A Wnt5 Activity Asymmetry and Intercellular Signaling via PCP Proteins Polarize Node Cells for Left-Right Symmetry Breaking

Graphical Abstract

Highlights

- A gradient of Wnt5 activity polarizes node cells along the A-P axis
- A Wnt5 activity asymmetry induces polarized localization of PCP proteins
- Prickle proteins play a non-cell-autonomous role acting downstream of Wnt5a asymmetry

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In Brief

Polarization of node cells along the anterior-posterior axis of mouse embryos is responsible for left-right symmetry breaking. Opposing gradients of Wnt5a and Wnt5b and of their Sfrp inhibitors, together with intercellular signaling via PCP proteins, polarize node cells along the anterior-posterior axis for breaking of left-right symmetry.
A Wnt5 Activity Asymmetry and Intercellular Signaling via PCP Proteins Polarize Node Cells for Left-Right Symmetry Breaking

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SUMMARY

Polarization of node cells along the anterior-posterior axis of mouse embryos is responsible for left-right symmetry breaking. How node cells become polarized has remained unknown, however, Wnt5a and Wnt5b are expressed posteriorly relative to the node, whereas genes for Sfrp inhibitors of Wnt signaling are expressed anteriorly. Here we show that polarization of node cells is impaired in Wnt5a−/−Wnt5b−/− and Sfrp mutant embryos, and also in the presence of a uniform distribution of Wnt5a or Sfrp1, suggesting that Wnt5 and Sfrp proteins act as instructive signals in this process. The absence of planar cell polarity (PCP) core proteins Prickle1 and Prickle2 in individual cells or local forced expression of Wnt5a perturbed polarization of neighboring wild-type cells. Our results suggest that opposing gradients of Wnt5a and Wnt5b and of their Sfrp inhibitors, together with intercellular signaling via PCP proteins, polarize node cells along the anterior-posterior axis for breaking of left-right symmetry.

INTRODUCTION

Breaking of left-right (L-R) symmetry in vertebrates takes place at an early stage of embryogenesis in a region known as the ventral node (or equivalent structure) (Blum et al., 2008; Shiratori and Hamada, 2006). Cells at the ventral node each possess a motile cilium, the rotation of which generates a unidirectional fluid flow in the node cavity (Nonaka et al., 1998). In the mouse embryo, motile cilia at the node rotate in a clockwise direction, generating a leftward flow as a result of a posterior tilt of the rotational axis (Nonaka et al., 2005; Okada et al., 2005). Node cells are polarized along the anterior-posterior (A-P) axis by the planar cell polarity (PCP) mechanism, which regulates the coordinated polarization of cells in the plane of a tissue (Bayly and Axelrod, 2011; Goodrich and Strutt, 2011; Singh and Mlodzik, 2012), with PCP core proteins such as Disheveled (Dvl), Vangl1, and Prickle showing an anteriorly or posteriorly shifted localization
Figure 1. Wnt5 and Sfrp Genes that Show Opposite Expression Patterns Are Required for Correct Positioning of the Basal Body in Node Cells

(A) Whole-mount in situ hybridization analysis of Wnt5a, Wnt5b, and Sfrp gene expression in mouse embryos at embryonic day 7.5 (E7.5). Arrowheads indicate the position of the node, which is shown outlined in the corresponding lower images. Scale bars, 100 μm.

(B) Immunofluorescence staining for the tight junction protein Zo-1 (red) and the basal body protein Odf2 (green) in mouse embryos of the indicated genotypes at the 3- to 5-somite stage. Only the node regions are shown. Whereas the basal body (green) is located at the posterior side of node cells in the WT embryo, it is located centrally in those of Wnt5a−/−Wnt5b−/− and Sfrp1−/−Sfrp2−/−Sfrp5−/− embryos. Two types of Wnt5a−/−Wnt5b−/− embryos, showing severe (ABP = 0.10) and less severe (ABP = 0.21) defects, are shown. Schematic representations of the Odf2 and Zo-1 staining patterns are shown in the insets. Scale bars, 5 μm.

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in each node cell (Antic et al., 2010; Hashimoto et al., 2010; Song et al., 2010). Such polarized localization of PCP core proteins positions the basal body of the motile cilium to the posterior side of node cells, giving rise to the posterior tilt of the node. Positioning of centrioles is also regulated by Frizzled (Fz)-PCP signaling in Drosophila wings, suggesting that it is a conserved readout of PCP signaling (Carravajal-Gonzalez et al., 2016).

How the polarization of node cells is initiated has remained unknown. Pre-existing A-P positional information is presumably translated by node cells to give rise to the polarized localization of PCP core proteins, but the identity of such A-P information and the mechanism of its translation are unclear. We have now examined the role of noncanonical Wnt signaling in node cell polarization in the mouse. Our results suggest that a combination of posteriorly shifted expression of Wnt5a and Wnt5b and anteriorly shifted expression of the Wnt antagonists Sfrps (secreted Frizzled-related proteins) generates asymmetry in Wnt5 activity with regard to the position of the node, and is responsible for polarization of node cells.

RESULTS

Role of Posteriorly Expressed Wnt5a and Wnt5b in Node Cell Polarization

We first examined whether Wnt proteins contribute to positioning of the basal body in node cells. Noncanonical Wnt signaling has recently been implicated in the establishment of PCP in various developmental contexts (Sokol, 2015), as exemplified by the role of Wnt1 in convergent extension during gastrulation (Heisenberg et al., 2000) as well as that of Wnt5a in inner ear formation (Qian et al., 2007) and limb patterning (Gao et al., 2011). At least three noncanonical Wnt genes—Wnt5a, Wnt5b, and Wnt11—are expressed near the node of the mouse embryo, and two of them (Wnt5a and Wnt11) are essential for axis elongation (Andre et al., 2015). Whereas Wnt11 is expressed uniformly in and around the node (Figure S1), Wnt5a and Wnt5b are expressed asymmetrically with regard to the position of the node, being found preferentially on the posterior side (Figure 1A), an expression pattern that might be expected to generate an asymmetric distribution of Wnt activity along the A-P axis.

We examined node cells of Wnt5a and Wnt5b mutant mice for the average basal body position (ABP) (Hashimoto et al., 2010), which represents the relative position of the basal body within each node cell along the A-P axis, with the anterior and posterior ends of a cell being defined as −1.0 and +1.0, respectively (Figure S2). The ABP of wild-type (WT) embryos is +0.3 (Hashimoto et al., 2010), reflecting the position of the basal body on the posterior side. The position of the basal body in Wnt5a−/−/Wnt5b−/− and Wnt5a+/−/Wnt5b+/− embryos was lost, with half (5/10) of Wnt5a+/−/Wnt5b−/− embryos showing an ABP below the control range (Figures 1B–1D), suggesting that the basal body failed to shift posteriorly in these embryos. Unidirectional laminar flow was also replaced with multiple vortical flows in half (2/4 of Wnt5a−/−/Wnt5b+/− embryos (Figures 2A–2E). Consistently, asymmetric Nodal expression was lost (1/5 embryos), downregulated (2/5 embryos), or normal (2/5 embryos) in Wnt5a−/−/Wnt5b−/− embryos (Figure 2K).

To determine whether Wnt ligands are also required for the polarized localization of PCP core proteins in node cells, we examined the localization of Vangl1, Vangl2, Prickle2, and Celsr1. By super-resolution imaging, Vangl1, Vangl2, and Prickle2 were found at the anterior side of node cells in WT embryos (Figures 3A–3F and S3; Movie S1). However, Vangl1 and Prickle2 were localized uniformly in Wnt5a−/−/Wnt5b−/− embryos (4/4 embryos for Vangl1) (Figures 3G–3I). Quantitative analysis of Vangl1 localization further supports that polarized localization of Vangl1 is impaired in Wnt5a−/−/Wnt5b−/− embryos (Figure 3H). Celsr1, which is found at the anterior and posterior sides of node cells in WT embryos, was also localized uniformly in Wnt5a−/−/Wnt5b−/− embryos (3/3 embryos) (Figure S3). These findings suggested that Wnt5a and Wnt5b are required for correct basal body positioning and for the polarized localization of PCP core proteins in node cells.

Anteriorly Expressed Sfrps Are Also Essential for Node Cell Polarization

The position of the basal body was affected by the lack of both Wnt5a and Wnt5b, but this defect was apparent in only half of the mutant embryos. These results suggested the existence of additional genes that contribute to A-P asymmetric distribution of Wnt activity. Candidates for such genes included those for Sfrps, which contain a cysteine-rich domain (CRD) with homology to the Wnt binding domain of Frizzled and function as antagonists of Wnt-β-catenin and PCP pathways (Cruciat and Niehrs, 2013; Mii and Taira, 2011; Satoh et al., 2008). Sfrp1, Sfrp2, and Sfrp5 are expressed in the developing mouse embryo (Matsumura et al., 2009), with Sfrp1 being expressed in the region anterior to the node—that is, on the side opposite to the expression domain of Wnt5a and Wnt5b (Figure 1A). Sfrp2 and Sfrp5 are also expressed preferentially in the region anterior to the node (Figure 1A). These expression patterns of Sfrp genes suggested that Sfrps may determine the level of Wnt5 activity in node cells.

To test this hypothesis, we examined the basal body position and the localization of PCP core proteins in node cells of Sfrp mutant embryos. The loss of these Sfrps had similar but more profound effects on node cell polarization compared with the lack of Wnt5a and Wnt5b. The position of the basal body was thus markedly altered in all (3/3) Sfrp1+/−/Sfrp2−/−/Sfrp5+/− embryos, and was also defective in many (6/8) of the Sfrp1+/−/Sfrp2+/−/Sfrp5−/− embryos examined (Figures 1B–1D). The PCP core proteins Vangl1, Vangl2, Prickle2, and Celsr1 were...
Figure 2. Nodal Flow and Nodal Expression Are Impaired in Wnt5, Sfrp and Prickle Mutant Mice

(A–J) Nodal flow was examined in wild-type (WT) (A), Wnt5a<sup>−/−</sup>Wnt5b<sup>−/−</sup> (B–E), Sfrp1<sup>−/−</sup>Sfrp2<sup>−/−</sup>Sfrp5<sup>−/−</sup> (F–H), and Prickle1<sup>Δex6/Δex6</sup>Prickle2<sup>Δex6/Δex6</sup> (I and J) mouse embryos at the 3- to 5-somite stage by particle image velocimetry (PIV) analysis with fluorescent microbeads. The white line indicates the outline of the node. Small arrows indicate the direction and velocity of the flow at the indicated positions. The relative color scale indicates the magnitude of the flow velocity (leftward in red and rightward in blue). Note that the leftward flow was detected in embryos shown in (A), (D), (E), and (I) but was lost in the remaining embryos.

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detected uniformly at the apical membrane of node cells, and thus failed to show a polarized localization, in the absence of Sfrp1, Sfrp2, and Sfrp5 (3/3 embryos for Vangl1) (Figures 3G–3J and S3). Consistent with these findings, nodal flow was severely impaired in all (3/3) Sfrp1−/−Sfrp2−/−Sfrp5−/− embryos, with the fluid flow consisting of both leftward and rightward elements but with rightward flow being dominant (Figures 2F–2H). Asymmetric Nodal expression was lost (1/3 embryos), downregulated on the left side (1/3 embryos), or reversed to the right side (1/3 embryos) in Sfrp1−/−Sfrp2−/−Sfrp5−/− embryos (Figure 2L). These results thus suggested that polarization of node cells along the A-P axis requires these three Sfrps.

Asymmetric Distribution of Wnt5 and Sfrp Proteins Is Required for Node Cell Polarization

The phenotype of the Wnt5a−/−Wnt5b−/− and Sfrp1−/−Sfrp2−/−Sfrp5−/− embryos did not distinguish whether the corresponding proteins act as permissive or instructive signals for basal body positioning. To clarify this issue, we first examined whether a uniform distribution of Wnt5a impairs basal body positioning in node cells. We cultured WT embryos with recombinant Wnt5a from the late streak stage, when the basal body is localized in the central region of node cells (Hashimoto et al., 2010), and then we evaluated the posterior positioning of the basal body at the 3- to 5-somite stage. Compared with the control condition, treatment with recombinant Wnt5a (1 μg/mL) significantly reduced the ABP value (Figures 4A–4C), whereas the morphology of the node remained normal (Figure S1C). These results thus indicated that the uniform distribution of the exogenous Wnt5a protein affected the position of the basal body in node cells. Furthermore, the recombinant Wnt5a did not rescue the basal body positioning defect of Wnt5a−/−Wnt5b−/− embryos (Figure 4C), suggesting that Wnt5a acts as an instructive signal not as a permissive factor. On the other hand, Wnt5a did not affect the basal body position in node cells at a concentration of 0.8 μg/mL that is known to induce Wnt signaling in the embryonic day 8 (E8.0) mouse embryo (Nakamura et al., 2012) (Figures 4D and S1D).

Culture of WT embryos with recombinant Sfrp1 (25 μg/mL) from the late streak stage also significantly reduced the ABP value (Figure 4E), suggesting that a uniform distribution of Sfrp1 protein impairs basal body positioning in node cells. The nonuniform distribution of Sfrps, rather than the presence of these proteins per se, thus also seems to be required for polarization of node cells.

We also induced Wnt5a expression in RosaWnt5a+/− embryos with three different types of Cre transgene. Expression of Wnt5a was thus induced either uniformly with Sox2-Cre (Cha et al., 2014) or evenly around the node with Noto-CreERT2 (Ukita et al., 2009). The ABP value was reduced in about half (3/7) of the RosaWnt5a+/−;Sox2-Cre embryos examined (Figures 4F and S4A–S4C). More than half (3/5) of tamoxifen-treated RosaWnt5a+/−;Noto-CreERT2 embryos also showed a reduced ABP value (Figures 4G and S4D). These data suggested that uniform Wnt5a expression with respect to the node impairs the posterior positioning of the basal body, and thus provided further support for the importance of an asymmetric distribution of Wnt5 activity along the A-P axis for the polarization of node cells. Furthermore, forced Wnt5a expression with Noto-CreERT2 did not rescue the basal body positioning defect of Wnt5a−/−Wnt5b−/− mice (Figure 4G), again suggesting that Wnt5a acts instructively rather than permissively. Finally, we tested the effect of Wnt5a expression in the region anterior to the node with the use of an Sfrp1-CreERT2 transgene (Figure S4E). Again, the ABP value was reduced in most (4/6) tamoxifen-treated RosaWnt5a+/−;Sfrp1-CreERT2 embryos (Figure 4H).

Role of PCP Core Proteins Prickle1 and Prickle2 in Node Cell Polarization

Prickle1 (Pk1) and Prickle2 (Pk2) are expressed in the ventral node (Figure S5A), and Pk2 protein shows a polarized localization at the anterior side of node cells (Antic et al., 2010) (Figure 3C). The function of Prickle proteins in the node has remained unknown, however. Deletion of Pk1 in mice results in embryonic death due to defects in apicobasal polarity in the epiblast and subsequent gastrulation defects (Tao et al., 2009). To determine whether Prickle proteins play a role in the positioning of node cilia, we generated Pk1 and Pk2 mutant mice by deletion of exon 6 (Δex6) of each gene (Figures S5B and S5E). Pk1ΔΔex6/Δex6 mice showed no obvious developmental defects. In contrast to the embryonic mortality of the previously described Pk1 null mice (Tao et al., 2009), Pk1ΔΔex6/Δex6 mice were born alive but died within 24 hr after birth, suggesting that the Δex6 allele of Pk1 is hypomorphic. In Pk1ΔΔex6/Δex6Pk2ΔΔex6/Δex6 embryos, nodal flow manifested multiple vortices (1/2 embryos; Figures 2I and 2J). Asymmetric Nodal expression was lost (1/3 embryos) or downregulated (2/3 embryos) in Pk1ΔΔex6/Δex6Pk2ΔΔex6/Δex6 embryos (Figure 2M). The ABP value was reduced in all (4/4) Pk1ΔΔex6/Δex6Pk2ΔΔex6/Δex6 embryos examined (Figures 5A–5C), indicating that the basal body failed to shift posteriorly in the absence of Prickle1 and Prickle2.

To clarify the relation between Prickle and Vangl1, we examined the localization of Vangl1 in node cells of Pk1ΔΔex6/Δex6Pk2ΔΔex6/Δex6 embryos. As described previously (Antic et al., 2010; Song et al., 2010), Vangl1 and Prickle2 are asymmetrically distributed at the anterior side of node cells in control embryos (Figures 3A, 3C, and 5D). In the node of Pk1ΔΔex6/Δex6Pk2ΔΔex6/Δex6 embryos, however, Vangl1 was detected uniformly around the cell–cell boundaries, although its apical localization was maintained (Figure 5D). Quantitative analysis of Vangl1 localization further supports that polarized localization of Vangl1 is impaired in Pk1ΔΔex6/Δex6Pk2ΔΔex6/Δex6 embryos (Figure 5E), indicating that Prickle1 and Prickle2 play a key role in the positioning of node cilia and are also required for the polarized localization of Vangl1 in node cells.

(K–M) Nodal expression in the lateral plate, which is left-sided in WT embryo, was examined for mutant embryos with the indicated genotype at the 5- to 6-somite stage. The frequency of the embryo exhibiting each expression pattern is shown in parentheses. In Wnt5a−/−Wnt5b−/− embryos (K), Nodal expression was lost (1/5 embryos), downregulated (2/5 embryos), or normal (2/5 embryos). In Sfrp1−/−Sfrp2−/−Sfrp5−/− embryos examined (L), Nodal expression was lost (1/3 embryos), weak Nodal expression was found on the right side (1/3 embryos) or weak expression was detected on the left side (1/3 embryos). In Pk1ΔΔex6/Δex6Pk2ΔΔex6/Δex6 embryos (M), left-sided Nodal expression was lost (1/3 embryos) or downregulated (2/3 embryos). Scale bar, 500 μm.

A, anterior; P, posterior; R, right; L, left.
Role of Intercellular Communication via PCP Core Proteins in Basal Body Positioning

In Drosophila, interaction between PCP core proteins plays an essential role in the establishment of PCP (Bayly and Axelrod, 2011; Fant and McNeill, 2004; Yang and Mlodzik, 2015). In mutant embryos such as Wnt5a"/"Wnt5b" embryos in which the ABP value was found to be reduced, the node contained cells both with a normal and an abnormal basal body position. These two cell populations were not randomly distributed within the node but tended to form clusters (Figure S6), suggestive of an influence of cell-cell interaction. To examine the possible role of cell-cell interaction in basal body positioning, we generated chimeric embryos that contained both WT cells and Pk1Δex6/Pk2Δex6 cells through aggregation of Pk1Δex6/Pk2Δex6 embryos with WT embryonic stem cells (Figure 6A). In chimeric embryos with a relatively small contribution of WT cells, individual WT cells were often surrounded by mutant cells. Importantly, the basal body in such WT cells failed to become positioned at the posterior side (16/21 cells; Figures 6B–6E). Polarized Vangl1 localization was often impaired in WT cells surrounded by Pk1Δex6/Pk2Δex6 cells (13/48 cells, Figure S7). This probably reflects the previous observations (Bastock et al., 2003) that Vangl protein physically interacts with PK proteins. In chimeric embryos with a higher contribution of WT cells, polarization of the WT cells was again impaired by neighboring Pk1Δex6/Pk2Δex6 cells even if the WT cells were not completely surrounded by the mutant cells (Figures 6F–6H). These results thus suggested that the absence of Prickle proteins influences the polarization of neighboring WT cells in a non-cell-autonomous manner. This is unexpected, as PK acts mostly cell-autonomously in the Drosophila wing (Tree et al., 2002). One possible difference is the way mosaics are generated in the two systems: coherent clones via mitotic divisions in Drosophila as opposed to chimeric mouse embryos where WT and mutant cells can mix.

Local Forced Expression of Wnt5a Impairs Polarization of Nearby Cells

Finally, we examined how locally enforced expression of Wnt5a in the node might influence polarization of nearby cells. Pregnant mice harboring RosaWnt5a"-Noto-CreERT2.CAG-CAT-EGFP embryos were administered a low dose of tamoxifen to induce Wnt5a expression in a subset of node cells. Subsequent examination of these embryos revealed two patterns of basal body localization. In the type I pattern (Figures 7A and 7E), node cells ectopically expressing Wnt5a (identified on the basis of EGFP expression) showed a centrally positioned basal body (41/58 cells), likely as a result of a uniform distribution of Wnt5a around these cells. The basal body of neighboring cells also remained in a central position (48/69 cells), possibly as a result of impaired signaling via PCP core proteins between the Wnt5a-expressing cell and the neighboring cell. In the type II pattern (Figures 7B and 7E), the position of the basal body was normal in node cells showing ectopic Wnt5a expression (17/58 cells) but was affected in neighboring cells (14/34 cells). Interestingly, in some cases (3/17 type II cells), neighboring cells posterior to the Wnt5a-expressing cell showed anterior localization of the basal body. In the case of isolated Wnt5a-expressing cells, neighboring cells with an abnormally positioned basal body were apparent over a distance of at least two or three cells from the Wnt5a-expressing cell (Figures 7A and 7B), suggesting that local expression of Wnt5a can influence cell polarization over a distance of several cells. Vangl1 localization was similarly impaired by local forced Wnt5a expression. Thus, Vangl1 localization was defective not only in Wnt5a-expressing cells (Figure 7C, type I) (19/26 cells: Figure 7E) but also in neighboring cells of type I (22/39 cells) and those of type II (7/11 cells) Wnt5a-expressing cells (Figures 7D and 7E).

DISCUSSION

The role of PCP core proteins has been well established for PCP in Drosophila. On the other hand, little is known of the potential existence of a positional cue acting upstream of PCP core proteins as well as the possible role of Wnt ligands in PCP. In the wing epithelium of Drosophila, protocadherins (Fat and Dachsous) have been proposed to function as a long-range cue that acts upstream of PCP core proteins (Ma et al., 2003), raising the possibility that Wnt ligands do not contribute to PCP. On the other hand, observations in Drosophila (Wu et al., 2013) and vertebrates (Gros et al., 2009) have provided support for an instructive role of Wnt ligands in PCP (Yang and Mlodzik, 2015).

Figure 3. Polarized Localization of PCP Core Proteins Is Dependent on Wnt5 and Sfrp Genes

(A–F) The subcellular localization of Vangl1, Prickle2, and Vangl2 in node cells of the WT embryo. Sagittal sections of the node stained for Vangl1 and Zo-1 (A), Vangl1 and E-cadherin (B), Prickle2 and Zo-1 (C), Prickle2 and E-cadherin (D), Vangl2 and Zo-1 (E), or Vangl2 and E-cadherin (F) were observed by super-resolution imaging. A higher-magnification image is also shown in each panel. White arrowheads indicate localization of these proteins to the anterior edge of the node cells. Vangl1, Prickle2, and Vangl2 proteins are apparently localized to the anterior side and apical side of node cells. Note that Vangl1 and Vangl2 show anterior localization even in the nonapical regions (open arrowheads in B and F).

(G) Localization of Zo-1 and Vangl1 in the node of WT, Wnt5a"/"Wnt5b", and Sfrp1"/"Sfrp2"/"Sfrp5"/" embryos at the 3- to 5-somite stage. The localization of Vangl1 in node cells is illustrated schematically in the insets in (G).

(H) Quantitative analysis of the Vangl1 localization pattern in individual embryos of the indicated genotypes. The angle (~90° to +90°) is determined by using quantitative analysis for each cell. In the rose diagram, angles are classified into 12 classes (15° for each class). The area size of each class indicates the cell numbers for each WT, Wnt5a"/"Wnt5b", and Sfrp1"/"Sfrp2"/"Sfrp5"/" embryos at the 3- to 5-somite stage. The localization of Vangl1 in node cells is illustrated schematically in the insets in (G).

(I) Localization of Zo-1 and Prickle2 in the green of the node of WT, Wnt5a"/"Wnt5b", and Sfrp1"/"Sfrp2"/"Sfrp5"/" embryos at the 3- to 5-somite stage. The localization of Zo-1 and Prickle2 is defective in the Wnts and Sfrp mutant embryos. Scale bars for (G)–(I), 5 μm. A, anterior; P, posterior. See also Figure S3.
Figure 4. Perturbation of the Asymmetric Distribution of Wnt5a and Sfrp1 Impairs Node Cell Polarization

(A and B) WT embryos were cultured with BSA or recombinant Wnt5a (1 μg/mL) from the late streak stage to the 3- to 5-somite stage. The relative position of the basal body (green dots in A) of node cells was then determined. Two different embryos, showing defective ABP and less defective ABP, are shown for Wnt3a-treated embryos. Data are shown for representative embryos in (A) and are summarized for the indicated numbers of embryos in (B) (red bars indicate mean values). Three open dots in (B) correspond to three embryos shown in (A). A, anterior; P, posterior.

(C) ABP values for Wnt5a–/– Wnt5b–/– embryos cultured with BSA or recombinant Wnt5a as in (A). Control embryos include Wnt5a+/–, Wnt5b+/–, Wnt5a–/–, and Wnt5a+/– Wnt5b–/– genotypes.

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Our data now suggest that asymmetric activity of Wnt5a and Wnt5b serves as an upstream regulator of PCP core proteins in the polarization of mouse node cells. The opposite patterns of Wnt5a/5b and Sfrp gene expression observed along the A-P axis of the node would thus be expected to generate a graded distribution of Wnt5a/5b activity and might thereby provide a global cue for positioning of the basal body of the motile cilium at the posterior side of each node cell. Individual node cells may sense the differential level of Wnt5a/5b activity at their anterior and posterior sides, resulting in the initiation of asymmetric localization of PCP core proteins such as Prickle1, Prickle2, Vangl1, and Dvl at the anterior or posterior side of the cell. Interaction between PCP core proteins, both at the intracellular level (such as between Prickle and Dvl) and intercellular level (such as between Vangl1/2 and Fz or through Celsr1) would then establish robust A-P polarity in node cells (Figure 7F). In this regard, it is important to know expression and localization of Wnt receptors, in particular Fz proteins, in node cells.

The precise mechanisms of intracellular and intercellular signaling mediated by PCP core proteins in node cells remain unknown. However, previous observations in various experimental systems provide a basis for speculation about such mechanisms. Given that Prickle2 and Vangl1 are both expressed at the anterior side of node cells and physically associate with each other (Ba-stock et al., 2003; Jenny et al., 2003), the absence of one might be expected to alter the localization of the other within a single cell, as we have now observed for the localization of Vangl1 in Pk1Δex6/Δex6 Pk2Δex6/Δex6 embryos. Intercellular signaling via PCP core proteins likely involves preferential association of such proteins in neighboring cells. The Frizzled-Dvl complex promotes enrichment of the Vangl2-Prickle complex across the cell junction with neighboring cells, which may account for the localization patterns of Prickle1/2, Vangl1, and Dvl in node cells. If this is the case, the absence of Prickle in a single cell would impair polarization not only of this cell but also of neighboring cells, as we have now observed with chimeric embryos consisting of both WT and Pk1Δex6/Δex6 Pk2Δex6/Δex6 cells. Theoretically, the absence of Prickle1/2 in a single cell could have a long-range effect, but such an effect may be prevented by the presence of the Wnt5a/5b activity gradient.

Among the various mutant embryos examined in the present study, the defect in basal body positioning was most pronounced in the Sfrp1Δ/Δ-Sfrp2Δ/Δ-Sfrp5−/− embryos. Thus, whereas this defect was variable in Wnt5a/wnt5b+/- embryos, it was apparent with an ABP of approximately 0.1 in all Sfrp1Δ/Δ-Sfrp2Δ/Δ-Sfrp5−/− embryos examined. These results suggest that the anteriorly expressed Wnt inhibitors (Sfrps) play a more profound role in the generation of asymmetric Wnt activity than do the posteriorly expressed Wnt5a and Wnt5b. They thus further suggest that Wnt ligands other than Wnt5a and Wnt5b may contribute to basal body positioning, although our data suggest that such a role for Wnt11 is unlikely. All Wnt genes expressed near the node, including those expressed equally on both sides of the node, may contribute to Wnt activity around the node. The level of Wnt activity would be A < P in total, but this asymmetry would be much sharpened by the anteriorly expressed Sfrps.

The observed opposing gene expression patterns for Wnt5a/5b and their Sfrp inhibitors would be expected to generate an asymmetric distribution of Wnt activity with regard to the node. However, it is currently technically difficult to directly visualize noncanonical Wnt activity in an embryo. Also unclear is how a node cell would sense different levels of Wnt activity at its anterior and posterior ends. The noncanonical Wnt signaling pathway may play a role, but it is not clear which receptor is responsible. It has been suggested that in the Drosophila wing the asymmetric Wnt exposure will tether Fz proteins toward the Wnt source and hence initiate the PCP factor interactions with a directional bias (Wu et al., 2013). However, involvement of Fz proteins in node cell polarization remains to be investigated. Finally, the basal body is initially present at a central position of each node cell but then moves toward the posterior side during a period of several hours (Hashimoto et al., 2010). It remains unknown how the basal body changes its position by interpreting the localization of PCP core proteins. While actin is a key mediator that positions centrioles in Drosophila wings (Carvajal-Gonzalez et al., 2016), it is unknown whether a similar mechanism operates in mouse node cells. These questions will need to be answered in future studies to provide a full understanding of the mechanism of L-R symmetry breaking.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Mice
- **METHOD DETAILS**
  - Whole-Mount In Situ Hybridization and X-gal Staining
  - Immunostaining
  - Super-Resolution Images
  - Whole-Embryo Culture and Recombinant Protein Treatment
  - Observation of Nodal Flow by PIV Analysis
  - Quantitative Analysis of Basal Body Position
  - Quantitative Analysis of PCP Core Protein Localization in Node Cells

(D) ABP values for WT embryos cultured with BSA or recombinant Wnt3a (0.8 μg/mL) from the late streak to 3- to 5-somite stages. Note that Wnt3a did not affect the basal body position in node cells.

(E) ABP values for WT embryos cultured with BSA or recombinant Sfrp1 (25 μg/mL) from the late streak to 3- to 5-somite stages.

(F) ABP values for RosaWnt5a/+ and RosaWnt5a−/−Sox2-Cre embryos, in the latter of which Wnt5a expression is induced uniformly.

(G) ABP values for tamoxifen-treated RosaWnt5a−/−-Noto-CreERT2 and Wnt5a+/-;Wnt5b−/-;RosaWnt5a−/−;Noto-CreERT2 embryos, in which Wnt5a expression is induced evenly around the node.

(H) ABP values for tamoxifen-treated RosaWnt5a−/−-Sfrp1-CreERT2 embryos, in which Wnt5a expression is induced in the region anterior to the node.

Dams were treated orally with tamoxifen (5 mg) both 24 and 12 hr before the late streak stage for embryos in (G) and (H). The p values for the indicated comparisons were determined with Student’s two-tailed t test. N.S., not significant. See also Figure S4.
Figure 5. Polarization of Node Cells Requires Prickle 1 and Prickle 2

(A) Immunostaining of the node region of \( Pk1^{Δex6/Δex6} Pk2^{Δex6/Δex6} \) (control) and \( Pk1^{Δex6/Δex6} Pk2^{Δex6/Δex6} \) mouse embryos at the 3- to 5-somite stage for Zo-1 (red) and Odf2 (green). A, anterior; P, posterior.

(B) Relative position of the basal body (green dots) summarized for individual node cells of embryos as in (A). A, anterior; P, posterior.

(C) ABP values for the indicated numbers of embryos as in (A). Red bars indicate mean values. Control embryos include \( Pk1^{Δex6/+} Pk2^{Δex6/+} \) and \( Pk2^{Δex6/+} \) genotypes.

(D) Immunostaining of \( Pk2^{Δex6/+} \) and \( Pk1^{Δex6/Δex6} Pk2^{Δex6/Δex6} \) embryos at the 3- to 5-somite stage for Zo-1 (red) and Vangl1 (green). Note that the polarized localization of Vangl1 is impaired in the latter embryo. The Vangl1 localization pattern is schematically illustrated below (shown in green).

(E) Quantitative analysis of the Vangl1 localization pattern in the Control (\( Pk1^{Δex6/+} Pk2^{Δex6/+} \)) and \( Pk1^{Δex6/Δex6} Pk2^{Δex6/Δex6} \) and \( Pk2^{Δex6/Δex6} \) embryos. The angle (–90° to +90°) is determined by using quantitative analysis for each cell. In the rose diagram, angles are classed into 12 classes (15° for each class). The area size of each class indicates the cell numbers for Control and \( Pk1^{Δex6/Δex6} Pk2^{Δex6/Δex6} \) embryos. The numbers of cells analyzed were 337 cells from three embryos for control and 654 cells from three embryos for \( Pk1^{Δex6/Δex6} Pk2^{Δex6/Δex6} \).

The p values were determined with Watson’s two-sample test of homogeneity. Scale bars, 5 μm. See also Figure S5.
Figure 6. The Absence of Prickle 1 and Prickle 2 in Node Cells Affects the Polarization of Nearby WT Cells

(A) Experimental design for the generation of chimeric embryos consisting of both WT and Pk1\textsuperscript{Dex6/Dex6}\textsuperscript{Dex6/Dex6} cells by aggregation of Pk1\textsuperscript{Dex6/Dex6}\textsuperscript{Dex6/Dex6} embryos (host) with Pk1\textsuperscript{+/+}\textsuperscript{+/+}\textsuperscript{+/+} embryonic stem cells (donor) that express EGFP. (B–H) E8 chimeric embryos generated as in (A) with a low (B–E) or high (F–H) contribution of WT cells were immunostained for Zo-1 (red), Odf2 (light blue), and EGFP (green). The node regions are shown in (B) and (F), with higher-magnification images presented in (C), (D), and (G). Arrowheads in (C) indicate the basal body of the donor cell. Schematic representations of basal body position in WT (EGFP-positive) and Pk1\textsuperscript{Dex6/Dex6}\textsuperscript{Dex6/Dex6} cells of corresponding chimeric embryos are shown in (E) and (H). In (H), posteriorly located and centrally located centrioles are shown in blue and red, respectively. Note that the basal body in WT cells failed to become positioned at the posterior side when the WT cells were surrounded by Pk1\textsuperscript{Dex6/Dex6}\textsuperscript{Dex6/Dex6} cells.

A, anterior; P, posterior. Scale bars, 5 μm. See also Figures S6 and S7.
Figure 7. Local Forced Expression of Wnt5a Impairs Polarization of Nearby Node Cells

(A–D) Pregnant mice harboring Rosa\textsuperscript{Wnt5a/\textsuperscript{\text{ERT2}}};Noto-Cre\textsuperscript{ERT2} ; CAG-CAT-EGFP embryos were treated with a low dose (1 mg) of tamoxifen at 12 hr before the late streak stage to induce Wnt5a expression in a subset of node cells. Embryos were recovered at the 3- to 5-somite stage and were immunostained for Zo-1 (red), Odf2 (green), and EGFP (blue) (A and B) or for Zo-1 (red), Vangl1 (green), and EGFP (light blue) (C and D). Node cells expressing Wnt5a were identified on the basis

(legend continued on next page)
QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2017.02.010.

AUTHOR CONTRIBUTIONS

K.M. designed, performed, and analyzed most of the experiments. K.S. performed particle image velocimetry analysis. M.H. contributed to the initial phase of this work. R.A., K.T., and H. Shiratori designed and analyzed some of the experiments such as those involving chimera embryos. Y. Ikawa, H.N., H.H., and M.H. designed and generated Prickle mutant mice. A.P.M., T.P.Y., R.A., H. Sasaki, and A.S. provided various mice used in this study and analyzed the data. K.W. prepared recombinant Wnt5a protein and analyzed the corresponding data. T.F. and T.O. analyzed the data involving Celsr1 and Prickle2 antibodies, respectively. Y.O. determined precise localization of Vangl1 and Prickle2 proteins. D.S. performed quantitative analysis of Vangl1 protein localization. Y. Igarashi generated a program that automatically analyzes the position of the basal body in node cells. H.H. and K.M. wrote the paper.

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REFERENCES


**STAR METHODS**

**KEY RESOURCES TABLE**

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Hiroshi Hamada (hiroshi.hamada@riken.jp).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

**Mice**

*Rosa<sup>Wnt5a/+</sup>* mice (Cha et al., 2014), *Sox2-Cre* mice (Hayashi et al., 2002), *Noto-Cre<sup>ERT2</sup>* mice (Ukita et al., 2009), CAG-CAT-EGFP (Kawamoto et al., 2000) mice, as well as *Wnt5a* (Yamaguchi et al., 1999), *Wnt5b* (Agalliu et al., 2009), *Wnt11* (Majumdar et al., 2003), and *Strep1, Strep2, and Strep5* (Satoh et al., 2006, 2008) mutant mice were described previously. *Prickle1<sup>Dex6/+</sup>* and *Prickle2<sup>Dex6/+</sup>* mice were generated by conventional gene targeting in ES cells (Figure S5). Specific polymerase chain reaction (PCR) primers for detection of WT and mutant *Prickle* alleles included Pk1-WT-F (5'-AATCGGCGAATGCCAATG-3') and Pk1-WT-R (GGATTTCATCCCTAACACACCAC) for the *Prickle1<sup>+</sup>* allele; Pk1-Mut-F (GTGACTGCAAACAAGCGAAA) and Pk1-Mut-R (GCATTGACTTGCCACCAGTA) for the *Prickle1<sup>Dex6</sup>* allele; Pk2-WT-F (CACATACATGCACACCCACAATTC) and Pk2-WT-R (TCAGGGTTGTTTTCAACTTCAAAGC) for the *Prickle2<sup>+</sup>* allele; and Pk2-WT-F and Pk2-Mut-R (GGCGCTCTATGGAAGGGAAAG) for the *Prickle2<sup>Dex6</sup>* allele. *Strep1-Cre<sup>ERT2</sup>* mice were generated with the use of a mouse *Strep1* bacterial artificial chromosome (BAC) clone in which the first exon of *Sfrp1* is replaced by *Cre<sup>ERT2</sup>*. All mouse experiments were approved by the relevant committees of Osaka University and RIKEN Center for Developmental Biology, license numbers FBS-12-019 and AH28-01.

**METHOD DETAILS**

**Whole-Mount In Situ Hybridization and X-gal Staining**

Whole-mount in situ hybridization was performed according to standard protocols with specific RNA probes for *Wnt5a, Wnt5b, Wnt11, Prickle1, Prickle2, Strep1, Strep2, and Strep5*. Expression of the lacZ transgene was detected by fixing transgenic embryos with paraformaldehyde and glutaraldehyde followed by staining transgenic embryos with X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside) as described previously (Saijoh et al., 1999).

**Immunostaining**

Dissected embryos were fixed with 4% paraformaldehyde, dehydrated with methanol, and permeabized with 0.1% Triton X-100 in phosphate-buffered saline. They were stained with primary antibodies and Alexa Fluor–conjugated secondary antibodies (invitrogen) by incubation overnight at 4°C. Primary antibodies included those to Zo-1 (mouse monoclonal, 1:10 dilution; kindly provided by S. Tsukita, Osaka University), to Vangl1 (rabbit polyclonal, 1:250 dilution; Sigma), Vangl2 (rabbit polyclonal, 1:250 dilution; Sigma), to Odf2 (rabbit polyclonal, 1:250 dilution; kindly provided by S. Tsukita or rabbit polyclonal, 1:250 dilution; Abcam), to Celsr1 (pig polyclonal, 1:100 dilution) (Shi et al., 2014), to EGFP (rat monoclonal, clone No. GF-090R, 1:100 dilution; Nacaai Tesque) and to Prickle2 (rabbit polyclonal, 1:100 dilution) (Hida et al., 2011). Fluorescence images were acquired with an FV-1000 confocal microscope (Olympus).
Super-Resolution Images

Two super-resolution methods were employed for the precise localization of PCP core proteins in node cells. In the first method, the sagittal sections (4 μm thick) were prepared from immunostained node samples and were observed by a Zeiss Elyra PS.1 system. Alternatively, the node regions of whole embryo were observed with a super-resolution microscopy based on the confocal microscopy optics (Hayashi and Okada, 2015). Briefly, the raw images were taken with the objective lens, HCPLAPO 40/1.10 W CORR CS2 on a SP-8 confocal microscope (Leica). The pinhole was reduced to the size corresponding 0.5 Airy unit at 580 nm. Three-dimensional twice oversampled data were further processed by the classical maximum likelihood estimation deconvolution algorithm (Huygens, Scientific Volume Imaging) to enhance the high resolution components beyond the classical diffraction limit of 200 nm. Two-dimensional maps are shown by projection along the axis parallel to the apico-basal axis of the node pit cells. The three-dimensional (3D) views of the node were reconstructed from serial images (Z-stack) obtained by super-resolution imaging.

Whole-Embryo Culture and Recombinant Protein Treatment

Mouse embryos were dissected into phenol red–free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), transferred to a 50-ml tube containing DMEM supplemented with 75% rat serum (prepared by us), and cultured with rotation in an incubator containing 5% CO₂ at 37°C. The embryos were cultured in the presence of BSA (control) or recombinant proteins from the late streak stage to the three- to five-somite stage. The final concentrations of the recombinant proteins were 200 ng/ml for Wnt5a (Green et al., 2013; Willert, 2008), 1.0 μg/ml for Wnt5a (645-WN, R&D Systems), 0.8 μg/ml for Wnt3a (5036-WN, R&D Systems), and 25 μg/ml for Sfrp1 (5396-SF-025, R&D Systems).

Observation of Nodal Flow by PIV Analysis

Nodal flow was observed by multipoint scanning confocal microscopy and particle image velocimetry (PIV) analysis as described previously (Shinohara et al., 2012). Dissected embryos were first cultured under 5% CO₂ at 37°C for ~30 min. The region containing the node was then excised, and the node cavity was filled with DMEM supplemented with 10% fetal bovine serum and fluorescent microbeads (0.2 μm in diameter) with excitation and emission wavelengths of 505 and 515 nm, respectively (Invitrogen). The motion of the beads was monitored in planes of +5 and +10 μm relative to the cavity for 10 s (30 frames per second) with the use of a CSU-X or CSU-W1 confocal unit (Yokogawa) and an iXon EMCCD camera (Andor Technology) connected to a DMI6000B microscope (Leica) equipped with a 63× glycerin-immersion objective lens. Time-series images for PIV analysis were captured at a resolution of 512 by 512 pixels and were processed with interrogation windows of 16 by 16 pixels with 50% overlap, corresponding to a spatial resolution of 4.3 by 4.3 μm. The time-averaged velocity distributions were calculated for 10-s intervals.

Quantitative Analysis of Basal Body Position

The average basal body position (ABP) represents the relative position of the basal body in each node cell along the A-P axis (vertical), as described previously (Hashimoto et al., 2010). Confocal images of the node immunostained for Odf2 and Zo-1 were obtained to determine the position of the basal body in each node cell (Figures S2A and S2B). For characterization of the shape and orientation of the node cells, the outline of each cell was calculated from the pattern of Zo-1 staining with the use of the ImageJ software plugin (http://rsb.info.nih.gov/ij/image) to apply watershed segmentation (binary watershed line image in Figure S2C). The basal body was traced manually according to the Odf2 staining image, and the x,y coordinates of each basal body were recorded using of the graphical user interface (GUI) in MATLAB software (Figure S2D). The relative value for the position of the basal body in each cell was calculated from the coordinate data with the anterior and posterior ends of a cell expressed as −1.0 and +1.0, respectively (Figure S2E). This approach was programmed by one of the authors, Y. Igarashi. To examine if changes in cell morphology may influence ABP, we have examined cell morphology by quantitatively measuring the ratio of X/Y axis of each node cell (Figure S6). Although the ratio (morphology) varies among node cells, there is no significant difference between wild-type, Wnt5a−/−Wnt5b−/− and Sfrp triple mutant embryos, suggesting that changes in cell shape do not account for the reduced ABP in these mutant embryos.

Quantitative Analysis of PCP Core Protein Localization in Node Cells

The principle of the image analysis method for quantification of the polarity of staining signals within cell contours has been previously described (Aigouy et al., 2010; Shi et al., 2014). The cell boundary was traced manually from the pattern of Zo-1 staining and the x,y coordinates of each cellular contour were recorded. The Vangl1 staining intensities in the pixels in the contour were collected as f(x, y). f(x, y) was transformed into f(r, θ), where r is the distance between the pixel and the center of the cell while θ is the angle indicating the position of the pixel. Then, we calculated the average value of f(r, θ) for each θ section with a 5° range. The average value was set to S(θm), where θm is the center θ value of the section. The polarity of the staining signals φ (−90° ≤ φ < 90°) was determined for each cell to fit: cos 2φ = Q₁/√Q₁² + Q₂² , sin 2φ = Q₂/√Q₁² + Q₂² where Q₁ = Σ S(θm)cos2θm, Q₂ = Σ S(θm)sin2θm. Processing was performed in Excel. This approach was programmed by one of the authors, Dongbo Shi. Rose diagrams were drawn by using MATLAB.
Cre-Mediated Recombination
Expression of Cre<sub>ERT2</sub> transgenes in embryos was induced by oral administration of tamoxifen (Sigma) in corn oil to pregnant mice either at a dose of 5 mg both 24 and 12 hr before the late streak stage (for the normal induction protocol) or at a dose of 1 mg 12 hr before the late streak stage (for suboptimal induction).

Generation of Chimeric Embryos
Mutant (P<sub>k1</sub>Δex6/Δex6P<sub>k2</sub>Δex6/Δex6) embryos at the eight-cell stage were aggregated with wild-type ES cells expressing EGFP. The aggregated embryos at the blastocyst stage were transferred to the uterus of pseudopregnant mice and allowed to develop until E8.0. Embryos between the 3- and 5-somite stage were used for further analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analysis was performed with Student’s two-tailed t test, Wilcoxon rank-sum test or Watson’s two-sample test of homogeneity by using R. A p value of <0.05 was considered statistically significant.
INVENTORY OF SUPPLEMENTAL INFORMATION

Supplemental Figures

Figure S1. Role of Wnt11, Wnt5a and Wnt3a in node cell polarization, related to Figure 1.
Figure S2. Quantitative analysis of basal body position with ImageJ software, related to Figure 1.
Figure S3. Polarized localization of PCP core proteins is dependent on Wnt5 and Sfrp genes, related to Figure 3.
Figure S4. Ectopic Wnt5a expression induced by Cre transgenes, related to Figure 4.
Figure S5. Generation of Prickle1 and Prickle2 mutant alleles, related to Figure 5.
Figure S6. Distribution and shape of node cells from Control, Wnt5a<sup>−/−</sup>Wnt5b<sup>−/−</sup> and Sfrp1<sup>−/−</sup>Sfrp2<sup>−/−</sup>Sfrp5<sup>−/−</sup> embryos, related to Figure 6 and Figure 7.
Figure S7. Effects of the absence of Prickle 1 and Prickle2 on neighboring WT cells, related to Figure 6 and Figure 7.

Supplemental Movie

Movie S1. The localization of Vangl1 observed by the super-resolution imaging, related to Figure 3.
Figure S1. Role of Wnt11, Wnt5a and Wnt3a in node cell polarization, related to Figure 1.

(A) Whole-mount in situ hybridization analysis of Wnt11 expression in a WT mouse embryo at the three- to five-somite stage. The node is indicated by the arrowhead in the upper panel and is shown outlined in the lower panel. (B) Summary of ABP values for embryos of the indicated genotypes. The data for control, Wnt5a+/–Wnt5b–/–, and Wnt5a–/–Wnt5b–/– embryos are the same as those in Figure 1d. Note that the ABP of Wnt5a–/–Wnt5b–/–Wnt11+/– embryos is similar to that for Wnt5a–/–Wnt5b–/– embryos. (C, D) WT embryos were cultured with BSA or either recombinant Wnt5a (1 µg/ml) (C) or recombinant Wnt3a (0.8 µg/ml) (D) from the late streak to three- to five-somite stages. They were then subjected to immunofluorescence staining for Zo-1 (red) and Odf2 (green). The upper images in (D) are shown at higher magnification in the lower panels. Note that the morphology of the node remained normal after incubation of embryos with Wnt5a or Wnt3. The position of the basal body was affected by Wnt5a but not by Wnt3a. Scale bars, 5 µm.
Figure S2. Quantitative analysis of basal body position with ImageJ software, related to Figure 1.

(A) Confocal image of the node region of a WT embryo immunostained for Odf2 (green) and Zo-1 (red). (B) The enlarged view of a region in (A). (C) The node image in (B) was processed with the ImageJ software plugin to apply watershed segmentation, generating a “binary watershed line image.” The outline of each cell was estimated from the pattern of Zo-1 staining. (D) Graphical user interface (GUI) view of the binary watershed line image in (C). The basal body was traced according to the Odf2 staining image. (E) Position of the basal body in a node cell relative to the most anterior and posterior positions, –1.0 and +1.0, respectively. (F) Distribution map for the basal body in the analyzed embryo. The Red bar indicates ABP.
Figure S3. Polarized localization of PCP core proteins is dependent on Wnt5 and Sfrp genes, related to Figure 3.

(A, B) Immunofluorescence staining of WT embryos at the three- to five-somite stage for Vangl1 (green) and Zo-1 (purple) (A) or for Prickle2 (green) and Zo-1 (purple) (B). The node region is shown for each embryo, with higher magnification images of the node being shown in the right panels. The subcellular localization of Vangl1 and Prickle2 was observed by super-resolution imaging, revealing that both proteins are localized to the anterior side of node cells (arrowheads). A, anterior; P posterior. (C) Three-dimensional reconstruction of Vangl1 localization images in the WT embryo obtained by super-resolution imaging. The view of the node from the anterior side (left panel) and the view from the posterior side (right panel) are shown here with a higher magnification image in the inset. Note that Vangl1 is detected at the anterior side of node cells. (D) Localization of Zo-1 (red) and Celsr1 proteins (green) in the node of WT, Wnt5a−/− Wnt5b−/−, and Sfrp1−/− Sfrp2−/− Sfrp5−/− embryos at the three- to five-somite stage.
Figure S4. Ectopic Wnt5a expression induced by Cre transgenes, related to Figure 4.

(A, B) Whole-mount in situ hybridization analysis of Wnt5a expression in Rosa<sup>Wnt5a</sup>+ (A) or Rosa<sup>Wnt5a</sup>+; Sox2-Cre (B) embryos at E7.5. Note that Wnt5a is expressed ubiquitously in the latter embryo. Scale bars, 100 µm. (C) Rosa<sup>Wnt5a</sup>+ and Rosa<sup>Wnt5a</sup>+; Sox2-Cre embryos at the three- to five-somite stage were immunostained for Zo-1 (red) and Odf2 (green), with the white line indicating the outline of the node (upper panels). The position of the basal body (green dots) in node cells is summarized for each embryo in the lower panels, with the corresponding ABP values indicated. A, anterior; P, posterior. Two independent embryos are shown for Rosa<sup>Wnt5a</sup>+; Sox2-Cre embryos, with one showing abnormal ABP (0.10) and the other showing relatively normal ABP (0.23). (D) Wnt5a<sup>+/−</sup>; Rosa<sup>Wnt5a</sup>+ or Wnt5a<sup>+/−</sup>; Wnt5b<sup>+/−</sup>; Rosa<sup>Wnt5a</sup>+; Noto-CreERT2 embryos isolated at the three- to five-somite stage from dams treated orally with tamoxifen (5 mg) both 24 and 12 h before the late streak stage were immunostained for Zo-1 (red) and Odf2 (green). Note that the basal body is positioned centrally in many node cells of the latter embryo. Scale bars, 5 µm. (E) Specificity of Sfrp1-Cre transgene expression. A Rosa<sup>lacZ</sup>+/Sfrp1-CreERT2 embryo isolated at the three- to five-somite stage from a dam treated orally with tamoxifen (5 mg) both 24 and 12 h before the late streak stage was stained with the β-galactosidase substrate X-gal. Note that X-gal staining is predominant in the region anterior to the node, although some cells around the node are also positive.
Figure S5. Generation of Prickle1 and Prickle2 mutant alleles, related to Figure 5.

(A) Expression of Prickle1 and Prickle2 in the E8.0 mouse embryo was examined by in situ hybridization. Note that both genes are expressed at the ventral node. (B, E) Generation of Prickle1 (B) and Prickle2 (E) mutant alleles with targeting vectors designed to delete exon 6 of each gene. (C, F) ES cell clones that had undergone homologous recombination were selected by Southern blot hybridization with specific probes (shown in B and E, respectively) that recognize external or internal sequences relative to the targeting vector. Tail DNA of mice of the indicated genotypes was similarly analyzed. (D, G) The genotype of offspring from heterozygote intercrosses was determined by PCR analysis of tail DNA with primer sets (shown in B and E, respectively) specific for the WT or Δex6 alleles. Given the exon-intron structure of Prickle1 and Prickle2, the mutant alleles can only produce a truncated protein lacking the large COOH-terminal region (636 and 645 amino acid residues encoded by exons 6 to 12 would be lacking out of 832 and 845 amino acid residues of the whole Pk1 and Pk2 protein, respectively).
Figure S6. Distribution and shape of node cells from Control, Wnt5a\textsuperscript{-/-} Wnt5b\textsuperscript{-/-} and Sfrp1\textsuperscript{-/-} Sfrp2\textsuperscript{-/-} Sfrp5\textsuperscript{-/-} embryos, related to Figure 6 and Figure 7.

(A-C) Node cells were grouped on the basis of their ABP: +1.0 ~ +0.6 (red), +0.6~+0.2 (orange), +0.2 ~ -0.2 (green), -0.2~-0.6 (light blue), and -0.6 ~ -1.0 (blue). Distribution of each group of cells was examined in Wnt5b\textsuperscript{-/-} (A), Wnt5a\textsuperscript{-/-} Wnt5b\textsuperscript{-/-} (B) and Sfrp1\textsuperscript{-/-} Sfrp2\textsuperscript{-/-} Sfrp5\textsuperscript{-/-} (C) embryos. Note that distribution is not random. Instead, cells with a similar ABP tend to form clusters. (D) To examine cell shape, the x length /y length ratio was measure for 1568 node cells from eight WT (Control) embryo, 1716 node cells from ten Wnt5a\textsuperscript{-/-} Wnt5b\textsuperscript{-/-} embryos, and 478 node cells from three Sfrp1\textsuperscript{-/-} Sfrp2\textsuperscript{-/-} Sfrp5\textsuperscript{-/-} embryos. Statistical significance was examined by Wilcoxon rank sum test. Although different cell shapes are found in the node, there is no significant differences among WT, Wnt5a\textsuperscript{-/-} Wnt5b\textsuperscript{-/-} and Sfrp1\textsuperscript{-/-} Sfrp2\textsuperscript{-/-} Sfrp5\textsuperscript{-/-} embryos.
Figure S7. Effects of the absence of Prickle 1 and Prickle 2 on neighboring WT cells, related to Figure 6 and Figure 7.

(A) Chimeric embryos were generated by aggregating EGFP-expressing $Pkl^{+/+}Pkl^{2+/+}$ ES cells (donors) with an embryo (host). Host embryo was either $Pkl^{Δex6/Δex6}Pkl^{Δex6/Δex6}$, or Control ($Pkl^{Δex6/+}Pkl^{Δex6/+}$ or $Pkl^{+/+}Pkl^{Δex6/Δex6}$). An illustration of a donor cell (asterisk) surrounded by host cells in a chimeric embryo. Donor cells that were completely (100%) or partially (>50%) surrounded by host cells were subjected to further analysis. (B) The chimeric embryos in which host cells are $Pkl^{Δex6/Δex6}Pkl^{Δex6/Δex6}$ were immunostained for Zo-1 (red), Vangl1 (green), and EGFP (light blue). Note that a donor cell (asterisk) that is surrounded by $Pkl^{Δex6/Δex6}Pkl^{Δex6/Δex6}$ cells exhibits abnormal Vangl1 localization. (C) The Vangl1 localization in (B) is illustrated schematically. (D) The frequency of Vangl1 localization patterns (normal, abnormal or unclear) when donor cells are surrounded by Control cells or by $Pkl^{Δex6/Δex6}Pkl^{Δex6/Δex6}$ cells. Note that the frequency of abnormal Vangl1 localization increased when donor cells were surrounded by $Pkl^{Δex6/Δex6}Pkl^{Δex6/Δex6}$ cells (13/48 vs. 5/94 cells).