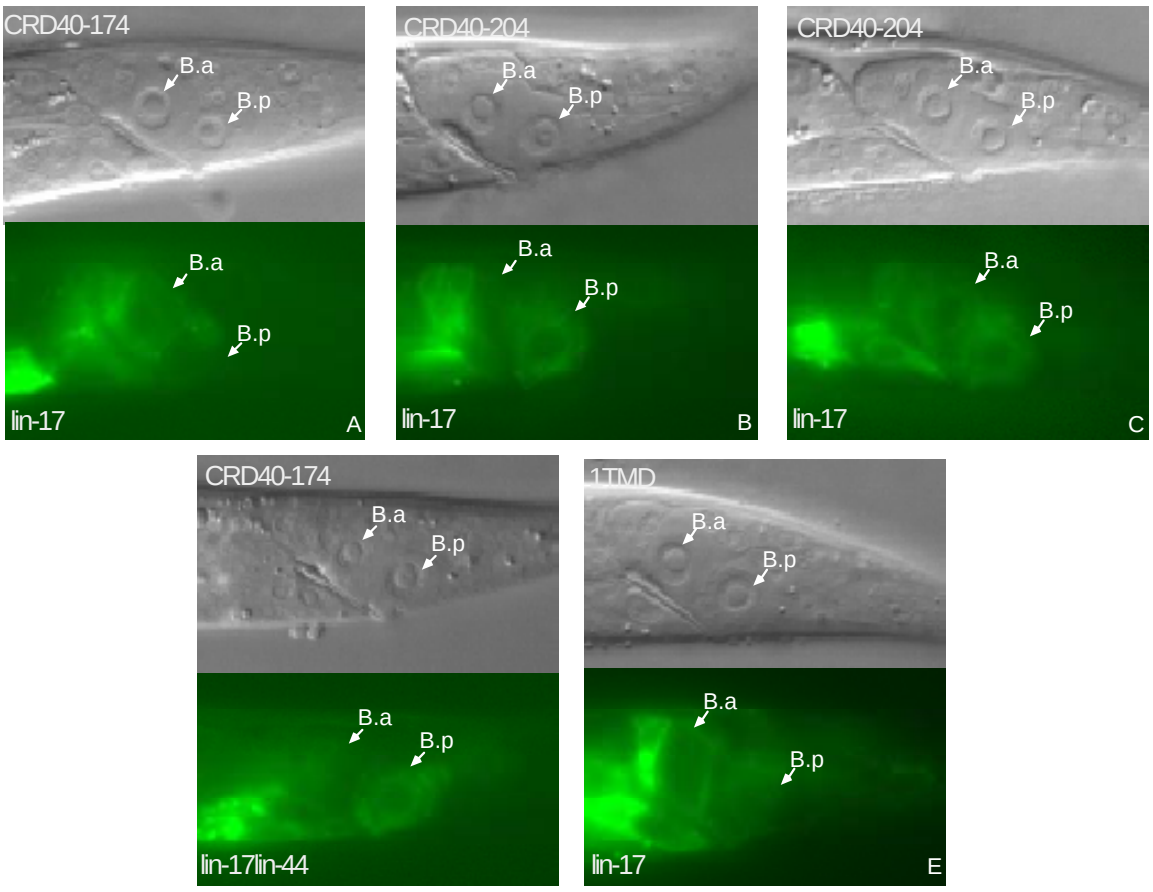

CRD40-174
B.a
B.p
B.a
B.p
lin-17
A
CRD40-204
B.a
B.p
B.a
B.p
lin-17
B
CRD40-204
B.a
B.p
B.a
B.p
lin-17
C
CRD40-174
B.a
B.p
B.a
B.p
lin-17lin-44
1TMD
B.a
B.p
B.a
B.p
lin-17
E

Figure 4 K and W in KTXXXW motif were required for LIN-17 membrane localization, function and also asymmetric distribution.

The mutated KTXXXW motif, the whole sequence of CTD and the alignment of CTDs in these truncated LIN-17::GFP were shown below. All the images shown were in the *lin-17* background.

KTVHAW
MTVHAW
KAVHAW
KTVHAG
RTVHAW
KTVAAW

KTWLIN-17::GFP: SAKTVHAWKNAA::GFP
ΔCTDLIN-17::GFP: SASGR::GFP
ΔKTWLIN-17::GFP: SASGRKNFIFCGMCSSAPVKNPIEPSTRPLLEPPTAPPPQPPVYMQMATNPQNSWRPSKVV::GFP
LIN-17::GFP: SAKTVHAWKNFIFCGMCSSAPVKNPIEPSTRPLLEPPTAPPPQPPVYMQMATNPQNSWRPSKVV::GFP

(A) K/M LIN-17::GFP displayed type III membrane localization. (B) T/A LIN-17::GFP displayed type II membrane localization and asymmetrically distributed to B daughter cells in *lin-17* males. (C) W/G LIN-17::GFP displayed type II membrane localization and was asymmetrically distributed to B daughter cells in *lin-17* males. ΔKTWLIN-17::GFP displayed asymmetric localization to B cell (D) or B.p cell (E) in *lin-17* males. ΔKTWLIN-17::GFP failed to rescue B cell polarity (F and G), but displayed normal asymmetric localization in *lin-17* males that displayed a loss of B cell polarity (F) or reversal of B cell polarity (G).

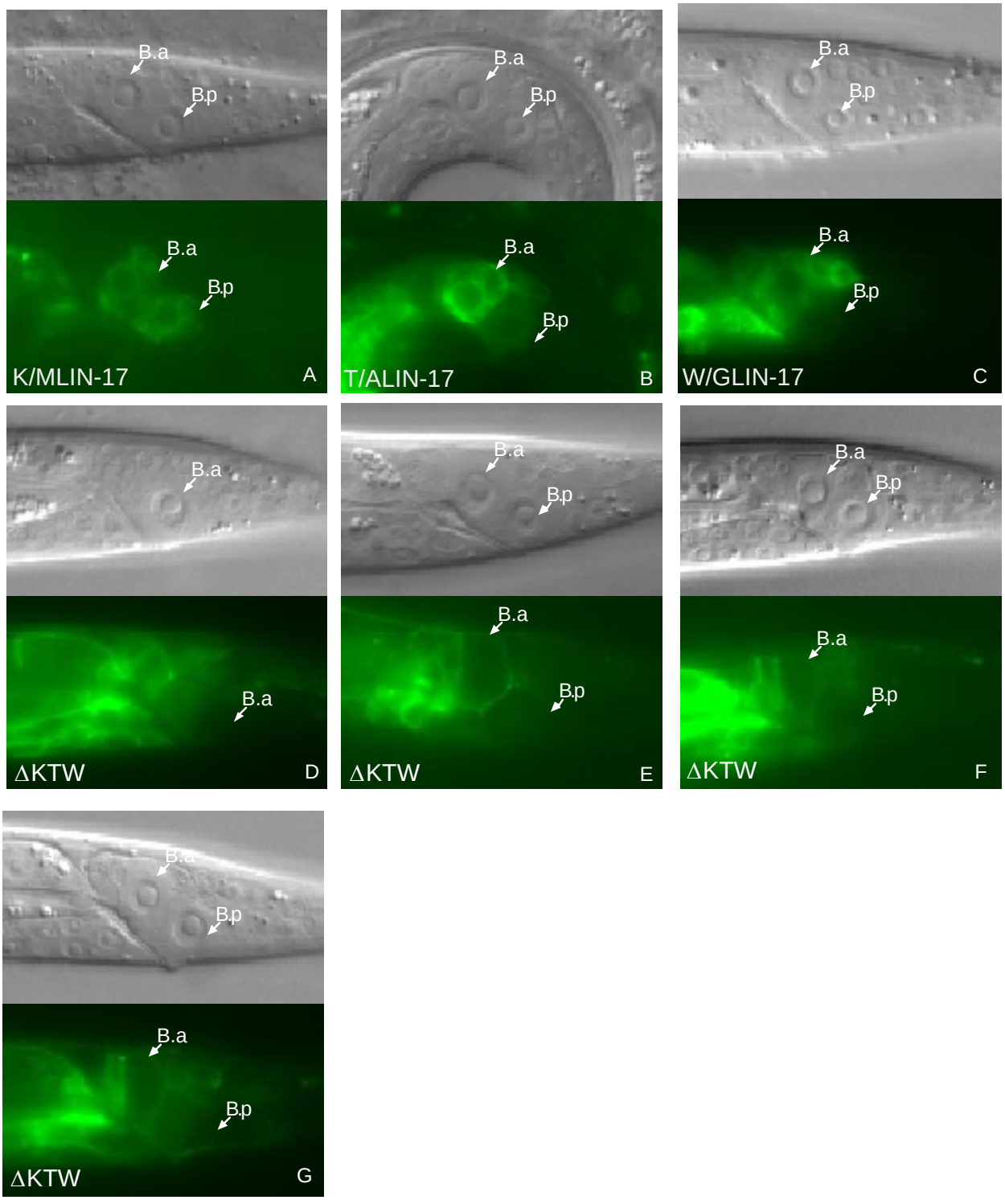

B.a
B.p
K/MLIN-17
A
T/ALIN-17
B
W/GLIN-17
C
ΔKTW
D
ΔKTW
E
ΔKTW
F
ΔKTW
G

Figure 5 Asymmetric subcellular localization of MIG-5::GFP prior to and after the B cell division was dependent upon *lin-17* but not *lin-44*.

(A) MIG-5::GFP accumulated to the anterior cortex of the B cell in a punctate pattern (small arrows). After division, in both wild type and *lin-44* males, there is puncta expression pattern and in wild type, puncta accumulated at posterior cortex of B.a and B.p cells (B), while in *lin-44*, puncta accumulated at anterior cortex of B.a nd B.p cells (D). (C) There was no membrane association in lin-17 males, but the level of MIG-5::GFP was higher in B.a than B.p.

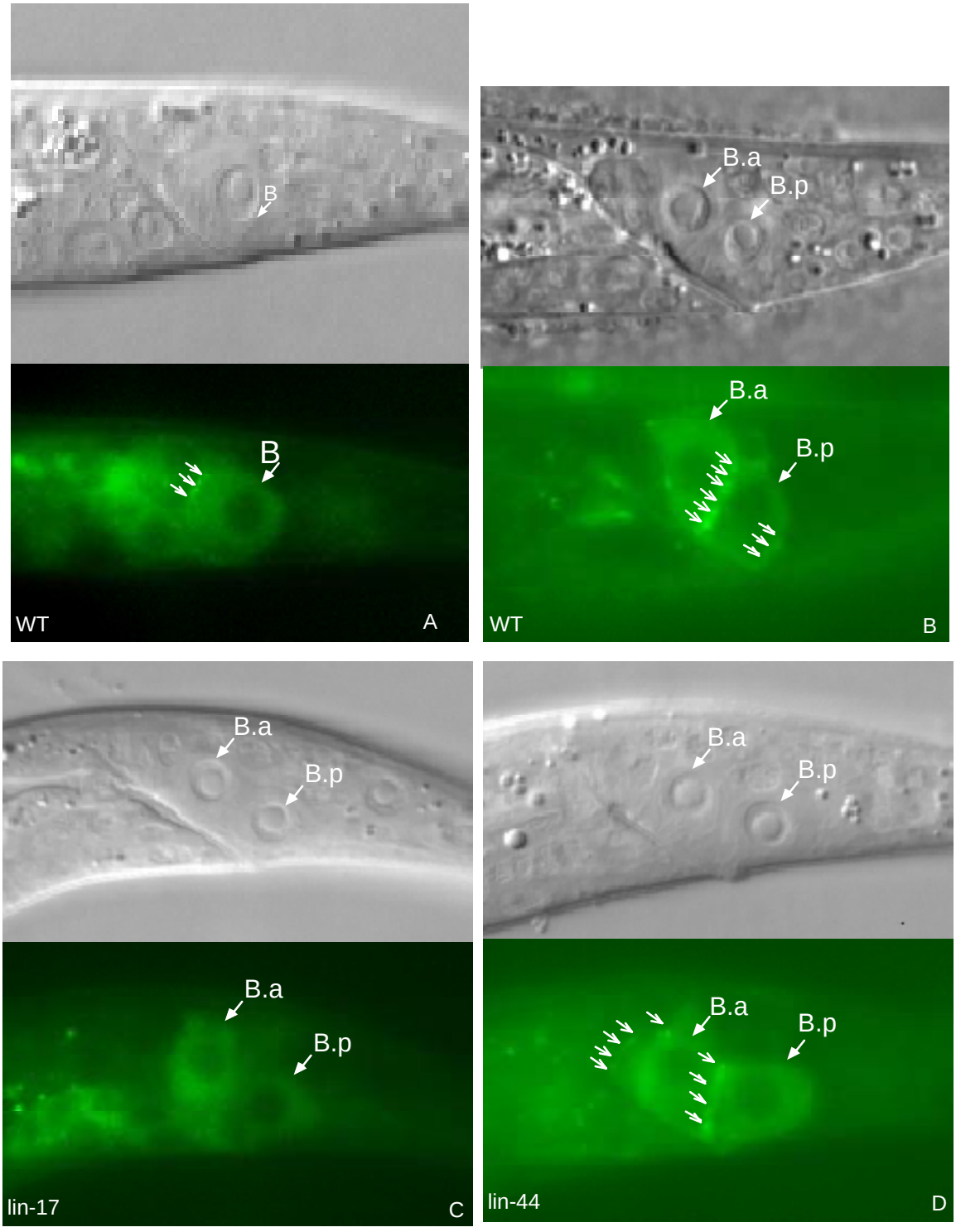

B
WT
A
B.a
B.p
WT
B
B.a
B.p
lin-17
C
B.a
B.p
lin-44
D

Figure 6 The DEP domain was required for MIG-5 membrane localization and the PDZ and DIX domains were required for MIG-5 asymmetric distribution to the B daughters.

(A) MIG-5::GFP accumulated to the anterior cortex of B cell in a punctate pattern (small arrows). (B) ΔDEPMIG-5::GFP did not display membrane association, but displayed an asymmetric distribution to the B daughters with B.a having a higher level than did B.p. (C) ΔPDZMIG-5::GFP displayed puncta expression pattern in B daughters. (D) ΔDIXMIG-5::GFP can associate with plasma membrane. (E) ΔDIXMIG-5::GFP was symmetrically distributed to B daughters in *lin-17* males.

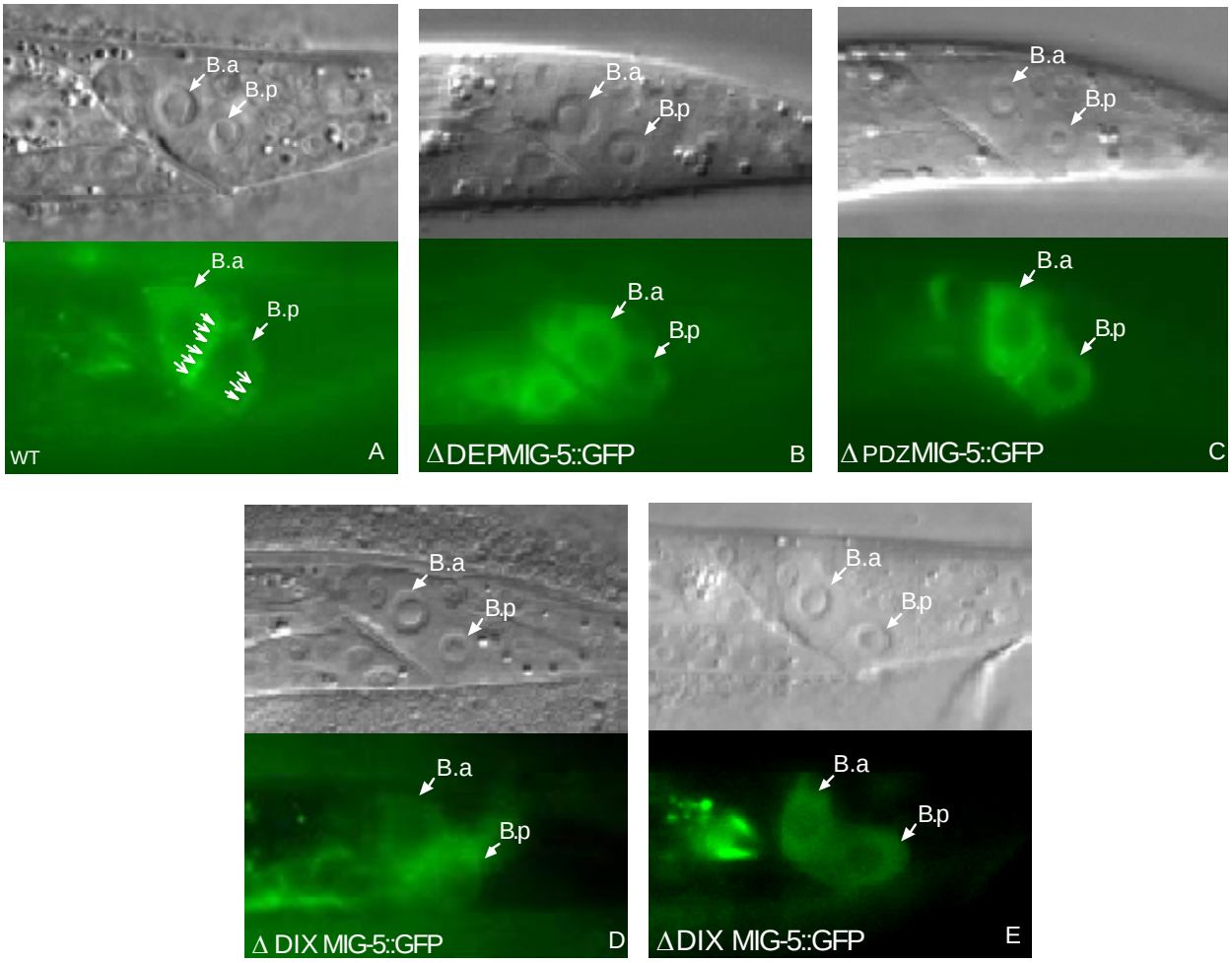
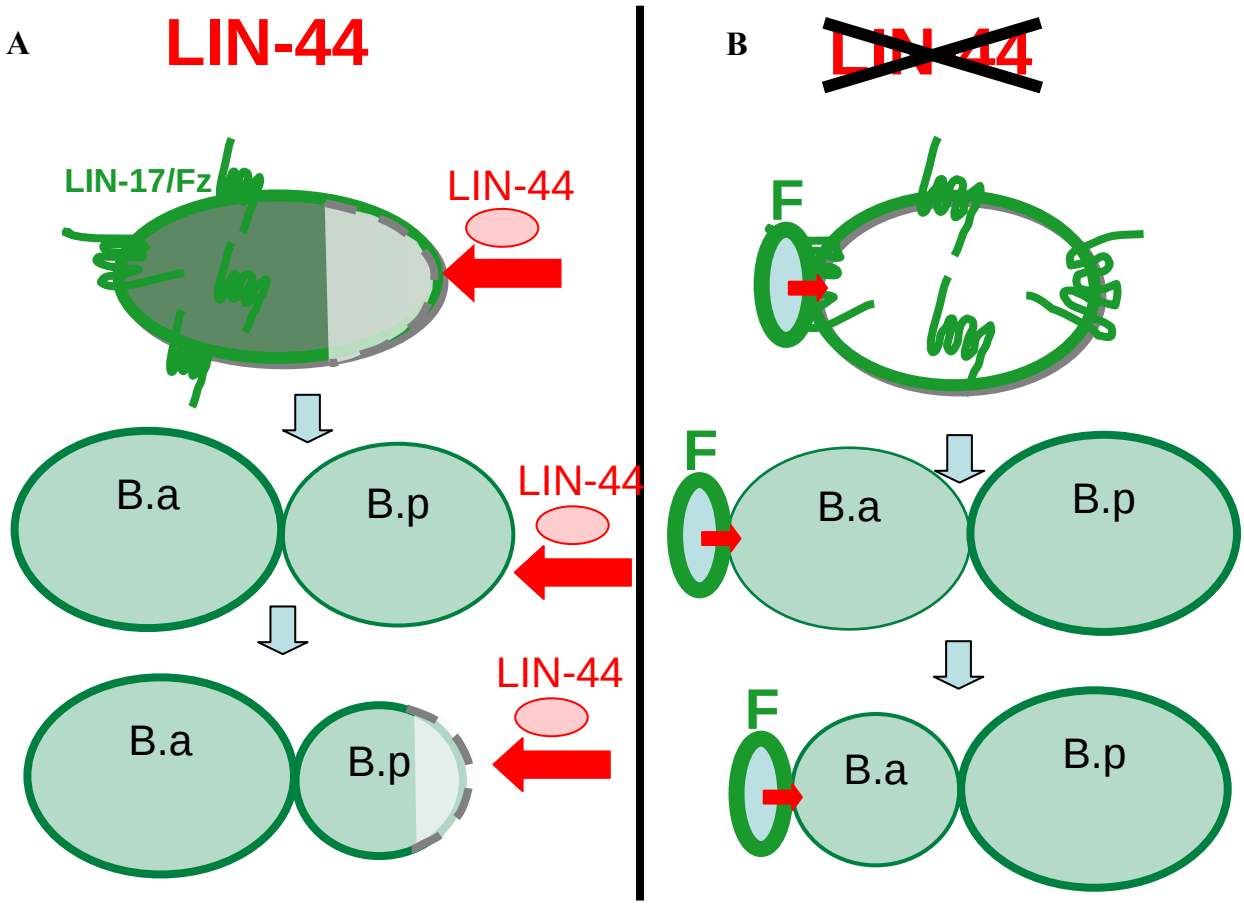

B.a
B.p
WT
A
ΔDEPMIG-5::GFP
B
ΔPDZMIG-5::GFP
C
Δ DIX MIG-5::GFP
D
ΔDIX MIG-5::GFP
E

Figure 7 Functional model: Asymmetric localization of LIN-17 and MIG-5 correlates with B cell polarity.

(A) When LIN-44/Wnt is present, LIN-44 binds to CRD and seven trans-membrane domain of LIN-17, which will recruit MIG-5 to the membrane and form a complex. This complex will be internalized and transported to the anterior cortex of B cell. The asymmetric localization of this complex will cause asymmetric distribution of cell fate determinants to the two daughters, which results in different cell fates and the one closer to Wnt signal will take B.p cell fate and the other will take B.a cell fate. The asymmetric localization of LIN-17::GFP results in asymmetric distribution to B daughters during mitosis with B.p cell membrane having a lower level than B.a cell. Shortly after cell division, B.p cell displays asymmetric localization. (B) When LIN-44 is absent, there is no accumulation of LIN-17 to the posterior of B cell membrane. In the F cell, which has a higher LIN-17 expression level than the B cell, might auto-activate and initiate signaling independent of LIN-44. The activation of signaling at the anterior of B cell will cause asymmetric distribution of cell fate determinants prior to B cell division, and cause the daughter cell that is closer to F cell will take B.p cell fate, while the other take B.a cell fate.


A
LIN-44
LIN-17/Fz
LIN-44
B.a
B.p
LIN-44
B.a
B.p
LIN-44
B
LIN-44
F
F
B.a
B.p
F
B.a
B.p