RESEARCH ARTICLE

The conserved transmembrane RING finger protein PLR-1 downregulates Wnt signaling by reducing Frizzled, Ror and Ryk cell-surface levels in C. elegans

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ABSTRACT

Wnts control a wide range of essential developmental processes, including cell fate specification, axon guidance and anteroposterior neuronal polarization. We identified a conserved transmembrane RING finger protein, PLR-1, that governs the response to Wnts by lowering cell-surface levels of the Frizzled family of Wnt receptors in Caenorhabditis elegans. Loss of PLR-1 activity in the neuron AVG causes its anteroposterior polarity to be symmetric or reversed because signaling by the Wnts CWN-1 and CWN-2 are inappropriately activated, whereas ectopic PLR-1 expression blocks Wnt signaling and target gene expression. Frizzleds are enriched at the cell surface; however, when PLR-1 and Frizzled are co-expressed, Frizzled is not detected at the surface but instead is colocalized with PLR-1 in endosomes. The Frizzled cysteine-rich domain (CRD) and invariant second intracellular loop lysine are crucial for PLR-1 downregulation. The PLR-1 RING finger and protease-associated (PA) domain are essential for activity. In a Frizzled-dependent manner, PLR-1 reduces surface levels of the Wnt receptors CAM-1/Ror and LIN-18/Ryk. PLR-1 is a homolog of the mammalian transmembrane E3 ubiquitin ligases RNF43 and ZNRF3, which control Frizzled surface levels in an R-spondin-sensitive manner. We propose that PLR-1 downregulates Wnt receptor surface levels via lysine ubiquitylation of Frizzled to coordinate spatial and temporal responses to Wnts during neuronal development.

KEY WORDS: Wnt signaling, Neuronal polarity, Membrane protein trafficking, C. elegans

INTRODUCTION

Wnts are secreted, hydrophobic glycoproteins that regulate a variety of developmental processes, including asymmetric cell division, cell fate determination and tissue polarity. Wnts interact with several cell-surface receptors, including Frizzleds, low-density lipoprotein receptor-related proteins (LRP)s, Ryk/Derailed tyrosine kinases and Ror tyrosine kinases (Angers and Moon, 2009). Wnt binding to Frizzled can activate a canonical β-catenin signaling cascade that controls gene transcription and several noncanonical signaling pathways that affect cytoskeletal organization.

Wnts are key organizers of the nervous system along the anteroposterior body axis of vertebrates and invertebrates and influence cell migration, neuronal polarity, axon guidance and synaptogenesis (Salinas and Zou, 2008). For example, in mouse, Wnts signal through Ryk to repel corticospinal tract axons toward the posterior (Liu et al., 2005) and act via Frizzled 3 to attract commissural axons toward the anterior after midline crossing (Lyuksyutova et al., 2003). In C. elegans, there are five Wnts, CWN-1, CWN-2, EGL-20, LIN-44 and MOM-2, and four Frizzleds, CFZ-2, LIN-17, MIG-1 and MOM-5. CWN-1 and EGL-20 act redundantly via the Frizzleds MIG-1 and MOM-5 to guide the processes of the AVM and PVM mechanosensory neurons toward the anterior (Pan et al., 2006; Prasad and Clark, 2006). Wnts also polarize neurons along the anteroposterior body axis. In select Wnt or Frizzled mutants, polarity of the mechanosensory neurons ALM or PLM is reversed: the anterior process adopts the length, branching pattern and synaptic characteristics of the wild-type posterior process and vice versa (Hilliard and Bargmann, 2006; Prasad and Clark, 2006). Lastly, Wnts direct long-range anteroposterior migrations of several neuroblasts; disruption or ectopic activation of Wnt signaling alters the final positions of these cells or their descendants (Whangbo and Kenyon, 1999; Zinovyeva and Forrester, 2005; Pan et al., 2006). The multifaceted role of Wnts during neuronal development illustrates dramatically the need for fine control over when and where Wnt signaling is activated.

Wnt signaling is regulated at several steps, from the processing and secretion of Wnts to modulation of the level and localization of Wnt receptors and downstream signaling molecules (Buechling and Boutros, 2011; Cruciat and Niehrs, 2013). Insufficient, excess or ectopic activation of Wnt signaling can lead to developmental defects or disease. Several regulators of Wnt receptor activity or surface levels have been identified. For example, the secreted Wnt inhibitor DKK1 acts by disrupting formation of the Wnt induced Frizzled-LRP6 complex (Semënov et al., 2001) and can induce LRP6 internalization when associated with Kremen (Mao et al., 2002). Correct folding and maturation of Wnt receptors is essential for release from the endoplasmic reticulum (ER). In Xenopus, the ER resident protein Shisa blocks both Wnt and FGF signaling by inhibiting maturation and export of Frizzled and the FGF receptor (Yamamoto et al., 2005). Conversely, the ER chaperone Boca/Mesd facilitates Wnt signaling by preventing aggregation of Arrow/LRP5/6 and permitting transport from the ER to the plasma membrane (Culi and Mann, 2003; Hsieh et al., 2003). Whereas Shisa and Boca/Mesd influence Wnt receptor transport to the surface, balanced ubiquitylation and deubiquitylation control Frizzled levels maintained at the surface (Mukai et al., 2010). Recently, the mammalian cell-surface transmembrane E3 ubiquitin ligases RNF43 and ZNRF3 were shown to downregulate the response to Wnts by inducing endocytosis of Frizzled and LRP6 (Hao et al., 2012; Koo et al., 2012). Inhibition of Wnt signaling by ZNRF3 is attenuated by the Wnt signaling enhancer R-spondin. R-
spondin triggers internalization and downregulation of ZNRF3 by inducing formation of a ternary complex with ZNRF3 and LGR4 (Hao et al., 2012).

We report here the identification and characterization of PLR-1, a C. elegans homolog of RNF43 and ZNRF3. Loss of plr-1 (cell polarity defective) causes the anteroposterior polarity of the interneuron AVG to be symmetric or reversed because of a failure to inhibit signaling by the Wnts CWN-1 and CWN-2, whereas ectopic PLR-1 expression blocks Wnt signaling and target gene expression. PLR-1 antagonizes Wnt signaling by lowering surface levels of Frizzled as well as the Wnt receptors CAM-1/Ror and LIN-18/Ryk in a Frizzled-dependent manner. The Frizzled cysteine-rich domain (CRD) and invariant second intracellular loop lysine are important for PLR-1 regulation. Our results define further the key features of Frizzleds needed for regulation and show that the PLR-1/RNF43/ZNRF3 family of transmembrane RING finger proteins found in vertebrates and invertebrates plays a conserved role in downregulating Wnt signaling.

RESULTS
PLR-1 controls anteroposterior neuronal polarity
We discovered plr-1 in a screen for mutations that disrupt anteroposterior polarity of the unpaired interneuron AVG using the green fluorescent protein (GFP) transcriptional reporter zdIs49[F59E11.7::gfp] (see Materials and Methods). AVG projects a short anterior process that forms gap junctions with an adjacent interneuron and a long posterior process that pioneers the ventral nerve cord and makes several chemical synapses along its path to the tail (White et al., 1976) (Fig. 1A,B). plr-1 mutations disrupt the lengths and synaptic characteristics of the AVG processes (Fig. 1C-H). In most animals, the anterior process extends a variable distance into the head and/or the posterior process stops prematurely, such that both are often similar in length. Moreover, in some animals, the posterior process is short like the wild-type anterior process and the anterior process is long like the wild-type posterior process; these long anterior processes enter the head and loop back toward the tail (Fig. 1D). Comparable defects in process length are evident in embryos and young larvae using zdIs57[inx-18::gfp], which expresses GFP in AVG earlier than zdIs49[F59E11.7::gfp], indicating that plr-1 mutations alter initial outgrowth as well as final patterning of AVG processes (Fig. 1E). To characterize the synaptic properties of the AVG processes, we visualized synaptic vesicles using a GFP-RAB-3 marker. Consistent with electron microscopy studies (White et al., 1976), synaptic vesicles in wild type are restricted to the posterior process; however, in plr-1 mutants, GFP-RAB-3 puncta are scattered throughout the anterior process when it is overextended or in both processes when they are intermediate in length (Fig. 1G,H). Thus, based on alterations in process length and synaptic properties, we conclude that the intrinsic anteroposterior polarity of AVG is inverted or symmetric in plr-1 mutants.

plr-1 encodes a conserved transmembrane RING finger protein
We cloned plr-1 by genetic mapping, germline rescue, RNAi experiments and sequence analysis (see Materials and Methods). We mapped plr-1 to the right of dpy-18 on LGIII and evaluated a candidate gene, Y47D3B.11, by analyzing genomic DNA from plr-1 mutants. Both alleles contain a G:C to A:T transition mutation in exon 4. Germline transformation using a 9.5 kb Y47D3B.11 genomic fragment rescues plr-1(zd165) mutants (3/3 independent transgenic lines) and reduction of Y47D3B.11 function by RNAi confers an AVG polarity defect (Fig. 1F). Therefore, Y47D3B.11 is plr-1.

Based on cDNA and genomic sequences, plr-1 encodes a 487 amino acid protein with an N-terminal signal sequence, a protease-associated (PA) domain, a single transmembrane region and a RING-H2 finger (Fig. 2A; supplementary material Fig. S1). First identified in two families of zinc proteases, PA domains are present in a variety of transmembrane proteins and are thought to mediate protein-protein interactions (Mahon and Bateman, 2000; Luo and Hofmann, 2001). RING fingers are hallmarks of one type of E3 ubiquitin ligase; E3s catalyze the final step of ubiquitylation by

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**Fig. 1. plr-1 mutations cause AVG anteroposterior polarity defects.** (A) AVG is located in the retrovesicular ganglion and extends a posterior process along the ventral nerve cord to the tail. The region depicted in the photomicrographs is boxed. (B) Wild-type zdIs49 expresses GFP in AVG starting during larval stages. (C) plr-1(zd165); zdIs49, symmetric polarity. (D) plr-1(zd165); zdIs49, reversed polarity. (E) plr-1(zd165); zdIs57 young larva, reversed polarity. (F) AVG processes were scored in young adults using zdIs49: wild-type, anterior and posterior processes are wild type in length; long anterior, overextended anterior process and wild-type posterior process; short posterior, wild-type anterior process and shortened posterior process; symmetric, overextended anterior process and shortened posterior process, often similar in length; reversed, anterior process is long like wild-type posterior process and posterior process is short like wild-type anterior process. n=100. Synaptic vesicles were visualized in AVG using inx-18::gfp-rab-3. (G) Wild type GFP-RAB-3 puncta in posterior process. (H) plr-1(zd165), GFP-RAB-3 vesicles are distributed throughout a long anterior process. Several head neurons express cytosolic GFP because of an unrelated reporter in the strain. The arrows indicate AVG processes.
A bicistronic operon that contains lin-11::SL2::plr-1::gfp differentiates using GFP in AVG and several other head neurons during early differentiation using lin-11::SL2::plr-1::gfp is expressed in AVG, DB, NSM, RIF or RIG (r), PHC and several motoneurons in head and ventral nerve cord. Expression is also detected in CAN, HSN, VC, excretory canal and tail epidermal cells (not shown). (E) Penetrance of polarity defective phenotype for plr-1(zd165) mutants and plr-1(zd165) animals harboring a rescuing lin-11::SL2::plr-1::gfp array. n=50-100. mn, motoneuron.

PLR-1 blocks Wnt signaling
Disruption of Wnt signaling causes ALM and PLM anteroposterior polarity to be reversed or symmetric. We therefore explored the possibility that Wnts influence AVG polarity and that PLR-1 affects polarity by perturbing Wnt signaling. For example, loss of PLR-1 might alter polarity by decreasing or increasing Wnt signaling. If loss of PLR-1 diminishes Wnt signaling, then Wnt loss-of-function mutations are expected to confer an AVG polarity defect like plr-1 mutants. Conversely, if loss of PLR-1 augments Wnt signaling, then eliminating the relevant Wnt is expected to suppress plr-1 defects. Indeed, deletion mutations of the Wnts cwn-1 and cwn-2 suppress the plr-1 polarity defect. In cwn-1; plr-1 double mutants, AVG polarity is restored and extension of the posterior process is nearly full length in 92% of the animals (Fig. 4). Severity of the polarity defect is greatly diminished in plr-1, cwn-2 double mutants, although posterior process extension remains defective. Because comparable suppression was observed when cwn-1 activity or both cwn-1 and cwn-2 activities were eliminated, we conclude that CWN-1 and CWN-2 act in a common pathway to affect AVG polarity in a plr-1 background. By contrast, mutation of the Wnt egl-20 or lin-44 fails to suppress plr-1 mutants. AVG polarity is wild type in cwn-1,
cwn-2, egl-20 and lin-44 single mutants (n=50). These data strongly support the idea that loss of PLR-1 disrupts polarity by causing an inappropriate activation of CWN-1 and CWN-2 signaling in AV and that PLR-1 acts to prevent Wnt signaling during AV differentiation.

Because loss of PLR-1 increases Wnt signaling, we tested whether ectopic PLR-1 expression decreases it. The mechanosensory neurons ALM, A VM, PLM and PVM respond to Wnts and do not express PLR-1. We expressed PLR-1 in the mechanosensory neurons and saw that ALM and PLM have anteroposterior polarity defects like Wnt or Frizzled mutants (Fig. 5). Ventrally directed growth of the A VM and PVM processes, which is guided by Netrin and Slit signaling (Hao et al., 2001), is unaffected. Therefore, ectopic PLR-1 expression blocks Wnt signaling and does not cause generalized defects in neuronal development or process outgrowth.

AVM and PVM are descendants of the neuroblasts QR and QL, respectively. QR and its descendants migrate to distinct anterior body positions, whereas QL and its descendants migrate to stereotypic posterior body locations. The Wnt EGL-20 controls cell fate specification and posterior migration of QL and its descendants by activating Hox gene mab-5 expression (Harris et al., 1996; Whangbo and Kenyon, 1999). egl-20 mutants fail to express mab-5:gfp in QL and its descendants, and egl-20 and mab-5 mutations cause PVM to migrate anteriorly instead of posteriorly. We expressed PLR-1-mCherry in QL and its descendants using egl-17 regulatory sequences and found that mab-5:gfp expression is abolished and that the QL descendant PVM often migrates anteriorly rather than posteriorly (Fig. 6). Our results show that ectopic PLR-1 expression inhibits Wnt signaling and target gene expression and, to our knowledge, provide the first evidence that QL can respond directly to Wnt signals.

To determine which PLR-1 domains are needed to antagonize Wnt signaling, we assayed truncated proteins for their ability to alter ALM and PLM polarity (Fig. 5E). Although removing the C-terminal region does not impair activity, proteins lacking the RING finger and C-terminal region (PLR-1ΔRING) or the PA domain (PLR-1ΔPA) fail to induce polarity defects. Thus, the luminal/extracellular PA domain and cytosolic RING finger are needed to block Wnt signaling.

**PLR-1 reduces Frizzled cell-surface levels**

Because PLR-1 acts in Wnt-responding cells, we investigated whether it affects Frizzled cell-surface localization. When expressed in the mechanosensory neurons, the Frizzleds CFZ-2, LIN-17, MIG-1 and MOM-5 are highly enriched at the plasma membrane (Fig. 7A; supplementary material Fig. S4A-C). By contrast, when co-expressed with PLR-1, CFZ-2, MIG-1 and MOM-5 are not detected at the surface but instead are colocalized with PLR-1 in endosomes (Fig. 7D-F,M-O; supplementary material Fig. S4D-F). LIN-17 is colocalized with PLR-1 in endosomes in all mechanosensory neurons except PLM. Interestingly, LIN-17 is still enriched at the surface of the posterior but not anterior PLM process (Fig. 7J-L; see Discussion). Furthermore, PLR-1ΔPA and PLR-1ΔRING, which do not antagonize Wnt signaling, fail to lower MOM-5 surface levels (Fig. 7S-X). We conclude that PLR-1 blocks Wnt signaling by reducing Frizzled surface levels and that the PA and RING finger domains are required for activity.
Our results argue that \textit{plr-1} polarity defects result from an increase in Frizzled surface levels and consequent heightened response to CWN-1 and CWN-2. To test this idea further, we investigated whether reducing Frizzled activity suppresses \textit{plr-1} defects and whether overexpressing Frizzled induces polarity defects. We found that a partial loss-of-function \textit{mom-5} allele strongly suppresses \textit{plr-1} polarity defects (Fig. 4). By contrast, mutation of \textit{cfz-2}, \textit{lin-17}, \textit{mig-1}, \textit{cam-1/Ror} or \textit{lin-18/Ryk} does not suppress such defects, although \textit{cfz-2} and \textit{cam-1} have genetic interactions with \textit{plr-1} (supplementary material Fig. S5). AVG polarity is wild type in \textit{cfz-2}, \textit{lin-17}, \textit{mig-1}, \textit{mom-5}, \textit{cam-1} and \textit{lin-18} single mutants (n=50). Using a \textit{mom-5::gfp} transcriptional reporter, we observed transient MOM-5 expression in AVG during embryogenesis. To test whether high levels of MOM-5 cause AVG polarity defects, we expressed MOM-5-GFP from high-copy embryogenesis. To test whether high levels of MOM-5 cause AVG polarity defects, we expressed MOM-5-GFP from high-copy extrachromosomal arrays and found that AVG polarity was often reversed or symmetric (Fig. 8). Together, these results strongly support the model that PLR-1 phenotypes result from an increase in Frizzled surface levels and enhanced response to Wnts.

**Frizzled invariant second intracellular loop lysine and CRD are important for PLR-1 regulation**

The PLR-1 RING finger and hence its predicted E3 ubiquitin ligase activity are required to block Wnt signaling and lower Frizzled surface levels. Ubiquitylation of Frizzled cytosolic lysines can regulate surface levels and internalization via endocytosis (Mukai et al., 2010). MIG-1 has a single cytosolic lysine (K326), whereas \textit{cfz-2}, \textit{LIN-17} and \textit{MOM-5} have nine, nine and six cytosolic lysines, respectively (supplementary material Fig. S6). To explore whether PLR-1 controls Frizzled surface levels via lysine ubiquitylation, we tested whether PLR-1 regulates MIG-1(K326R)-mCherry, MOM-5(6K->6R)-mCherry or LIN-17(9K->9R)-mCherry, in which all Frizzled cytosolic lysines are replaced by arginines. MIG-1(K326R), MOM-5(6K->6R) and LIN-17(9K->9R) are enriched at the surface like wild-type Frizzleds (Fig. 7B,C; supplementary material Fig. S4G). However, their surface levels are unchanged when they are co-expressed with PLR-1, indicating that cytosolic lysines are needed for PLR-1 downregulation (Fig. 7G-I, P-R; supplementary material Fig. S4J-L). These results are consistent with Frizzleds being direct targets of PLR-1-mediated lysine ubiquitylation and that ubiquitylation reduces Frizzled surface accumulation.

Lysine residues in the second and third intracellular loops and in the C-terminal tail of Frizzleds are highly conserved, but only a single lysine located in the second intracellular loop is invariant (Wang et al., 2006; Konikoff et al., 2008). Intriguingly, MIG-1 only has the invariant intracellular loop lysine (K326), and it is necessary and sufficient for PLR-1 regulation. To address whether the comparable lysine of LIN-17 and MOM-5 is important for PLR-1 regulation, we tested whether PLR-1 affects surface levels of LIN-17(K319R), LIN-17(8K->8R), in which all cytosolic lysines except K319 are changed to arginine, MOM-5(K341R), and MOM-5(5K->5R), in which all cytosolic lysines except K341 are changed to arginine. The mutant LIN-17 and MOM-5 proteins accumulate at the surface when expressed alone but are not detected at the surface when co-expressed with PLR-1 (Fig. 9A,B,D-I; supplementary material Fig. S4H,I,M-R). Thus, K319 of LIN-17 and K341 of MOM-5 are sufficient yet not necessary for PLR-1 downregulation. Together, these data indicate that the invariant second intracellular loop lysine is important for PLR-1 regulation.

We also tested whether the Frizzled CRD is required for PLR-1 regulation. MIG-1ΔCRD, which lacks the CRD, is enriched at the surface like wild-type MIG-1, but its surface levels are unaffected when co-expressed with PLR-1 (Fig. 9C,J-L). Thus, the CRD is necessary for PLR-1-induced downregulation.

**PLR-1 accumulates at the cell surface in the absence of Frizzled**

When expressed in \textit{plr-1-} or \textit{mec-4-}expressing cells, PLR-1 is enriched in endosomes and not detected at the cell surface. We investigated whether endogenous Frizzleds influence PLR-1 localization. \textit{cfz-2}, \textit{lin-17} and \textit{mig-1} single or triple mutants are viable. Because strong \textit{mom-5} mutations cause maternal effect lethality, we used a cell-specific RNAi strategy to lower \textit{mom-5} activity in only the mechanosensory neurons (Esposito et al., 2007). In wild-type, \textit{mec-4::mom-5(RNAi)} and \textit{mig-1 lin-17; cfz-2} triple mutant animals, PLR-1 exhibits a similar punctate localization pattern and is not detected at the surface (Fig. 9M; data not shown). By contrast, PLR-1 accumulates at the surface when expressed in a \textit{mig-1 lin-17; cfz-2; mec-4::mom-5(RNAi)} background (Fig. 9N).
Thus, elimination or reduction of endogenously expressed Frizzleds causes cell-surface accumulation of PLR-1. To determine whether ubiquitylation of PLR-1 is needed for its localization or activity, we assayed an altered PLR-1 that lacks all cytosolic lysines. In PLR-1(13K>11R2N), eleven cytosolic lysines are replaced by arginines and the two lysines within the RING finger are converted to asparagines. Examination of RING finger sequences most similar to the PLR-1 RING finger revealed that asparagine is occasionally present instead of lysine at these positions, so we thought that a lysine to asparagine substitution would not be likely to disrupt RING finger function. PLR-1 and PLR-1(13K>11R2N) exhibit a similar localization pattern and ability to reduce Frizzled surface levels (Fig. 9O-R). Thus, if ubiquitylation of cytosolic lysines occurs, it is not needed for PLR-1 endosomal enrichment or activity.

PLR-1 lowers CAM-1/Ror and LIN-18/Ryk cell-surface levels

Because Ror and Ryk receptor tyrosine kinases function as Wnt receptors or co-receptors with Frizzleds (Angers and Moon, 2009), we investigated whether PLR-1 affects CAM-1/Ror and LIN-18/Ryk surface levels. Both CAM-1 and LIN-18 are enriched at the plasma membrane (Fig. 10A,B). When co-expressed with PLR-1, CAM-1 and LIN-18 are not detected at the surface but rather colocalize with PLR-1 in endosomes (Fig. 10D-I). As these neurons endogenously express Frizzleds, we tested whether PLR-1 modulation of CAM-1 and LIN-18 surface levels requires Frizzled. As described above, PLR-1 is enriched at the surface in the absence of Frizzled. In a mig-1 lin-17; cfz-2; mec-4::mom-5(RNAi) background, CAM-1 and LIN-
18 are enriched at the surface when expressed alone or when co-expressed with PLR-1 (Fig. 10J-O; data not shown). By contrast, PLR-1 still lowers MOM-5 surface levels in a \textit{cam-1; lin-18} background (Fig. 10C,P-R). Thus, reduction of CAM-1 and LIN-18 surface levels by PLR-1 requires Frizzled, but PLR-1 downregulation of Frizzled does not require CAM-1 or LIN-18. Lastly, we tested whether PLR-1 lowers surface levels of an unrelated transmembrane protein, the Netrin receptor UNC-40. UNC-40 is localized at the surface when expressed alone or when co-expressed with PLR-1, indicating that PLR-1 does not influence UNC-40 trafficking and probably acts selectively on Frizzled and associated Wnt co-receptors (Fig. 10S-U).

\section*{DISCUSSION}

Our results show that PLR-1 downregulates the cellular response to Wnts during neuronal development. Loss of PLR-1 in AVG causes anteroposterior polarity defects that are mimicked by overexpression of Frizzled MOM-5 and that are suppressed by mutation of \textit{cwn-1}, \textit{cwn-2} or \textit{mom-5}. Ectopic PLR-1 expression causes phenotypes characteristic of Wnt and Frizzled loss-of-function mutations, including inversion of ALM and PLM anteroposterior polarity and failure to upregulate Wnt target \textit{mab-5} expression in QL and its descendants. Together, these results indicate that PLR-1 is a negative regulator of Wnt signaling and that loss of PLR-1 increases CWN-1 and CWN-2 signaling in AVG.

CWN-1 controls several embryonic processes, including establishment of ALM and PLM anteroposterior polarity and long-range migrations of ALM, HSN and CAN along the anteroposterior body axis (Zinovyeva and Forrester, 2005; Hilliard and Bargmann, 2006; Pan et al., 2006; Prasad and Clark, 2006). During embryogenesis, CWN-1 expression is initially detected in the posterior (Pan et al., 2006; Song et al., 2010) and extends anteriorly as development continues (Harterink et al., 2011). CWN-2 is expressed in the developing intestine and pharyngeal muscle during the embryonic comma stage (Kennerdell et al., 2009; Song et al., 2010). AVG is born before the comma stage in an anterior position and differentiates as the embryo elongates (supplementary material Fig. S7). We propose that transient PLR-1 expression prevents AVG from responding to CWN-1 and CWN-2 because activation of Wnt signaling at this stage of differentiation inappropriately alters its
polarization. PLR-1 is expressed in additional cells embryonically and post-embryonically, and we imagine that PLR-1 acts similarly to inhibit premature, excessive or ectopic activation of Wnt signaling in these cells.

PLR-1 blocks Wnt signaling by lowering Frizzled cell-surface levels. The PLR-1 RING finger and hence its predicted E3 ubiquitin ligase activity are essential for function. Ubiquitylation controls protein transport between membrane compartments by acting as a sorting signal on protein cargo and by regulating activity of trafficking machinery (Hicke and Dunn, 2003). In particular, monoubiquitylation at one or more lysine residues of a transmembrane protein can trigger internalization via endocytosis (Hicke, 2001; d’Azzo et al., 2005). Indeed, balanced ubiquitylation and deubiquitylation of Frizzled controls its surface levels and the cellular response to Wnts (Mukai et al., 2010). PLR-1 does not downregulate Frizzleds lacking cytosolic lysines. Our data support the model that Frizzleds are direct targets of PLR-1-mediated lysine ubiquitylation and that ubiquitylation leads to internalization via endocytosis.

Recently, two mammalian transmembrane E3 ubiquitin ligases, RNF43 and ZNRF3, were found to induce endocytosis of Frizzled and LRP6 by direct ubiquitylation (Hao et al., 2012; Koo et al., 2012). PLR-1 is a homolog of RNF43 and ZNRF3 and is also related in sequence and structure to *Xenopus* GREUL1 (Borchers et al., 2002). Ectopic GREUL1 expression induces anteriorization of ectodermal tissue, which is consistent with a disruption of Wnt signaling. Our independent identification and analysis of PLR-1 reveal that the PLR-1/RNF43/ZNRF3 family of transmembrane RING proteins and Frizzleds depends in part on interactions between their luminal/extracellular domains.

Many E3 ubiquitin ligases can self ubiquitylate (Fang and Weissman, 2004). We found that lysine ubiquitylation of PLR-1, if it were to occur, is not essential for PLR-1 activity or endosomal enrichment. In contrast to RNF43 and ZNRF3, PLR-1 is only detected at the cell surface when endogenous Frizzleds are eliminated. One possibility is that PLR-1 is rapidly internalized from the surface in a complex with monoubiquitylated Frizzled and that the PLR-1 internalization rate is reduced when Frizzleds are absent. Alternatively, a PLR-1 and Frizzled complex might assemble during transport along the secretory pathway and be directly targeted to endosomes by monoubiquitylated Frizzled. Reduction of CAM-1/Ror and LIN-18/Ryk surface levels by PLR-1 requires Frizzled. We propose that internalization of these Wnt co-receptors might require association with monoubiquitylated Frizzled or formation of a ternary complex with Frizzled and PLR-1. ZNFR3 downregulates the Wnt co-receptor LRP6, although it is unknown whether Frizzled is required (Hao et al., 2012).

In mammals, the Wnt signaling enhancer R-spondin interacts with ZNRF3 to form a ternary complex with LGR4 that leads to ZNRF3 internalization and downregulation (Hao et al., 2012). As such, R-spondin is proposed to potentiate Wnt signaling by eliminating the negative regulator ZNRF3 and increasing Frizzled and LRP6 surface levels. Although R-spondin and LGR4/5 are not conserved in *C. elegans* (unpublished observations), other mechanisms might exist in *C. elegans* to modulate PLR-1 activity or localization. Based on our *plr-1::SL2::gfp* transcriptional reporter, PLR-1 is expressed...
transiently in AVG, indicating that transcription might be the primary mechanism controlling PLR-1 activity and that Wnt signaling is inhibited constitutively when PLR-1 is expressed.

Wnts can influence Frizzled localization (Hilliard and Bargmann, 2006; Wu and Herman, 2007). In PLM, LIN-17 surface levels are higher in the posterior process compared with the anterior process (Hilliard and Bargmann, 2006). Asymmetric LIN-17 enrichment depends on the Wnt LIN-44, which is expressed in four tail epidermal cells located behind PLM. When PLR-1 and LIN-17 are co-expressed in PLM, LIN-17 is detected at the surface of the posterior process but not the anterior. Thus, the LIN-44-regulated mechanism that increases LIN-17 surface levels in the posterior process can overcome or bypass PLR-1 downregulation, indicating that PLR-1 and Wnt-mediated trafficking processes might act in concert to modulate Frizzled surface levels and asymmetric enrichment to fine-tune cellular responses to Wnts.

MATERIALS AND METHODS
Genetics
Worms were raised at 20°C and cultured as described (Brenner, 1974). We used the following strains: LG: mig-1(e1787), lin-17(n677), lin-44(n1792), mom-5(gm469) (S.-C. Chien and G. Garriga, personal communication), mec-4::gfp(zds15), cwm-1(ak37, gm122), mab-5::gfp(muls16); LGIII: dpy-18(e364), unc-25(e156); LGIV: eri-1(mg366), egl-20(n585), cwm-2(ak895); LGV: cfz-2(ok1201), inx-18::gfp(zds57); LGX: lin-18(e620), lin-15(n765), F59E1.7::gfp(zds49).

We screened for mutants with defects in AVG development using zdIs49, which expresses GFP in AVG starting during early larval development. We treated zdIs49 animals with ethyl methanesulfonate, transferred F1 animals to separate plates and examined their progeny by epifluorescence microscopy. We isolated six mutations from screening 3944 haploid genomes and mapped them by two-factor crosses using visible markers or microscopy. We isolated six mutations from screening 3944 haploid genomes and mapped them by two-factor crosses using visible markers or single nucleotide polymorphisms between N2 and CB4856 (Wicks et al., 2001). These mutations define four genes: klp-7(zd101, zd160), plr-1(zd164, zd165), rpm-1(zd196), and zd195.

plr-1 cloning
We mapped plr-1 to the right of dpy-18 on LGIII [dpy-18 1/73 plr-1(zd165) 72/73 unc-25]. Coding and splice junction regions of Y47D3B.11 were amplified and sequenced from wild-type, zd164 and zd165 genomic DNA. Both alleles contain a mutation in exon 4 [zd164: TGT>TAT (LGIII: 11,461,185); zd165: TGG>TGA (LGIII: 11,461,276)]. Sequence analysis of EST clones yk1060e03 and yk1051e07 confirmed plr-1 gene structure (WormBase version WS238; www.wormbase.org).

A 9.5-kb fragment containing Y47D3B.11 was amplified from wild-type genomic DNA and cloned into pBluescript to generate pSK242. For rescue experiments, we co-injected plr-1(zd165); zdIs49 animals with pSK242 and the dominant marker gcy-36::dsRedT4 (pSK223), which labels URX, AQR and PQR, or rol-6(su1004)(pRF4). We performed PLR-1 RNAi experiments by injecting dsRNAs generated from a Y47D3B.11 cDNA into RNAi-sensitized strain eru-1 (Kennedy et al., 2004).

Molecular biology and transgenic animals
We employed standard cloning and polymerase chain reaction (PCR) methods to create transcriptional and translational reporters and cell-specific
expression constructs using vectors provided by A. Fire (Stanford University School of Medicine, CA, USA). Constructs were either standard ‘promoter-gene’ fusions or synthetic bicistronic operons. Some *C. elegans* genes reside in operons in which a multigene primary transcript is produced and processed by trans-splicing to yield separate mRNAs encoding each gene product (Blumenthal, 2012). Sequences required for polyadenylation and trans-splicing to spliced leader 2 (SL2) are located between genes in an operon. We placed the intergenic region from the *lin-15A* and *lin-15B* operon (Clark et al., 1994) between the 3′ end of *plr-1* and 5′ end of *gfp to create *plr-1*:SL2::gfp (pSK230). We generated *lin-11*:SL2::plr-1-gfp (pSK231), egl-17::SL2::mCherry (pSK250) and egl-17::SL2::plr-1-mCherry (pSK251) using *lin-11* (Sarafi-Reinach et al., 2001) or egl-17 genomic sequences (Brand and Stern, 2000). The bicistronic construct *plr-1*(5′):SL2::mom-5-gfp (pSK249) only expresses mom-5-gfp because a frameshift mutation was introduced in *plr-1* genomic sequences that eliminated rescuing activity. We used *plr-1* genomic (pSK242) and *gfp* coding sequences to produce the full-length, functional PLR-1-GFP translational fusion construct *plr-1*:plr-1-gfp (pSK232). We performed localization studies in AVG using *inx-18*:plr-1-gfp (pSK233) or *inx-18*:plr-1-mCherry (pSK234) that contain *inx-18* regulatory sequences (Wenick and Hobert, 2004) and late endosomal marker *inx-18*:rme-8-gfp (pSK236) (Zhang et al., 2001), early endosomal marker *inx-18*:mrfp-eea-1 (pSK237) (Sato et al., 2005), Golgi marker *inx-18*:mans-yfp (pSK235) (Rolls et al., 2002), lysosomal marker *inx-18*:lmp-1::gfp (pSK238) (Kostich et al., 2000), or synaptic vesicle marker *inx-18*:gfp-rab-3 (pSK240) (Mahoney et al., 2006), mec-4::plr-1-yfp (pSK241), mec-4::plr-1-mCherry (pSK243), mec-4::lin-17A-mCherry (pSK214), mec-4::mig-1A-mCherry (pSK245), mec-4::mom-5-mCherry (pSK246), mec-4::cfz-2A-mCherry (pSK247), mec-4::cam-1B-gfp (pSK256), mec-4::lin-18-gfp (pSK257) and mec-4::unc-40-gfp (pSK248) were made using mec-4 promoter (Clark and Chiu, 2003) and cDNAs (*cam-1B*, *lin-17A*, *mig-1A*, *plr-1*, *unc-40*) or coding regions amplified by PCR from genomic DNA (*cfz-2*, *lin-18*, *mom-5*).
Deletion and mutant gene constructs were created using standard cloning approaches and synthetic or PCR-generated DNA fragments: nec-4::plr-1A4-mCherry (pSK253), nec-4::plr-1A4-yfp (pSK254), nec-4::mig-1A1CRD-mCherry (pSK259), nec-4::lin-17A(5K > 8R)-mCherry (pSK260), nec-4::lin-17A(3K19R)-mCherry (pSK261), nec-4::lin-17A(8K > 8R)-mCherry (pSK262), nec-4::mig-1A(3K26R)-mCherry (pSK263), nec-4::mom-5(4K1B)-mCherry (pSK271), nec-4::mom-5(5K > 5R)-mCherry, nec-4::mig-5(6K > 6R)-mCherry (pSK273), nec-4::pat-3(TM1)-mCherry (pSK264), nec-4::pl-1(3K > 1R2N)-yfp (pSK267), nec-4::pat-3(TM1)-pl-1cytosolic-yfp (pSK274), nec-4::pL1CTerminal-yfp (pSK276), mom-5 RNAi constructs nec-4::mom-5 (forward) (pSK265) and nec-4::mom-5 (reverse) (pSK266) contain a ~0.5 kb mom-5 exon 1 DNA fragment.

Constructions were injected into lin-15 animals using lin-15(+1) (pSK1) as a rescue marker (Clark et al., 1994) or into integrated GFP transgenic strains using gcy-36::dsRed14 or unc-122::gfp, which labels coelomocytes, as co-expression markers. Two to five independent, extrachromosomal array lines were created for each experiment, and 100-200 animals were scored for each line. Array lines for each experiment produced comparable expression and localization patterns. Images shown represent typical expression and localization patterns that were evident in ~95% of the animals, except for nec-4::mom-5 (RNAi) lines, in which PLR-1 surface localization was ~40% because of apparent variability in RNAi-mediated reduction of mom-5 activity.

Microscopy

Confocal images were obtained using a Zeiss LSM510, Olympus FV1000 or Leica TCS SP8. Fluorescence images were acquired using a Hamamatsu Orca II CCD camera, CARV spinning disk confocal and Leica DMIRE microscope and edited using Imrovision Openlab and Adobe Photoshop.

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