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β-Catenin asymmetry is regulated by PLA_1 and retrograde traffic in *C. elegans* stem cell divisions

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Asymmetric division is an important property of stem cells. In *Caenorhabditis elegans*, the Wnt/β-catenin asymmetry pathway determines the polarity of most asymmetric divisions. The Wnt signalling components such as β-catenin localize asymmetrically to the cortex of mother cells to produce two distinct daughter cells. However, the molecular mechanism to polarize them remains to be elucidated. Here, we demonstrate that intracellular phospholipase A_1 (PLA_1), a poorly characterized lipid-metabolizing enzyme, controls the subcellular localizations of β-catenin in the terminal asymmetric divisions of epithelial stem cells (seam cells). In mutants of *ipla-1*, a single *C. elegans* PLA_1 gene, cortical β-catenin is delocalized and the asymmetry of cell-fate specification is disrupted in the asymmetric divisions. *ipla-1* mutant phenotypes are rescued by expression of *ipla-1* in seam cells in a catalytic activity-dependent manner. Furthermore, our genetic screen utilizing *ipla-1* mutants reveals that reduction of endosome-to-Golgi retrograde transport in seam cells restores normal subcellular localization of β-catenin to *ipla-1* mutants. We propose that membrane trafficking regulated by *ipla-1* provides a mechanism to control the cortical asymmetry of β-catenin.

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Introduction

Asymmetric division is an attractive means for stem cells to balance self-renewal and differentiation (Morrison and Kimble, 2006). To divide asymmetrically, stem cells become polarized prior to asymmetric divisions; *Drosophila* neural stem cells, for example, establish an axis of polarity to localize cell-fate determinants to one side of the cells and to orient the mitotic spindle correctly (Betschinger and Knoblich, 2004; Yu *et al*, 2006).

In *Caenorhabditis elegans*, the polarity of the most asymmetric divisions is regulated by the Wnt signalling pathway, dubbed as the 'Wnt/β-catenin asymmetry pathway' (Thorpe *et al*, 2000; Herman, 2002; Mizumoto and Sawa, 2007b). In the canonical Wnt pathway, binding of Wnt to its receptor, Frizzled (Fz), results in activation of Dishevelled (Dsh), which in turn inactivates the β-catenin destruction complex, leading to β-catenin stabilization. The stabilized β-catenin enters the nucleus, where it interacts with TCF transcription factors and converts them from repressors to activators (Logan and Nusse, 2004; Clevers, 2006). In the Wnt/β-catenin asymmetry pathway, Wnt signalling components become polarized in the mother cells by Wnt proteins, and then polarized Wnt signalling produces two daughter cells with distinct transcriptional activities of TCF *pop-1*. Model asymmetric divisions for the Wnt/β-catenin asymmetry pathway include that of the T blast cell. Before T cell division, the Wnt protein *lin-44*, which is expressed posterior to the T cell, induces asymmetric cortical localizations of the canonical Wnt pathway components; Fz *lin-17*, Dsh *dsh-2* and Dsh *mig-5* localize to the posterior side of the T cell, and β-catenin *wrm-1* and some of the destruction complex proteins localize to the anterior cortex (Takeshita and Sawa, 2005; Goldstein *et al*, 2006; Mizumoto and Sawa, 2007a). In telophase, WRM-1 accumulates into the posterior nucleus, where it appears to promote the nuclear export of POP-1 (Lo *et al*, 2004), and the nuclear level of POP-1 becomes asymmetric between two daughter cells (POP-1 asymmetry). Recently, it was shown that another β-catenin, SYS-1 acts as a coactivator for POP-1 (Kidd *et al*, 2005), and that in many A–P divisions, the nuclear localization of SYS-1 is also asymmetric in a manner reciprocal to POP-1 asymmetry (Huang *et al*, 2007; Phillips *et al*, 2007). This reciprocal asymmetry of POP-1 and SYS-1 provides a robust change in POP-1 transcriptional activity and seems to be a universal mechanism for cell-fate specification during animal development (Huang *et al*, 2007; Phillips *et al*, 2007). Among the cells that undergo asymmetric divisions during *C. elegans* development, the lateral epithelial seam cells are unique in that they have characteristics of stem cells. First, seam cells repeatedly divide to generate one daughter with a seam cell fate (self-renewal) and one daughter that fuses with the hypodermal syncytium

(differentiation). Second, like many stem cells in other organisms, seam cells can also divide symmetrically to expand in number during development. Therefore, seam cells can provide a good model for the study of stem cells (Mizumoto and Sawa, 2007b).

Phospholipids, in addition to acting as structural components of cell membrane, also regulate many biological processes by acting as lipid mediators, second messengers and subcellular microenvironment. In many cases, these functions are mediated by a diverse group of phospholipases (PLs) that are classified into four groups (PLA, PLB, PLC and PLD) according to the bond hydrolysed on phospholipid substrates. PLA is represented by the two isoenzymes PLA_1 and PLA_2 that differ in the fatty acid they remove from a glyceride; PLA_1 hydrolyses *sn*-1 fatty acids attached to phospholipids to produce 2-acyl-lysophospholipids, whereas PLA_2 hydrolyses *sn*-2 fatty acids. PLA_2 enzymes, the largest group of PLs, have essential functions in the generation of lipid mediators such as prostaglandins and leukotrienes. In contrast, the molecular identity of PLA_1 enzymes was not clarified until recently (Higgs *et al*, 1998). In mammals, at least three members of the intracellular PLA_1 family exist, namely PA-PLA_1, KIAA0725 and p125, none of which show sequence homology with other known PLs (Inoue and Aoki, 2006). Intracellular PLA_1 is highly conserved in a wide range of eukaryotic organisms from yeast, plants to mammals. However, most of their physiological functions remain to be elucidated. Our laboratory is currently interested in studying the intracellular PLA_1 family. Using a reverse genetic approach, we show here an intracellular PLA_1, *ipla-1*, as a regulator of asymmetric divisions in *C. elegans*. Disruption of the *ipla-1* gene causes defects in spindle orientation, asymmetric cell-fate determination and asymmetric cortical localization of WRM-1 in the terminal asymmetric divisions of seam cells. We have also used a forward genetic approach to identify genes that functionally interact with *ipla-1* in the asymmetric divisions, and provide evidence to suggest that *ipla-1* regulates membrane trafficking to control cortical asymmetry of WRM-1 during the terminal asymmetric divisions of seam cells.

Results

Isolation and initial characterization of ipla-1 mutants

Our database searches identified one intracellular PLA_1 family member, which we named *ipla-1*, in *C. elegans*. *ipla-1* is predicted to encode a protein of 840 amino acids, including a serine esterase consensus sequence motif (GxSxG) and the DDHD domain, and shows 31% identity to human PA-PLA_1 (Supplementary Figure 1B and C).

To investigate the functional roles of *ipla-1*, we isolated two *ipla-1* deletion alleles, designated *ipla-1(xh13)* and *ipla-1(tm471)*, by PCR-based screening of UV-TMP-mutagenized libraries. The *xh13* allele deleted 1138 bp encoding exons 6 and 7, whereas *tm471* harboured a 1172 bp deletion at exon 8 (Supplementary Figure 1A). None of the two alleles of *ipla-1* showed detectable IPLA-1 protein by western blot, suggesting that they are strong loss-of-function or null alleles (Supplementary Figure 1D). These two *ipla-1* mutants were viable and fertile; however, some of *ipla-1* mutants exhibited vulval defects, including a protruding vulva and occasional vulval bursting (Supplementary Figure 2A and B). Because *xh13* and *tm471* were phenotypically indistinguishable in the initial characterization, we used *ipla-1(xh13)* mainly in subsequent analyses.

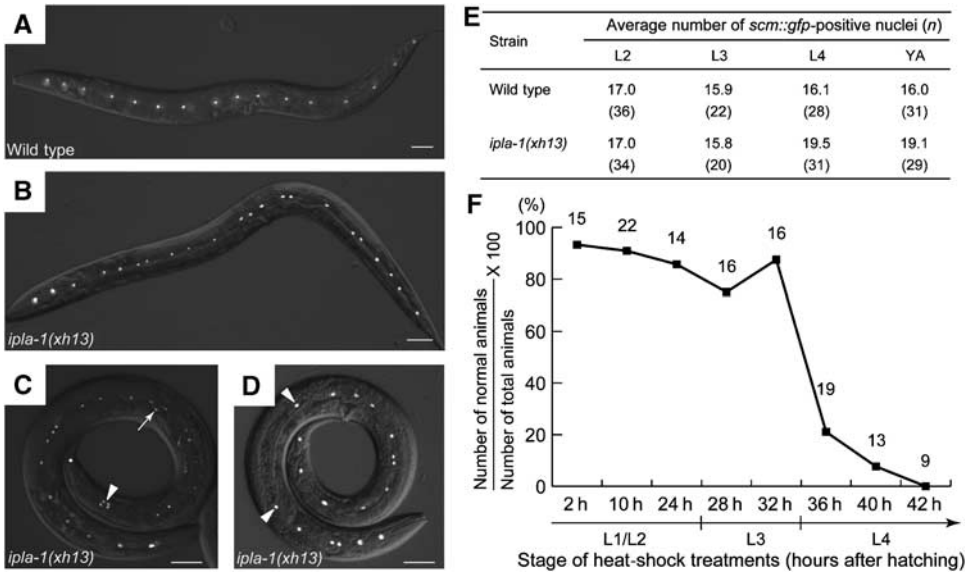

Figure 1 *ipla-1* mutants have aberrant number and alignment of seam cells. (A–D) Seam cells in adult hermaphrodites were visualized by a *seam cell marker::gfp* (*scm::gfp*) fusion. (**A**) Wild-type. Evenly spaced 16 *scm::gfp*-positive nuclei are observed. (**B–D**) *ipla-1(xh13)*. More than 16 seam cell nuclei align unevenly. *scm::gfp*-positive nuclei are occasionally observed outside the row of seam cells (C, arrow). *ipla-1(xh13)* mutants show loss of *scm::gfp*-positive nuclei (D, delimited by arrowheads). (**E**) The number of *scm::gfp*-positive nuclei in each developmental stage. On average, more *scm::gfp*-positive nuclei were observed in *ipla-1(xh13)* than in wild-type animals at the late L4 stage and the young adult stage (YA). L2 cell counts were often high by one cell because of the comparatively late division of cells of the T lineage, causing both daughters to be scored. The numbers in parentheses indicate the number of animals scored. (**F**) Stage-specific rescue of seam cell defects of *ipla-1(xh13)*. Synchronized *ipla-1(xh13)* animals carrying *hsp::ipla-1* were heat shocked at various developmental times. Rescue was obtained by heat-shock treatments before the L4 stage. The number above each point is the number of animals scored. Scale bars are 50 μm in (A–D).

ipla-1 expression in seam cells is sufficient for proper morphology of the vulva

The vulva is formed from the descendants of three vulval precursor cells (VPCs). The fates of the VPCs are specified by an inductive signal from the gonadal anchor cell, which appears to be controlled by an inhibitory signal from the major hypodermal syncytium (hyp7) (Sternberg, 2005). In addition, recent studies have suggested that maintenance of seam cells is important for structural integrity of the vulva (Pellis-van Berkel *et al*, 2005; Smith *et al*, 2005). To identify the cells in which *ipla-1* is required for proper morphology of the vulva, we expressed an *ipla-1* minigene in subsets of these cells using specific promoters. Proper vulval morphology was restored to *ipla-1* mutants by expression of *ipla-1* with the *dpy-7* promoter, which drives expression in hyp7 and weakly in seam cells (Gilleard *et al*, 1997), and with the seam cell-specific *scm* promoter (Koh and Rothman, 2001). In contrast, expression of *ipla-1*, under the control of either the hyp7-specific *egl-15* enhancer elements (Huang and Stern, 2004) or the VPC-specific *lin-31* promoter (Tan *et al*, 1998) failed to rescue the vulval defects (Supplementary Figure 2C). These results suggest that defects in seam cells cause abnormal vulval morphogenesis in *ipla-1* mutants.

ipla-1 functions cell-autonomously to regulate the terminal asymmetric divisions of seam cells

The lateral seam cells are specialized epithelial cells. During each larval stage, seam cells divide asymmetrically in a stem cell-like manner producing an anterior daughter cell that fuses with hyp7 and loses the expression of the seam cell marker (*scm::gfp*, transgene *wIs51*), and a posterior daughter cell that assumes the seam cell fate again and continues to express *scm::gfp* (Figure 2A and D).

To understand the nature of seam cell defects in *ipla-1* mutants, we first analysed the number of seam cells using *scm::gfp*. Wild-type adult hermaphrodites usually contain evenly spaced 16 *scm::gfp*-positive nuclei on each side of the animals, derived from the 10 embryonically derived blast cells H0, H1, H2, V1-6 and T (Figures 1A and 2A). *ipla-1* mutants, by contrast, contained more than 16 unevenly spaced seam cell nuclei (Figure 1B–D; Table I, see group A), some of which lay outside a row of seam cells (Figure 1C, arrow), and some of which lay close to each other (Figure 1C, arrowhead). Furthermore, *scm::gfp*-positive nuclei were occasionally lost (Figure 1D, arrowheads), and in such area seam cells were separated by gaps as indicated by a break in *ajm-1::gfp*, *cdh-3::gfp* and lateral alae (Supplementary Figure 3). To determine the cause of the missing or additional seam cells in *ipla-1* mutants, we then followed the development of seam cells during each larval stage. Although *ipla-1* mutants showed normal number and alignment of seam cells during the L2 and L3 stages, they exhibited aberrant number and alignment of seam cells at the L4 stage (Figure 1E). This suggests that *ipla-1* expression is required for the terminal asymmetric divisions of seam cells that occur at the beginning of the L4 stage, which we call hereafter the 'S4 divisions' (Seam cell divisions at the L4 stage). To confirm this idea, we expressed *ipla-1* cDNA by the heat-shock promoter at specific developmental stages. We found that heat-shock treatments before but not after the S4 divisions (even at the end of the L3 stage) restored proper number and alignment of seam cells to *ipla-1* mutants (Figure 1F). Thus, presence of IPLA-1 protein during the S4 divisions is sufficient for proper number and alignment of seam cells at the adult stage.

We next conducted cell-specific rescue experiments to confirm the cell autonomy of *ipla-1* function. The seam cell defects were fully rescued by expression of *ipla-1* under the *dpy-7* promoter and the seam cell-specific *scm* promoter. No rescue was obtained when *ipla-1* was expressed with the hyp7-specific *egl-15* enhancer elements (Table I, see group A). Furthermore, expression of catalytically inactive mutant IPLA-1 (*ipla-1* S489A, see Supplementary data, Supplementary Figure 1A and C, for details) by the *scm* promoter did not rescue the seam cell defects of *ipla-1* mutants (Table I, see group A). These results indicate that *ipla-1* functions cell-autonomously in seam cells to regulate their S4 divisions through its enzymatic activity.

ipla-1 is required for spindle orientation and cell-fate determination in the S4 divisions

We next analysed seam cells undergoing the S4 divisions and performed lineage analyses. In wild-type animals, the S4 divisions invariably occurred parallel to the anterior–posterior axis (A–P axis) (Figure 2B). In *ipla-1* mutants, however, orientation of the S4 divisions was essentially randomized relative to the A–P axis (asterisks in Figure 2C). To quantify this defect, we measured the angle between a line connecting the two daughter nuclei and the A–P axis as depicted in Figure 2F (see Supplementary data). In wild-type animals, the measured angle was always less than 10° ($n = 110$; Figure 2G). In *ipla-1* mutants, the majority of seam cells underwent the S4 divisions in various directions (52%, $n = 107$; Figure 2H). These results suggest that *ipla-1* mutants have defects in spindle orientation during the S4 divisions. Furthermore, detailed lineage analyses revealed that in *ipla-1* mutants, the asymmetry of the divisions was often disrupted (62%, $n = 53$), leading to the transformation of the anterior daughters from hyp7 cells to seam cells or to the reversal of these cell fates (Figure 2E and I). It is noted that seam cells that divided normally in the A–P direction did have defects in the asymmetry of the divisions (Figure 2I; types I and II), indicating that defects in cell-fate determination are not secondary to the spindle orientation phenotype. We conclude that *ipla-1* mutants are defective in both spindle orientation and cell-fate determination in the S4 divisions.

ipla-1 regulates the cortical asymmetry of β-catenin wrm-1 prior to the S4 divisions

In *C. elegans*, many asymmetric divisions are controlled by the Wnt/β-catenin asymmetry pathway, including β-catenin *wrm-1* and TCF *pop-1* transcription factor (Mizumoto and Sawa, 2007b). In several asymmetric divisions, including those of seam cells at the L1 stage, WRM-1 localizes asymmetrically to the anterior cortex in mother cells (Nakamura *et al*, 2005; Takeshita and Sawa, 2005; Mizumoto and Sawa, 2007a). After the asymmetric divisions, the anterior daughters have more nuclear GFP::POP-1 than posterior daughters. This phenomenon has been dubbed 'POP-1 asymmetry' (Lin *et al*, 1998).

To test whether *ipla-1* mutation influences the Wnt/β-catenin asymmetry pathway in the S4 divisions, we determined the subcellular localization of WRM-1::GFP and GFP::POP-1. In wild-type animals, WRM-1::GFP always localized to the anterior cortex in seam cells before the S4

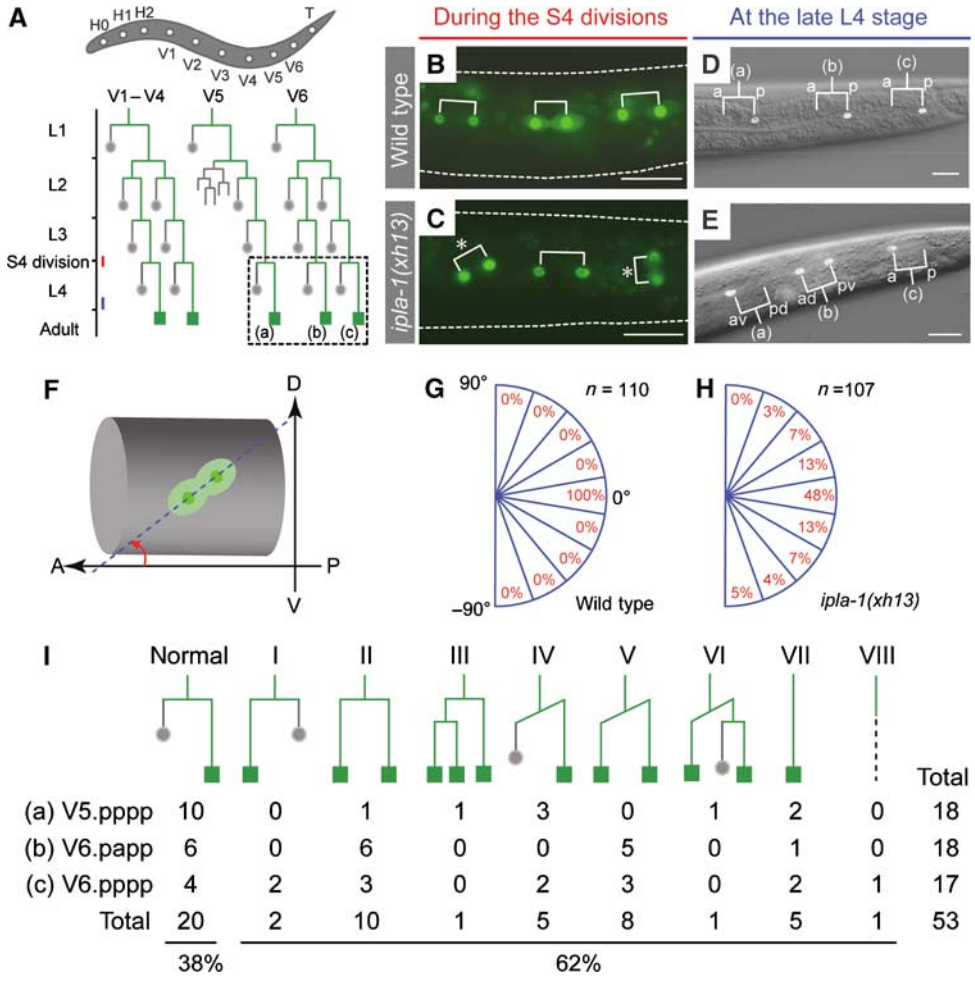

Figure 2 *ipla-1* mutants disrupt the orientation and polarity of the S4 divisions. (**A**) A schematic drawing of seam cell lineages. The V lineage seam cells are shown. Lineage branches expressing *scm::gfp* are shown in green. The dotted box marks the lineages analysed in this study; V5.pppp lineage (a), V6.papp lineage (b) and V6.pppp lineage (c). Grey circles, anterior daughters that fuse with the hyp7; green squares, posterior daughters that assume the seam cell fate again. Red and blue lines indicate the developmental stages corresponding to those of (B, C), and (D, E), respectively. (**B**, **C**) Fluorescent images of seam cells during or just after the S4 divisions visualized by *scm::gfp*. Three pairs of daughter cells are shown with brackets for each panel. (B) Wild-type. The three seam cells divide parallel to the A–P axis. (C) *ipla-1(xh13)*. Seam cells, as shown by asterisks, divide in various directions. (**D**, **E**) Seam cells of late L4 hermaphrodite visualized by *scm::gfp*. (D) Wild-type. The posterior daughters assume the seam cell fate in all the three lineages. (E) *ipla-1(xh13)*. The seam cell fates are adopted by either or both of the anterior and posterior daughters and the mitotic spindle is misoriented. The seam cell lineages are shown in the images. The letters (a), (b) and (c) correspond to those of (A). Results are summarized in (I). The characters indicate the directions of the S4 divisions; a, anterior; p, posterior; av, anterior-ventral; ad, anterior-dorsal; pv, posterior-ventral and pd, posterior-dorsal. (**F**) Schematic for measurement of spindle orientation. A, anterior; P, posterior; D, dorsal; and V, ventral. (**G**, **H**) Quantification of spindle orientation of the S4 divisions. The percentages are determined from a random sample of angles from wild-type (G) or *ipla-1(xh13)* (H) seam cells measured as depicted in (F). (**I**) Abnormal V-cell lineages in *ipla-1(xh13)* after the S4 divisions. The numbers of seam cells that showed the lineages are indicated below the diagrams. The letters (a), (b) and (c) correspond to those of (A). Spindle orientation is defective in types IV–VI (indicated with diagonal lines). Additional cell divisions occur just after the first divisions in types III and VI. The S4 divisions do not occur in types VII and VIII. The dotted line in type VIII indicates that the *scm::gfp* fluorescence disappears during the L4 stage. Anterior is oriented towards the left. Scale bars in (B–E) are 20 μm.

divisions ($n = 21$; Figure 3A, B and D). However, the cortical localization of WRM-1 was randomized in *ipla-1* mutants; WRM-1::GFP was symmetrically localized (22%), absent from the cortex (34%) and occasionally enriched posteriorly (6%) ($n = 34$; Figure 3C and D). We also found that *ipla-1* mutants had defects in the POP-1 asymmetry just after the S4 divisions; the levels of POP-1 were equally high in the two daughters (32%), and higher in the posterior daughters (36%) in *ipla-1* mutants ($n = 25$; Figure 3G and H), whereas the level of GFP::POP-1 was always higher in the anterior daughters in wild-type animals ($n = 19$; Figure 3E, F and H). The frequencies of these mislocalization phenotypes were similar to that of defects in cell-fate determination after the S4 divisions in *ipla-1* mutants (62%; Figure 2I). These results indicate that *ipla-1* is required for the formation and/or maintenance of cortical asymmetry of WRM-1 prior to the S4 divisions.

A genetic screen for mutations that suppress the ipla-1 seam cell defects

To understand the molecular mechanism underlying the regulation of cortical WRM-1 asymmetry mediated by *ipla-1*, we carried out genetic screens for mutations that suppress the seam cell defects of *ipla-1* mutants (see Supplementary data). In a screen of 3000 haploid mutagenized *ipla-1(xh13);wIs51* genomes, we isolated two strong suppressor

Table I Seam cell phenotypes

Group	Genotype	No. of seam cell nuclei	High	Low	*scm::gfp* alignment[a]		*N*	*P*-Values
A	N2	15.9	17	15	N	(0)	19	—
	ipla-1(xh13)	19.1	26	16	A	(100)	29	<0.0001[b]
	ipla(xh13);Ex[dpy-7p::ipla-1]	16.1	18	14	N	(8)	13	<0.0001[c]
	ipla(xh13);Ex[scm::ipla-1]	16.3	17	16	N	(0)	12	<0.0001[c]
	ipla(xh13);Ex[scm::ipla-1 S489A]	19.4	24	14	A	(100)	25	0.6489[c]
	*ipla(xh13);Ex[Pe15*2::ipla-1]*[d]	17.2	23	13	A	(100)	21	0.0050[c]
B	*mon-2(xh22)*	16.0	17	15	N	(0)	21	—
	ipla-1(xh13);mon-2(xh22)	16.2	17	14	N	(7)	18	<0.0001[c]
	ipla-1(xh13);mon-2(RNAi)[e]	16.2	18	14	N	(7)	28	<0.0001[c]
	ipla-1(xh13);mon-2(xh22);Ex[scm::mCherry::mon-2]	18.2	23	15	A	(94)	18	<0.0001[f]
	N2; *Ex[scm::mCherry::mon-2]*	16.0	17	15	N	(0)	26	0.7588[b]
C	*tbc-3(xh23)*	16.0	17	16	N	(0)	30	—
	ipla-1(xh13);tbc-3(xh23)	15.9	18	15	N	(4)	24	<0.0001[c]
	ipla-1(xh13);tbc-3(RNAi)[e]	16.2	17	15	N	(0)	16	<0.0001[c]
	ipla-1(xh13);tbc-3(xh23);Ex[scm::mCherry::tbc-3a]	16.0	17	15	N	(0)	18	0.7709[g]
	ipla-1(xh13);tbc-3(xh23);Ex[scm::mCherry::tbc-3b]	18.5	21	15	A	(89)	19	<0.0001[g]
	N2; *Ex[scm::mCherry::tbc-3b]*	16.0	17	15	N	(0)	29	0.7588[b]
D	*sid-1(qt2)*	16.1	15	17	N	(0)	31	—
	ipla-1(xh13);sid-1(qt2)	20.4	26	14	A	(100)	27	—
	ipla-1(xh13);sid-1(qt2);Ex[scm::tat-5 RNAi]	15.8	17	15	N	(0)	14	<0.0001[h]
	*ipla-1(xh13);sid-1(qt2);Ex[Pe15*2::tat-5 RNAi]*[d]	18.5	21	15	A	(100)	18	0.0012[h]
	ipla-1(xh13);sid-1(qt2);Ex[scm::pad-1 RNAi]	15.9	16	15	N	(0)	11	<0.0001[h]
	*ipla-1(xh13);sid-1(qt2);Ex[Pe15*2::pad-1 RNAi]*[d]	18.8	23	14	A	(100)	15	0.0141[h]
	ipla-1(xh13);sid-1(qt2);Ex[scm::vps-35 RNAi]	16.1	18	15	N	(7)	30	<0.0001[h]
	*ipla-1(xh13);sid-1(qt2);Ex[Pe15*2::vps-35 RNAi]*[d]	18.7	23	15	A	(92)	25	0.0012[h]

[a]Percentage of animals with aberrant alignment is shown in parentheses; N, normal; A, aberrant.
[b]Compared with N2.
[c]Compared with *ipla-1(xh13)*.
[d]*Pe15*2* represents the tandem *egl-15* enhancer elements (Huang and Stern, 2004).
[e]Feeding RNAi.
[f]Compared with *ipla-1(xh13); mon-2(xh22)*.
[g]Compared with *ipla-1(xh13); tbc-3(xh23)*.
[h]Compared with *ipla-1(xh13); sid-1(qt2)*.
All strains contain seam cell marker *wIs51(scm::gfp)*. The number of *scm::gfp*-positive nuclei was scored in young adult hermaphrodites.

mutations. The two recessive suppressor alleles, *xh22* and *xh23*, mapped to chromosome IV and did complement each other, indicating that they identify two distinct loci. On their own, these suppressor alleles displayed wild-type seam cell number and alignment and had no obvious morphological defects (Table I, see groups B and C; and data not shown). However, they effectively suppressed the seam cell phenotype of *ipla-1* mutants with almost complete penetrance (Table I, see groups B and C). This suppression was not simply due to cell cycle arrest or reducing seam cell number before the S4 divisions, because evenly spaced seam cells divided asymmetrically and parallel to the A–P axis in both *sup* and *ipla-1;sup* double mutants during the S4 divisions (Figure 4F and data not shown; *sup* refers to any suppressor mutation). Notably, both *ipla-1; xh22* and *ipla-1; xh23* double mutants had normal vulvae, supporting the notion that abnormal vulval morphology is caused by the seam cell defects in *ipla-1* mutants (Supplementary Figure 2 and data not shown).

ipla-1 suppressor mutations affect the Arf GEF-like *mon-2* and the Rab GAP *tbc-3*

We mapped *xh22* and *xh23* to 0.5-cM intervals on the right arm of chromosome IV. Of the genes predicted to reside in each region, inactivation of *mon-2* and *tbc-3* by RNAi gave rise to suppression of *ipla-1* seam cell defects (Table I, see groups B and C; see Supplementary data). We subsequently found by germline transformation experiments that *mon-2* and *tbc-3* genes can rescue the suppression phenotype in *ipla-1;xh22* and *ipla-1;xh23*, respectively (see Table I groups B and C, and below for rescue by mCherry fusion genes).

mon-2 encodes a homologue of the yeast Mon2p, which is highly conserved through evolution and is related to the GBF and BIG family ADP ribosylation factor (Arf) guanine nucleotide exchange factors (GEFs) (D'Souza-Schorey and Chavrier, 2006) over much of its length but lacks the catalytic domain (Figure 4E) (Efe *et al*, 2005; Gillingham *et al*, 2006). Sequencing of the *mon-2* gene in *xh22* mutants identified a nonsense mutation in the middle of the protein (Trp787Stop) (Figure 4D and E). This mutation would result in loss of the C-terminal region that is reported to be essential for yeast Mon2p function (Efe *et al*, 2005). Meanwhile, *tbc-3* encodes a member of the highly conserved TBC (Tre-2, Bub2 and Cdc16) family of GTPase-activating proteins (GAPs) specific for Rab/Ypt GTPase (Lafourcade *et al*, 2004), and is expressed as two alternatively spliced forms, *tbc-3a* and *tbc-3b* according to the information in the *C. elegans* EST database (http://www.wormbase.org/) (Figure 4A and B). *xh23* mutants harboured a missense mutation in the TBC domain (Asp234Asn) in the *tbc-3* gene. This Asp residue is three residues next to the catalytic Arg237 and is completely conserved among the TBC family proteins (Figure 4C) (Pan *et al*, 2006). On the basis of molecular nature of the mutations

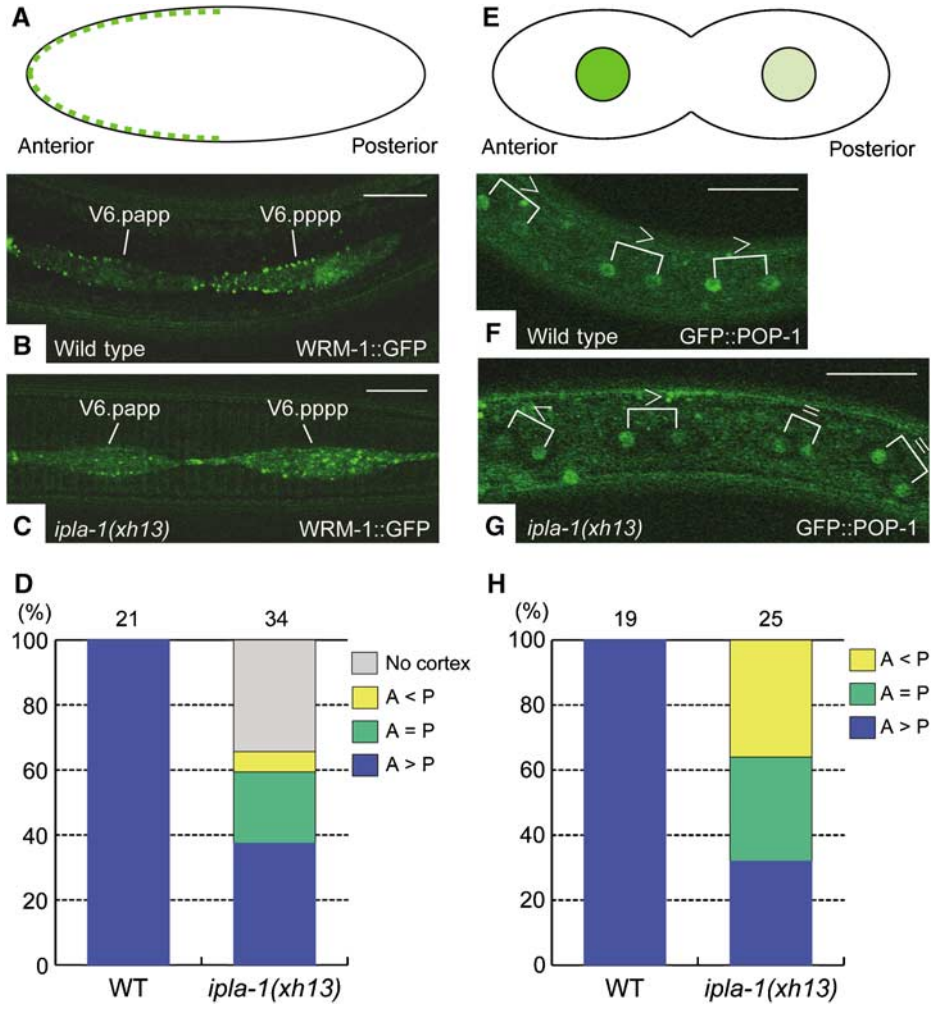

Figure 3 *ipla-1* regulates polarity of the seam cells in the S4 divisions. (**A**) Schematic for the seam cells before the S4 divisions. WRM-1 localization is shown in green; WRM-1 localizes to the anterior cortex of the seam cells. (**B**, **C**) Confocal images showing the localization of WRM-1::GFP in the V6.papp and V6.pppp cell of wild-type (B) and *ipla-1(xh13)* (C). (**D**) Frequency of the localization patterns of WRM-1::GFP. The number of samples is shown above each column. The equality and inequality signs indicate relative intensities of cortical WRM-1::GFP. A, intensity of WRM-1::GFP at the anterior cortex; P, intensity of WRM-1::GFP at the posterior cortex. No cortex means that WRM-1::GFP signal is absent from the cortex of the cells. (**E**) Schematic for the seam cells just after the S4 divisions. POP-1 localization is shown in green; the anterior daughters contain more nuclear POP-1 than posterior daughters. (**F**, **G**) Confocal images showing the localization of GFP::POP-1 in wild-type (F) and *ipla-1(xh13)* (G). Pairs of daughter nuclei are shown with brackets for each panel. The equality and inequality signs indicate relative intensities of GFP::POP-1 levels between two daughters. (**H**) Frequency of the localization patterns of GFP::POP-1. The number of samples is shown above each column. A, intensity of nuclear GFP::POP-1 in the anterior daughter; P, intensity of nuclear GFP::POP-1 in the posterior daughter. Scale bars are 10 μm in (B, C), or 20 μm in (F, G). Anterior is oriented towards the left; ventral is oriented towards the bottom.

and the fact that reduction of *mon-2* and *tbc-3* by RNAi resulted in suppressing the seam cell phenotype of *ipla-1* mutants (Table I, see groups B and C), we conclude that suppression of *ipla-1* seam cell defects is due to loss or strong reduction of function in these genes.

mon-2 and tbc-3 function cell-autonomously in seam cells

As mentioned above, *ipla-1* functioned cell-autonomously in seam cells (Table I, see group A). To understand in which cells *mon-2* and *tbc-3* interact with *ipla-1*, we performed rescue experiments using the *scm* promoter in the *ipla-1;sup* backgrounds. Expression of *mon-2* and *tbc-3b* fully rescued the suppression of the *ipla-1* mutant phenotype by *mon-2* and *tbc-3* mutations, respectively (Table I, see group B and C, respectively). In contrast, no rescue was obtained by expression of *tbc-3a* in *ipla-1;tbc-3* mutants. These results are consistent with our observation that *tbc-3b* but not *tbc-3a* is expressed in seam cells during the S4 divisions (Supplementary Figure 4). Taken together, all these results indicate that *mon-2* and *tbc-3* function cell-autonomously in seam cells for their interactions with *ipla-1*.

Reducing the expression of the retrograde transport pathway genes restores proper subcellular localization of WRM-1 to ipla-1 mutants

In yeast, the homologues of *mon-2* (Mon2p) and *tbc-3* (Gyp1p) are required for endosome-to-Golgi retrograde transport (Lafourcade *et al*, 2004; Efe *et al*, 2005; Gillingham *et al*, 2006). These previous reports led us to hypothesize that reducing the function of the retrograde transport pathway suppresses the seam cell phenotype of *ipla-1* mutants. To test this possibility, we knocked down the expression of genes whose homologues are known to regulate endosome-to-Golgi

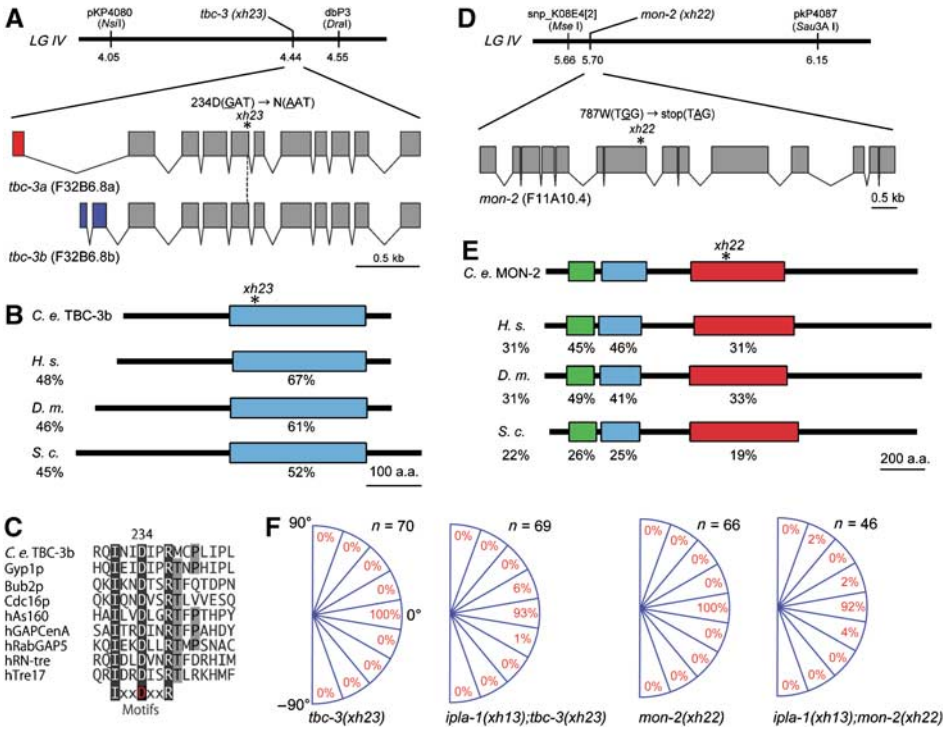

Figure 4 Structure of the *ipla-1* suppressor locus, gene and protein. (**A**) *xh23* locus. *xh23* mutation maps to *LG IV* between snip-SNPs markers pkP4080 and dbP3. Boxes, exons; lines, introns; red, an exon specific for *tbc-3a*; blue, exons specific for *tbc-3b*. *xh23* is a G-to-A base change that generates a missense mutation predicted to change an aspartic acid-234 to asparagine. (**B**) Domain structure, mutation and multiple sequence alignment of TBC-3b and TBC1D22 homologues. TBC domains, light blue. The overall amino-acid identities between TBC-3b and its homologues in human, *Drosophila* and yeast are shown as well as the identities between the TBC domains. *C. e.*, *C. elegans* F32B6.8b (NP_001023165); *H. s.*, *Homo sapiens* TBC1D22B (NP_060242); *D. m.*, *Drosophila melanogaster* CG5745-PA (NP_650941); *S. c.*, *Saccharomyces cerevisiae* Gyp1p (NP_014713). (**C**) Multiple sequence alignment of Rab GAP proteins. The aspartic acid residue, which is mutated in *xh23*, is completely conserved across the Rab GAP family and indicated in red (Pan *et al*, 2006). (**D**) *xh22* locus. *xh22* mutation maps to *LG IV* between snipSNP markers snp_K08E4[2] and pkP4087. Boxes, exons; lines, introns. *xh22* is a G-to-A base change that predicts translation of a stop codon instead of tryptophan-787. (**E**) Domain structure, mutation and multiple sequence alignment of MON-2 and Mon2 homologues. DCB domain, green; HUS domain, light blue; HDS1-3 domain, red (Mouratou *et al*, 2005). The overall amino-acid identities between MON-2 and its homologues in human, *Drosophila* and yeast are shown as well as the identities between the conserved domains. *C. e.*, *C. elegans* F11A10.4 (NP_502295); *H. s.*, *Homo sapiens* MON2 homologue (NP_055841); *D. m.*, *Drosophila melanogaster* CG8683-PA (NP_001033884); *S. c.*, *Saccharomyces cerevisiae* Mon2p (NP_014102). (**F**) Quantification of spindle orientation in *tbc-3(xh23)*, *ipla-1(xh13);tbc-3(xh23)*, *mon-2(xh22)* and *ipla-1(xh13);mon-2(xh22)*. The angles are measured as depicted in Figure 2F.

retrograde transport in other species (Bonifacino and Rojas, 2006). We also reduced the expression of the genes whose homologues show physical or genetic interactions with Mon2p and Gyp1p in yeast (Lafourcade *et al*, 2004; Wicky *et al*, 2004; Efe *et al*, 2005; Gillingham *et al*, 2006).

We tested 50 genes listed in Supplementary Table 1 and identified seven genes that could suppress the seam cell phenotype of *ipla-1* mutants. Among the newly identified suppressor genes listed in Table II, the functions of four genes have been previously well described: *vps-26*, *vps-29*, *vps-35* and *snx-1*. The proteins encoded by these four genes form a large complex, termed the retromer complex, which is involved in retrograde transport of intracellular sorting receptors such as yeast Vps10p and mammalian mannose 6-phosphate receptors from endosomes to the trans-Golgi network (TGN) (Seaman, 2005). *snx-3* is a *C. elegans* homologue of yeast Grd19p, which is required for the sorting of the t-SNARE Pep12p and the aminopeptidase Ste13p from late endosomes to the TGN (Voos and Stevens, 1998; Hettema *et al*, 2003). *tat-5* and *pad-1* are *C. elegans* homologues of yeast Neo1p (a putative aminophospholipid translocase) and Dop1p (a member of the Dopey leucine zipper-like family), respectively, both of which interact physically with Mon2p

Table II Genetic suppressors of *ipla-1* mutants

Systematic name	Standard name	Structural description	Cell autonomy
F11A10.4	*mon-2*	Arf GEF-like protein	Yes
F32B6.8	*tbc-3*	Rab GAP protein (TBC domain)	Yes
T20D3.7	*vps-26*	Retromer complex	Yes[a]
ZK1128.8	*vps-29*	Retromer complex	ND[b]
F59G1.3	*vps-35*	Retromer complex	Yes
C05D9.1	*snx-1*	Retromer complex (sorting nexin)	ND[b]
W06D4.5	*snx-3*	Sorting nexin	Yes[a]
F36H2.1	*tat-5*	Putative aminophospholipid translocase	Yes
Y18D10A.13	*pad-1*	Dopey family Leucine zipper-like	Yes

[a]Data not shown.
[b]ND, not determined.

(Wicky *et al*, 2004; Efe *et al*, 2005; Gillingham *et al*, 2006). We also knocked down the expression of genes involved in other transport pathways and found that *ipla-1* mutant

phenotype was not suppressed by reduction of such genes (Supplementary Table 1 and data not shown). Next, we investigated the cell autonomy of these suppressor genes by tissue-specific RNAi strategy using RNAi-spreading defective *sid-1* mutants (see Supplementary data). We knocked down the expression of each suppressor gene (*tat-5*, *pad-1* and *vps-35*) in seam cells as well as in hyp7 in a cell-specific manner, and found that, in addition to *mon-2* and *tbc-3*, these three genes functioned cell-autonomously in seam cells (Table I, see group D). Finally, we studied the subcellular localization of WRM-1 in *ipla-1;sup* backgrounds (*sup* refers to any suppressor gene). Knockdown of each suppressor gene (*mon-2*, *tbc-3* and *snx-1*) in *ipla-1* mutants revealed that in all cases, WRM-1 localized properly to the anterior cortex of seam cells prior to the S4 divisions (Figure 5A–D). Taken together, we conclude that WRM-1 mislocalization phenotype in *ipla-1* mutants depends on the presence of the endosome-to-Golgi retrograde trafficking genes in seam cells.

Discussion

The assembly of Wnt signalling components at specific subcellular locations is an important aspect of asymmetric divisions in *C. elegans*. We described here a previously poorly characterized phospholipase, *ipla-1*, that regulates the subcellular localizations of β-catenin *wrm-1* in the terminal asymmetric divisions of the stem cell-like epithelial seam cells (the S4 divisions). We also showed that *ipla-1* genetically interacts with a group of genes involved in endosome-to-Golgi retrograde trafficking, including the retromer complex genes. Our results strongly support the notion that *ipla-1* functions as a regulator of membrane trafficking in the polarization of β-catenin *wrm-1* before asymmetric divisions.

We have shown that *ipla-1* mutants were affected in cell-fate specification after the S4 divisions. The major cause of this phenotype appears to be misregulation of cortical polarity of β-catenin *wrm-1* prior to the S4 divisions, which is proposed to be translated into asymmetry of nuclear TCF *pop-1* level between two daughter cells (POP-1 asymmetry) (Mizumoto and Sawa, 2007b). Indeed, the POP-1 asymmetry was also disrupted in *ipla-1* mutants.

We provided two lines of evidence indicating the involvement of endosome-to-Golgi retrograde trafficking in the formation and/or maintenance of cortical asymmetry of β-catenin *wrm-1*. First, cell polarity defects of *ipla-1* mutants were suppressed by mutations of the two genes, Arf GEF-like *mon-2* and Rab GAP *tbc-3*, both of which are reported to be involved in endosome-to-Golgi retrograde transport (Lafourcade *et al*, 2004; Efe *et al*, 2005; Gillingham *et al*, 2006). Although the yeast homologue of *mon-2* is reported to

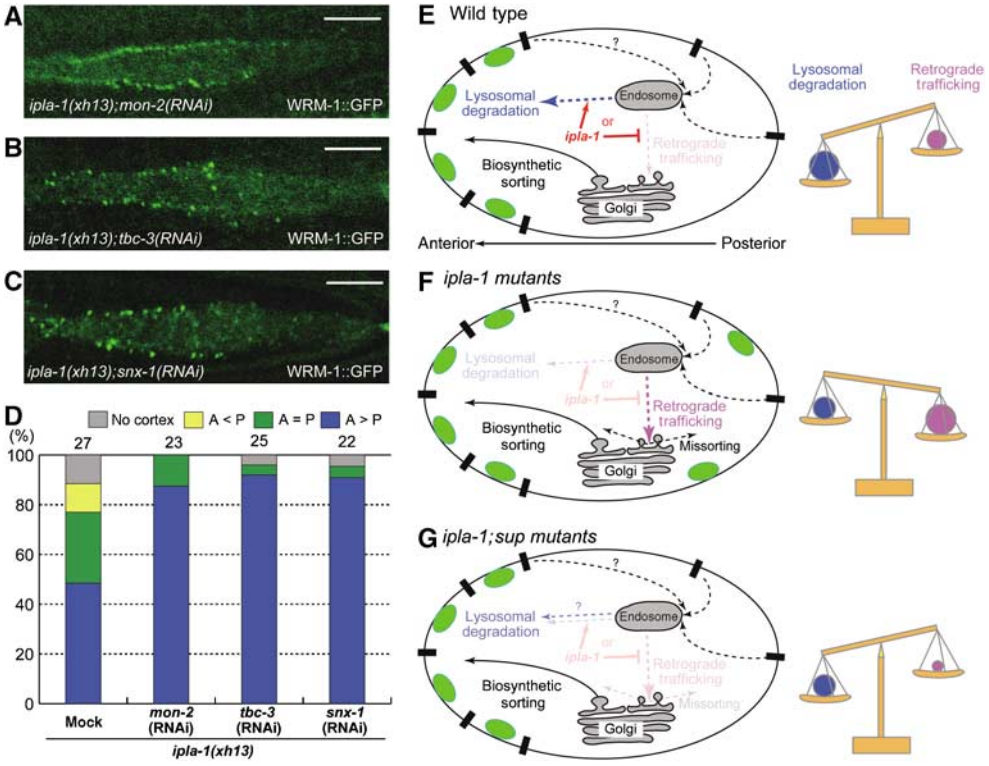

Figure 5 Retrograde trafficking is involved in cortical asymmetry of WRM-1 before the S4 divisions. (**A**–**C**) Confocal images showing the localization of WRM-1::GFP in the V6.pppp cell of *ipla-1(xh13);mon-2(RNAi)* (A), *ipla-1(xh13);tbc-3(RNAi)* (B) and *ipla-1(xh13);snx-1(RNAi)* (C). Scale bars are 5 μm. (**D**) Frequency of the localization patterns of WRM-1::GFP. The number of samples is shown above each column. (**E**) Wild-type. The A–P polarity is formed through biosynthetic sorting from the Golgi (black arrow), and is maintained by protein degradation pathway (blue dotted arrow); proteins on plasma membrane (black boxes) are endocytosed and degraded through an *ipla-1*-dependent mechanism (black dotted arrows). *ipla-1* promotes degradation pathway directly, or indirectly by inhibiting the endosome-to-Golgi retrograde pathway that requires the retromer complex, *mon-2* and *tbc-3b* (purple dotted arrow). As a result, β-catenin *wrm-1* localizes to the anterior cortex (green ellipses). (**F**) *ipla-1* mutants. The endocytosed proteins are not degraded, but transported to the Golgi by the retromer, *mon-2* and *tbc-3b*, and then missorted to various plasma membrane domains. As a result, cortical asymmetry of β-catenin *wrm-1* is disturbed. (**G**) *ipla-1;sup* double mutants, or *ipla-1;sup* (RNAi). The endocytosed proteins are retained in the endosome or progress towards the lysosome through an *ipla-1*-independent mechanism (shown with an additional blue dotted arrow).

function in the cytoplasm-to-vacuole transport pathway (the Cvt pathway) besides retrograde trafficking (Efe *et al*, 2005), *ipla-1* seam cell defects were not suppressed by knockdown of other Cvt pathway genes (Supplementary Table 1, see group F). Second, knockdown of the retromer complex genes (*vps-26*, *vps-29*, *vps-35* and *snx-1*) also resulted in suppression of WRM-1 mislocalization phenotype of *ipla-1* mutants. The retromer is a coat-like complex that is required for efficient retrograde traffic from the endosome to the Golgi (Seaman, 2005). Cell-specific RNAi experiments revealed that the retrograde trafficking acts cell-autonomously in seam cells to modulate the asymmetric cortical localization of WRM-1.

The asymmetric distribution of proteins on distinct plasma membrane domains is a fundamental property of polarized epithelial cells and is formed in part by biosynthetic sorting from the Golgi (Mostov *et al*, 2003; Rodriguez-Boulan *et al*, 2005). In addition, a recent study has demonstrated that lysosomal degradation of endocytosed proteins is important for maintenance of cell polarity; a failure in endosomal entry and progression towards lysosomal degradation cause an expansion of the apical determinant proteins to basolateral surfaces in *Drosophila* epithelial cells (Lu and Bilder, 2005). Meanwhile, the endosome-to-Golgi retrograde transport pathway genes have roles in preventing lysosomal sorting and degradation of the cargo proteins (Arighi *et al*, 2004; Lafourcade *et al*, 2004; Seaman, 2004); in other words, the retrograde transport and the lysosomal degradation are alternative pathways, and thus anything that directly affects one is likely to affect the other indirectly. We showed in this study that expression of the WRM-1 mislocalization phenotype in *ipla-1* mutants depends on the genes implicated in the retrograde transport pathway. In view of these results, we propose, as a possible explanation of our observations, that in wild-type seam cells *ipla-1* promotes degradation of endocytosed proteins directly, or indirectly by inhibiting the retrograde transport pathway, to maintain the cortical asymmetry of β-catenin *wrm-1* (schematized in Figure 5E). In *ipla-1* mutants, endocytosed proteins may not be degraded, but alternatively transported to the Golgi, and then mistransported to various plasma membrane domains (Figure 5F). When *sup* genes are mutated or inactivated in *ipla-1* mutants, such mistargeting may not occur and endocytosed proteins may be retained in the endosome or progress towards the lysosome, resulting in suppression of WRM-1 mislocalization phenotype (Figure 5G; *sup* refers to any suppressor gene listed in Table II). What are the proteins that are transported via an *ipla-1*-dependent mechanism in the S4 divisions? Possible candidates are the proteins that can mediate the cortical localization of β-catenin *wrm-1*. Interestingly, WRM-1::GFP is also localized as dots of fluorescence in the cytoplasm, which colocalizes with APC *apr-1* and Axin *pry-1* (Mizumoto and Sawa, 2007a), raising the possibility that vesicles containing these proteins are transported to the anterior cortex in mother cells. Other possible candidates are proteins that function upstream of β-catenin *wrm-1*, such as Dsh and Fz. Interestingly, Dsh *dsh-2* can physically interact with subunits of the clathrin adaptor AP-1, which participates in membrane traffic between the endosome and the Golgi (Yu *et al*, 2007). Further experiments are needed to investigate our hypothesis, but this work is the first, to our knowledge, to raise the possibility that membrane-trafficking machinery can modulate the Wnt/β-catenin asymmetry pathway in a cell-autonomous manner.

We also found that *ipla-1* mutants were defective in spindle orientation. It is believed that during asymmetric divisions, spindle positioning is governed by the interaction of astral microtubules with cortical factors that are localized asymmetrically in accordance with the polarity axis. In the asymmetric divisions of the EMS blastomere, spindle orientation is controlled by Fz *mom-5* and Dsh *dsh-2*, but not by downstream nuclear effectors such as β-catenin *wrm-1* and TCF *pop-1* (Schlesinger *et al*, 1999; Walston *et al*, 2004). In the present study, we have shown that the asymmetry of cell fates was abolished in the S4 divisions that occurred properly along the A–P axis in *ipla-1* mutants, which suggests that spindle orientation is controlled independently of the asymmetry of cortical β-catenin *wrm-1* and nuclear TCF *pop-1* in the S4 divisions. Given our results that spindle orientation phenotype of *ipla-1* mutants was also almost completely suppressed by the mutations in both *mon-2* and *tbc-3*, it is possible that a still unidentified cortical factor(s) which interacts with astral microtubules may be targeted by a similar mechanism to that of β-catenin *wrm-1*.

The retromer complex is reported to function non-cell-autonomously in Wnt-producing cells to control the polarity of the V5 seam cell division at the L1 stage, by creating a long-range gradient of Wnt *egl-20* (Coudreuse *et al*, 2006). In addition, very recent studies have revealed that the retromer promotes Wnt secretion by effectively recycling Wntless *mig-14*, a putative cargo receptor for Wnt, from the endosome to the Golgi (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Pan *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008). As mentioned above, we have demonstrated that the retromer complex genes functioned cell-autonomously in seam cells for controlling the polarity of the S4 divisions. Moreover, *ipla-1* seam cell defects were not suppressed by an *egl-20 (n585)* mutation or by RNAi of *mig-14* (Supplementary Figure 5), suggesting that suppression of the *ipla-1* mutant phenotypes is not due to reduced secretion of Wnt ligands. Thus, our findings show that the retromer complex not only functions in the Wnt-producing cells, but also in the Wnt-receiving cells.

The S4 divisions were specifically affected in *ipla-1* mutants. We did not observe any defects in three well-studied asymmetric divisions: those of the EMS blastomere, the somatic gonadal precursor cells and the T-blast cell (Supplementary Figure 6 and data not shown). We currently have little experimental data that can explain this specificity; it is unlikely that *ipla-1* is specifically expressed in seam cells at the timing of the S4 divisions as such stage-specific expression of IPLA-1 was not seen in our immunostaining experiments (Supplementary Figure 7 and Supplementary Movie 1); similarly to *ipla-1*, *tbc-3* is not specifically expressed in seam cells just prior to the S4 divisions but expressed in seam cells in the earlier stages (Supplementary Figure 4). Furthermore, previous studies have demonstrated that the expression of *vps-35,* one of the suppressor genes for *ipla-1* mutation, is not only limited to seam cells, but is also observed in body wall muscle, intestine, somatic gonad, pharynx and neuronal cells (Coudreuse *et al*, 2006; Prasad and Clark, 2006). During animal development, membrane traffic pathways are modified to accommodate the specific needs of individual cell types (Mostov *et al*, 2003). As seam

cells in the S4 divisions are larger in size as compared with those in the asymmetric divisions at early larval stages, an additional mechanism provided by *ipla-1* may be required for maintenance of the cortical polarity of β-catenin *wrm-1*.

In a genetic screen for shoot gravitropism mutants in *Arabidopsis*, a homologue of intracellular PLA_1 (SGR2) and a SNARE protein (SGR4) were found to be important for gravity sensing (Kato *et al*, 2002). A mammalian intracellular PLA_1 family member, p125 was previously shown to interact with the COPII component Sec23 (Tani *et al*, 1999). These studies, together with our findings, strongly suggest that *ipla-1* acts as a regulator of membrane trafficking. A key emerging question is how a lipid-metabolizing enzyme, intracellular PLA_1 controls membrane trafficking. It is notable that we identified *tat-5*, a putative aminophospholipid translocase, as a genetic suppressor of *ipla-1* mutants. Aminophospholipid translocases, which specifically transport aminophospholipids such as phosphatidylserine and phosphatidylethanolamine from the outer to the inner leaflet of the membrane, are believed to drive the formation of vesicles either by inducing the bending of membrane to facilitate vesicle budding, or by recruiting proteins for vesicle budding (Graham, 2004). It is tempting to speculate that *ipla-1* may control endosome-to-Golgi retrograde transport by hydrolysing the aminophospholipids that are transported to the inner leaflet by the activity of *tat-5*. To support this idea, we have recently shown that aminophospholipids are preferred substrates for intracellular PLA_1s (Nakajima *et al*, 2002; Morikawa *et al*, 2007).

Wnt signalling is implicated in the maintenance of stem cells that undergo asymmetric divisions (Aubert *et al*, 2002; Reya *et al*, 2003). Moreover, given *ipla-1*'s striking evolutionary conservation, as well as the conservation of all the suppressor genes identified in this study, our results raise the possibility that intracellular PLA_1 and the retrograde transport pathway genes control asymmetric divisions of stem cells in other animals.

Materials and methods

Strains and alleles

Maintenance and genetic manipulation of *C. elegans* were carried out as described (Brenner, 1974). The following mutations and integrated transgenes were used: *ipla-1(xh13, tm471)II*, *mon-2(xh22)IV*, *tbc-3(xh23)IV*, *sid-1(qt2)V*, *wIs51[scm::gfp]*, *osIs10[scm::wrm-1::venus]* and *qIs74[gfp::pop-1]*. Detailed information is included in the Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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