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Cilia at the node of mouse embryos sense fluid flow for left-right determination via Pkd2

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Abstract

Unidirectional fluid flow plays an essential role in the breaking of left-right (L-R) symmetry in mouse embryos, but it has remained unclear how the flow is sensed by the embryo. We report that the Ca^{2+} channel Pkd2 is required specifically in the peri-nodal crown cells for sensing the nodal flow. Examination of mutant forms of Pkd2 shows that the ciliary localization of Pkd2 is essential for correct L-R patterning. Whereas *Kif3a* mutant embryos, which lack all cilia, failed to respond to an artificial flow, restoration of primary cilia in crown cells rescued the response to the flow. Our results thus suggest that nodal flow is sensed in a manner dependent on Pkd2 by the cilia of crown cells located at the edge of the node.

(Please start the introduction with a more general sentence about LR asymmetry to draw in the broad reader —which organs are asymmetric, etc.— and define the ventral node for the nonspecialist.) Most of the visceral organs in vertebrates exhibit left-right (L-R) asymmetry

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in their shape or position. The breaking of L-R symmetry in the embryos of many vertebrates is mediated by a unidirectional fluid flow in the ventral node (an embryonic cavity at the midline filled with extra-embryonic fluid) or its equivalent structure (1, 2). The flow may transport a determinant molecule or provide mechanical force. However, it has remained unclear how the flow is sensed. We have now addressed this issue with the use of mice lacking Polycystin-2 (Pkd2, also known as TRPP2), a gene encoding a Ca^{2+}-permeable cation channel implicated in polycystic kidney disease in humans(3, 4).

Pkd2 is required only in peri-nodal crown cells

The *Pkd2*$^{-/-}$ mouse was previously shown to lose *Nodal* expression in the lateral plate mesoderm (LPM) and to exhibit typical L-R patterning defects(5). *Pkd2* is expressed ubiquitously at the early somite stage of mouse embryos(5) (fig. S1); however, it has been unclear whether the function of Pkd2 in L-R patterning is executed in the node, LPM, or other region of the embryo. We first examined if the LPM of the *Pkd2*$^{-/-}$ embryo is competent for Nodal signaling. When an expression vector for Nodal was introduced to a small region of the right LPM of the *Pkd2*$^{-/-}$ embryo, expression of endogenous *Nodal* gene was induced in the entire region of the right LPM (fig. S2), suggesting that the LPM of the *Pkd2*$^{-/-}$ embryo remains competent to express Nodal. To further localize the site of Pkd2 action, we established three types of transgenic mice that express *Pkd2* specifically in the node and examined whether such transgenes were able to prevent the L-R defects of the *Pkd2*$^{-/-}$ mouse (Fig. 1; fig. S3). In one transgene, *Foxa2NNE-hsp-Pkd2-IRES-LacZ* (Fig. 1A), *Pkd2* expression is driven by a node/notochord-specific enhancer of the mouse *Foxa2* gene. This transgene is expressed in the midline including the node (Fig. 1A), and it restored left-sided expression of *Nodal* and *Pitx2* in the LPM of *Pkd2*$^{-/-}$ embryos (rescued/tested embryos: 6/6 for *Nodal* and 6/6 for *Pitx2*) (Fig. 1C).

The ventral node contains two different types of ciliated cells: pit cells located in the central region of the node and crown cells located at the edge (fig. S3A)(1). The second *Pkd2* transgene studied, *NDE-hsp-Pkd2-IRES-LacZ* (Fig. 1B), is driven by the node-specific enhancer (NDE) of *Nodal*, which is active specifically in the crown cells. This transgene was able to rescue left-sided expression of *Nodal* and *Pitx2* in the LPM of *Pkd2*$^{-/-}$ embryos (6/6 and 3/3, respectively) (Fig. 1C). Finally, expression of Pkd2 specifically in the pit cells with *dgFoxa2-hsp-Pkd2-IRES-LacZ* (fig. S3B) was unable to rescue the left-sided expression of *Nodal* and *Pitx2* (0/1 and 0/3 embryos, respectively) (fig. S3C). These results thus suggested that Pkd2 is required exclusively in crown cells of the node for correct L-R determination.

Cerl2 is the major target of Pkd2-mediated signaling

Initial breaking of L-R symmetry by leftward nodal flow is followed by asymmetric (R>L) expression of *Cerl2* in crown cells(6, 7). Given that Pkd2 is required specifically in crown cells, we examined crown cell–specific markers in *Pkd2*$^{-/-}$ embryos. *Gdf1*, which is bilaterally coexpressed in crown cells and is required for subsequent *Nodal* expression in left LPM(8, 9), was normally expressed in *Pkd2*$^{-/-}$ embryos (fig. S4A). However, L-R asymmetric gene expression in crown cells was impaired in the mutant embryos. Expression of *Cerl2* in crown cells is greater on the right side in wild-type embryos (fig. S4A)(6), but it was expressed equally on the two sides in *Pkd2*$^{-/-}$ embryos (fig. S4A). *Nodal* expression, which shows a subtle asymmetry in the wild-type embryo (right expression lower than left) (10), was bilaterally equal in *Pkd2*$^{-/-}$ embryos (fig. S4A). These results suggested that crown cells are correctly specified but lose asymmetric gene expression in the absence of Pkd2.

The higher expression of *Cerl2* on the right in crown cells is required for subsequent asymmetric expression of *Nodal* in LPM(6). To clarify the relation between *Pkd2* and *Cerl2*, we examined possible interaction between the two genes by analyzing $Pkd2^{-/-}$;$Cerl2^{-/-}$ double-mutant embryos (fig. S4B). *Nodal* expression in LPM was always absent in $Pkd2^{-/-}$ embryos (5/5), whereas it was either left-sided (2/11), right-sided (3/11), bilateral (2/11), or absent (4/11) in $Pkd2^{-/-}$;$Cerl2^{-/-}$ embryos (fig. S4B, C). The *Nodal* expression pattern in the double mutant was thus similar to that in $Cerl2^{-/-}$ embryos (fig. S4B, C), indicating that *Cerl2* is the major target of Pkd2-mediated signaling for L-R asymmetric patterning (fig. S4D).

Pkd2 in crown cells is required for sensing nodal flow

Particle image velocimetry (PIV) revealed that nodal flow is maintained in the $Pkd2^{-/-}$ mutant (Fig. 2A), suggesting that Pkd2 might serve to sense a flow-derived signal(s) in crown cells. We tested this possibility with the use of a transcriptional enhancer that responds to nodal flow. This enhancer (ANE) is derived from the human *Lefty1* gene and exhibits asymmetric (L>R) activity in crown cells (Fig. 2B)(11). Furthermore, ANE was found to respond to artificial flow. The relative L-R activity of ANE was thus reversed when a rightward flow was imposed on the wild-type embryo (Fig. 2C, D). In $Pkd2^{-/-}$ embryos, ANE activity was R = L (Fig. 2B), despite the fact that the mutant possesses morphologically normal cilia (fig. S5) and functional flow (Fig. 2A), suggesting that crown cells of $Pkd2^{-/-}$ embryos fail to sense the flow.

Given that *Pkd2* encodes a Ca^{2+} permeable ion channel, we examined Ca^{2+} signaling in crown cells by generating a transgenic mouse that expresses Ca^{2+} indicator GCaMP2, which exhibits highly cooperative Ca^{2+} binding over the physiological range of cellular free Ca^{2+} (KD= 146 nM)(12), specifically in crown cells. Indeed Ca^{2+} signaling was detected in crown cells, but it was present bilaterally and was retained in $Pkd2^{-/-}$ embryos (fig. S6). It should be noted that Ca^{2+} asymmetry previously observed with exogenous dyes (13, 14) occurs in the endoderm, a different tissue around the node. To clarify the role of Ca^{2+} signaling, transgenic embryos harboring *ANE-lacZ* were incubated with various Ca^{2+} signaling blockers. $GdCl_3$(an inhibitor of stretch-sensitive TRP channels), 2-ABP (an inhibitor of IP_3 receptor) and Thapsigargin (an inhibitor of Ca^{2+}-ATPase in ER) disrupted L>R asymmetry of ANE activity, whereas Ruthenium Red (a potent inhibitor of intracellular Ca^{2+}release by ryanodine receptors) did not (Fig. 3). Treatment with Thapsigargin did not significantly reduce the level of Ca^{2+} signaling within crown cells (fig. S7), likely because a single inhibitor would only affect a portion of Ca^{2+} signaling within a cell. Rotational movement of pit cell cilia and ciliary localization of Pkd2 were maintained by treatment with $GdCl_3$and Thapsigargin (fig. S8). These results suggest that Ca^{2+} signaling mediated by Pkd2 and IP_3 receptor is essential for generating L-R asymmetry at the node (Fig. 3B).

Ciliary localization of Pkd2 is essential for correct L-R decision

Pkd2 protein resides in the primary cilia of renal epithelial cells(15). In crown cells as well as pit cells of the node, endogenous Pkd2 was also localized in the cilia (fig. S9A; (13)). We also examined the subcellular localization of Pkd2 with the use of a transgenic mouse that expresses a Pkd2::Venus fusion protein in crown cells (Fig. 4A). This fusion protein was shown to be functional by the observation that its expression in crown cells corrected the L-R defects of $Pkd2^{-/-}$ embryos, and was preferentially localized to the cilia of crown cells (Fig. 4A). Expression of Pkd2::Venus protein did not influence motility of cilia (movie S1). Notably, live imaging of Pkd2::Venus-labeled cilia at the presomite stage showed that most of the cilia were immotile (fig. S10; movie S2). The frequency of motile cilia gradually increased between the presomite stage and the three-somite stage (fig. S10). However, most

(90%~) crown cell cilia were immotile while ~60% of pit cell cilia are already motile at the presomite stage (fig. S10), the stage at which *Cerl2* expression in crown cells begins to exhibit L-R asymmetry (11).

Ciliary localization of Pkd2 may depend on its interaction with Pkd1l1, another ciliary protein whose deficiency in mice and zebrafish results in L-R defects similar to those of the *Pkd2*$^{-/-}$ mutant(16, 17). To test if Pkd2 may function in cilia of crown cells, various mutant forms of Pkd2 were examined for their abilities to localize to crown cell cilia and to correct the L-R defects of *Pkd2*$^{-/-}$ embryos (Fig. 4B-E; fig. S9; fig. S11). Although a missense mutation of Pkd2 (E442G) induces L-R defects identical to those of *Pkd1l1* mutant mouse(18), a Pkd2(E442G)::Venus fusion protein was unable to localize to the cilia of crown cells (Fig. 4B, C) and cilia of cultured LLC-PK1 cells (fig. S12). Pkd2(E442G) can interact with Pkd1l1(fig. S13), suggesting that ciliary localization of Pkd2 requires Pkd1l1-independent mechanisms. Pkd2(R6G), a missense mutant unable to localize to primary cilia in cultured LLC-PK1 cells(19), was found to localize to the cilia of crown cells (fig. S9B) and rescued L-R patterning in the *Pkd2*$^{-/-}$ mutant (fig. S11). Pkd2(5–73), which lacks the NH_2-terminal region (residues 5 to 73) including the R_6VxP motif(19), was also able to localize to the cilium when expressed in crown cells (fig. S9C). Pkd2(R6G-G819X), which harbors the R6G missense mutation and lacks the COOH-terminal region, was unable to localize to the crown cell cilium (fig. S9D) or to correct the L-R defects of the *Pkd2*$^{-/-}$ mutant (fig. S11). Similarly, Pkd2(D509V), a missense mutant associated with polycystic kidney disease in humans(20), failed both to localize to cilia of crown cells (Fig. 4B) and to normalize the *Pkd2*$^{-/-}$ phenotype (fig. S11). Two mutants that were unable to localize to crown cell cilia (E442G and R6G-G819X) were examined for their channel activity (Fig. 4D). Although Pkd2(R6G-G819X) lost channel activity, Pkd2(E442G) retained it (Fig. 4D). Our findings that Pkd2(E442G) retains channel activity yet is unable to localize to crown cell cilia (Fig. 4B, C) indicate that ciliary localization of Pkd2 in crown cell is essential for correct L-R decision.

Crown cell cilia function as sensors of nodal flow

Finally, we examined directly whether cilia of the crown cells function as sensors of nodal flow with the use of an IFT (intraflagellar transport) mouse mutant. *Kif3a* and *Kif3b* are expressed ubiquitously in mouse embryo and encode motor proteins that are required for formation of cilia(21–23). *Kif3a*$^{-/-}$ mutant embryos thus lack all node cilia, including those of both pit and crown cells and exhibit L-R defects(22, 23). A transgene that confers *Kif3a* expression specifically in crown cells (*NDE-hsp-Kif3a-IRES-LacZ*) restored cilia formation exclusively in these cells (Fig. 5A). Although *Nodal* expression in LPM was bilateral (L=R) in *Kif3a*$^{-/-}$ embryos (8/8), restoration of cilia formation in crown cells by the *NDE-Kif3a* transgene rescued left-sided *Nodal* expression in LPM in *Kif3a*$^{-/-}$ embryos (3/3) (Fig. 5B, C). PIV analysis revealed a weak leftward flow as well as vortical flow in the node of such embryos (fig. S14), likely because the number of motile cilia among crown cells is much smaller than that of immotile cilia (fig. S10). This result is consistent with our recent finding that as few as two rotating cilia are sufficient for symmetry breaking(24).

We then examined whether the embryos with cilia only in the crown cells are able to respond to artificial fluid flow. The *Kif3a*$^{-/-}$;*NDE-Kif3a* embryos, as well as control *Kif3a*$^{-/-}$ embryos, were thus subjected to rightward artificial flow (Fig. 5B), and L-R patterning as revealed by *Pitx2* expression in LPM was examined. Whereas *Kif3a*$^{-/-}$ embryos (5/5) failed to respond to the rightward flow, the *Kif3a*$^{-/-}$;*NDE-Kif3a* embryos (3/3) did respond by showing right-sided *Pitx2* expression in LPM (Fig. 5B, C). These results thus demonstrated that nodal flow is indeed sensed by crown cell cilia located at the edge of the node.

Our results indicate that the fluid flow in the node is sensed by cilia of peri-nodal crown cells via ciliary-localized Pkd2. Many proteins are known to be localized to primary cilia, and they are implicated in cilium-mediated signaling. However, the role of their ciliary localization has not been rigorously established. Identification of Pkd2(E442G), a L-R defect-causing mutant form of Pkd2 that retains Ca^{2+} channel activity yet is unable to localize to the cilia, provides the direct evidence showing that ciliary localization of a protein is indeed essential for cilium-mediated signaling.

Several questions remain unanswered. It is still not clear what the cilia sense in the breaking of L-R symmetry: Do they sense flow-transported chemicals or flow-generated mechanical forces? Pkd2 is localized to the primary cilium of kidney cells and vascular endothelial cells and mediates mechanosensation(25, 26). Circumstantial evidence, including our recent observation that as few as two rotating cilia are sufficient for L-R symmetry breaking(24), favors the possibility that crown cell cilia sense mechanical force. Also unknown is how cilia signal to *Cerl2*, the major target of the signal transmitted by nodal flow and Pkd2. Finally, our results support the previous proposal (13, 27) that there are two populations of cilia in the node, motile and immotile cilia, and that the former generate nodal flow whereas the latter sense the flow. Given that most cilia of crown cells are immotile at the time when L-R symmetry is broken, it is likely that the flow is sensed by immotile cilia, although we are not able to rigorously exclude the possibility that motile cilia of crown cells also sense the flow. Further studies with diverse approaches are needed to address these issues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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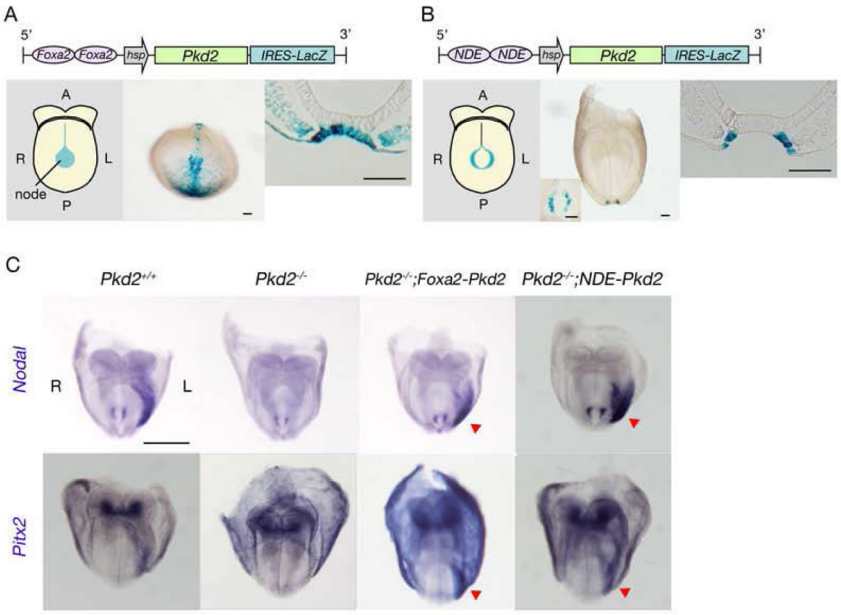


Figure 1. Specific requirement for Pkd2 in crown cells of the node for correct L-R patterning

(**A, B)** Schematic representation of two types of node-specific *Pkd2* transgeneare shown on the top. Their expression patterns were examined by staining of transgenic embryos at embryonic day (E) 8.0 with X-gal. Schematic (left panels) and actual (middle panels) views of whole embryos, and transverse sections of the node (right panels) are shown. Inset of the middle panel in B shows node from ventral view at higher magnification. Note that expression of the NDE-driven transgene is highly specific to crown cells of the node (**B**). A, anterior; P, posterior; L, left; R, right. Scale bars, 50μm. (**C**) Whole-mount *in situ* hybridization analysis of the expression of *Nodal* and *Pitx2* in E8.0 embryos of the indicated genotypes. Left-sided expression of *Nodal* and *Pitx2* in LPM is lost in *Pkd2*$^{-/-}$ embryos but is restored (red arrowheads) in *Pkd2*$^{-/-}$;*Foxa2-Pkd2* and *Pkd2*$^{-/-}$;*NDE-Pkd2* embryos. Scale bar, 500μm.

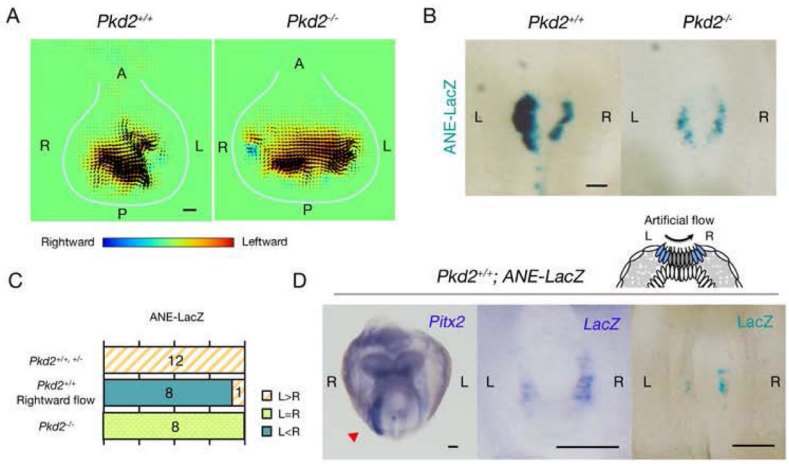


Figure 2. Pkd2 is necessary for sensing of nodal flow

(**A)** Nodal flows in wild-type and *Pkd2*$^{-/-}$ embryos were examined by PIV analysis at E8.0. Flow is maintained in the *Pkd2*$^{-/-}$ embryo. Each arrowhead denotes the direction and speed of the flow at the indicated position. The color scale indicates the direction and magnitude of the flow velocity (leftward in yellow and red, rightward in blue). White lines indicate the outlines of the node. Scale bar, 10μm. (**B)** E8.0 *Pkd2*$^{+/+}$ and *Pkd2*$^{-/-}$ embryos harboring the *ANE-LacZ* transgene were stained with X-gal. Scale bar, 50μm. (**C**) The L-R patterns of ANE activities in embryos of the indicated genotypes either *in situ* or under conditions of artificial rightward flow *in vitro* are summarized. The numbers of embryos showing each pattern are indicated. (Adjust the color/pattern for C. The small box definitions of colors will not show the pattern of top line after figure reduction. ---response: color/patterns have been changed and it is easy to see the difference in color/pattern) (**D)** *Pkd2*$^{+/+}$ embryos with *ANE-LacZ* were cultured under the influence of a rightward artificial flow from early headfold to six-somite stages. Artificial rightward flow reversed the pattern of *Pitx2* expression in LPM (left panel, red arrowhead) and that of ANE activity in crown cells, as detected by *in situ* hybridization of *LacZ* mRNA (middle) and X-gal staining (right). Scale bars, 100μm. Why does the “L” and “R” designation switch from left and right between the figure panels? Figure 1 shows “L” on the right side of figure; Figure 2 shows “L” and “R” in different panel sides in the figure. ---Response: embryos showing gene expression in the lateral plate such as ones in Fig. 1C are frontal views, so that “L ” is shown on the right side. Embryos showing gene expression in the node such as ones in Fig. 2B were back views, so that “L ” was shown on the left side. To avoid confusion, however, all embryo photos are shown in such a way that the right side of an embryo is always shown on the left side.) (Define N and C.---response: N & C are now deleted)

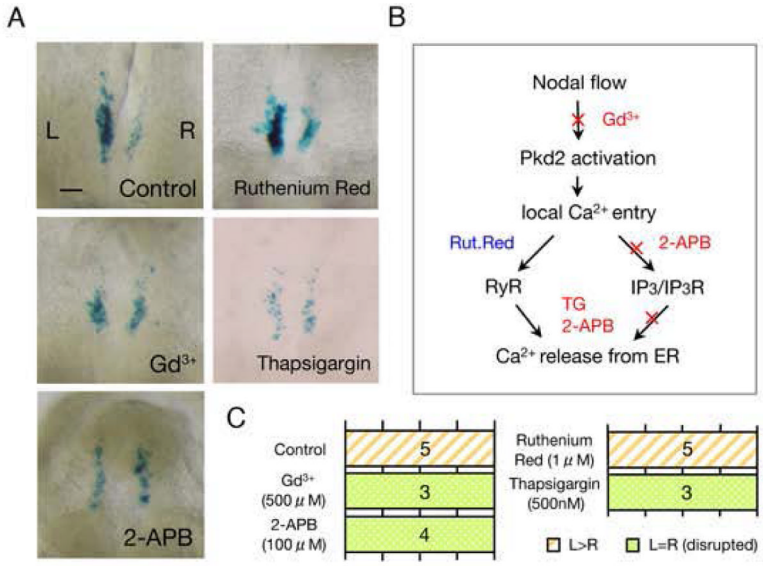
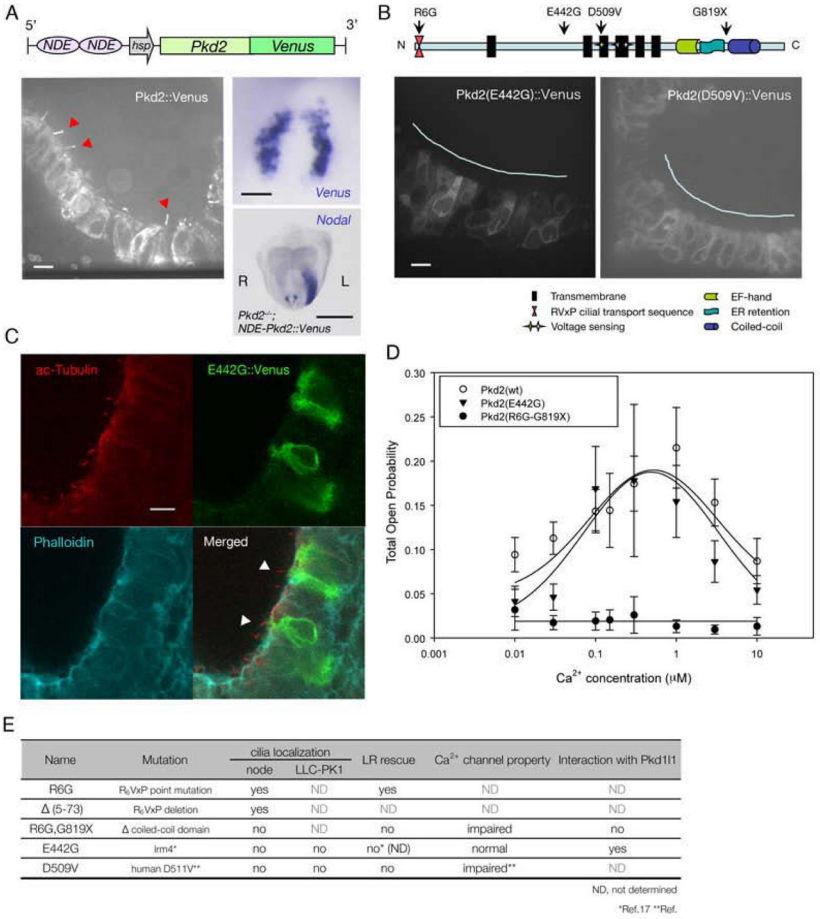


Figure 3. Calcium signal is essential for L-R symmetry breaking in the node crown cells

(**A)** Activity of ANE is disrupted when Ca^{2+} signal blockers are treated. Two pathways that lead to Ca^{2+} release from endoplasmic reticulum were examined (**B**). L>R asymmetric ANE activity is maintained in control and Ruthenium Red- treated embryos, whereas it is disrupted with Gd^{3+}, 2-aminoethoxydiphenyl borate (2-APB) or Thapsigargin (TG). Scale bar, 50μm. (**C)** The effects of each reagent on L-R asymmetric ANE activity are summarized. The numbers of embryos showing each pattern are indicated.



E

Name	Mutation	cilia localization		LR rescue	Ca^{2+} channel property	Interaction with Pkd1l1
		node	LLC-PK1			
R6G	R_6VxP point mutation	yes	ND	yes	ND	ND
Δ (5-73)	R_6VxP deletion	yes	ND	ND	ND	ND
R6G,G819X	Δ coiled-coil domain	no	ND	no	impaired	no
E442G	Irm4*	no	no	no* (ND)	normal	yes
D509V	human D511V**	no	no	no	impaired**	ND

ND, not determined

*Ref.17 **Ref.

Figure 4. Ciliary localization of Pkd2 is required for correct L-R determination

(**A**) Schematic representation of NDE-driven *Pkd2::Venus* transgene is shown on the top. Crown cell-specific expression of the transgene in an E8.0 embryo was confirmed by whole-mount *in situ* hybridization with a *Venus* probe (upper right panel). Scale bar, 50μm. Left-sided expression of *Nodal* was restored in $Pkd2^{-/-}$; *NDE-Pkd2::Venus* embryo (lower right panel), suggesting that Pkd2::Venus is functional. Scale bar, 500μm. Note that Pkd2::Venus protein is preferentially localized to cilia (left panel, red arrowheads). Scale bar, 10 μm. (**B**) Transgenic embryos expressing Pkd2(E442G)::Venus or Pkd2(D509)::Venus in crown cells. Note that both proteins are unable to localize to cilia. Scale bar, 10 μm. (Define N and C. ---response: N & C are removed) (**C**) Localization of Pkd2(E442G)::Venus was confirmed by immunofluorescence staining with antibodies to acetylated tubulin (red—difficult to see/read tubulin---response: we have changed the color brighter so that it is easier to see), to Venus; GFP (green) and phalloidin (cyan). Scale bar, 10μm. (**D**) Open probability of Pkd2(wt), Pkd2(E442G) and Pkd2(R6G-G819X) channels in the presence of increasing cytosolic Ca^{2+} concentration. Note that Pkd2(E442G) retains normal channel activity whereas Pkd2(R6G-G819X) loses it. Error bars represent ± SEM. (**E)** The relationship between ciliary localization, Ca^{2+} channel activity, ability of L-R rescue and interaction with Pkd1l1 is summarized for various Pkd2 mutants. (Please remove E from the figures section and make it a separate Table.---response: we have made E a separate Table).

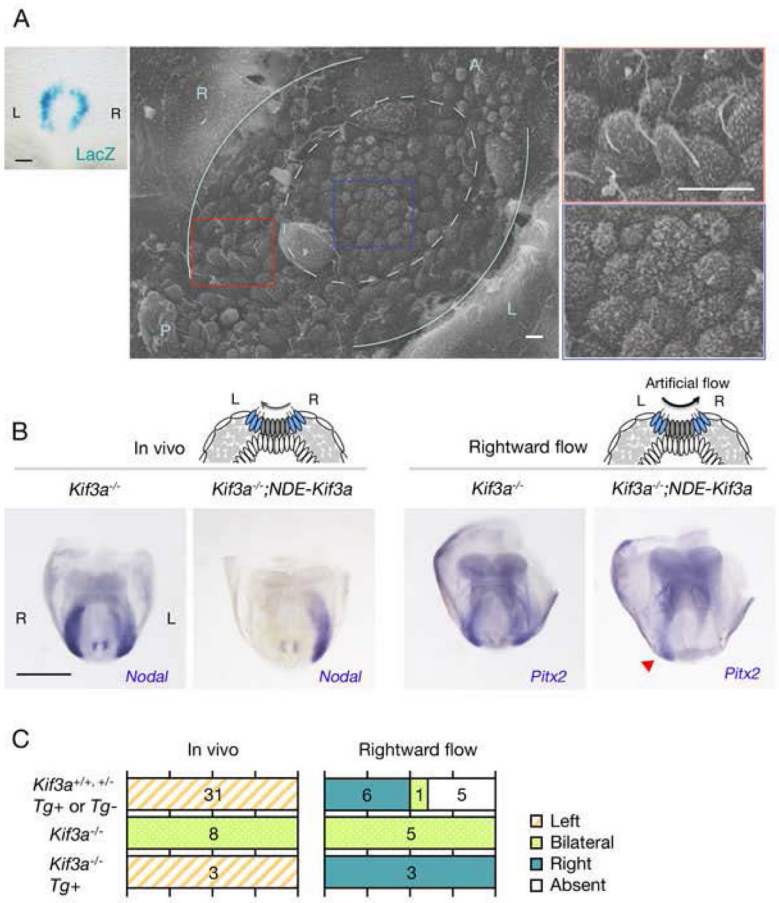


Figure 5. Nodal flow is sensed by the cilia of crown cells

(**A**) An E8.0 transgenic embryo harboring *NDE-Kif3a-IRES-LacZ* was stained with X-gal, revealing crown cell specific expression of the transgene (left). Scale bar, 50μm. In scanning electron microscopy of the node of an E8.0 *Kif3a*$^{-/-}$ embryo with *NDE-Kif3a*, cilia are apparent at the edge (boxed by the red line), but not at the center (boxed by the blue line) of the node. Pale blue lines indicate the border between the endoderm and crown cells, with the dotted circle enclosing pit cells. The boxed regions on the left are shown at a higher magnification on the right. Scale bars, 5 μm. (**B)** Expression of L-R marker genes in embryos of the indicated genotypes that were examined at E8.0 (*in vivo*) or cultured under the influence of a rightward artificial flow before analysis. Note that *Pitx2* expression pattern of the *Kif3a*$^{-/-}$;*NDE-Kif3a* embryo responded to the flow and is right-sided (red arrowhead). Scale bar, 500 μm. (**C**) The numbers of embryos showing each pattern of gene expression are summarized.

Table 1

Properties of various Pkd2 mutants

Name	Mutation	cilia localization		LR rescue	Ca^{2+}channel property	Interaction with Pkd1l1
		node	LLC-PK1			
R6G	R_6VxP point mutation	yes	ND	yes	ND	ND
Δ (5–73)	R_6VxP deletion	yes	ND	ND	ND	ND
R6G,G819X	Δ coiled-coil domain	no	ND	no	impaired	ND
E442G	Irm4 *	no	no	no * (ND)	normal	yes
D509V	human D511V **	no	no	no	impaired **	ND

ND, not determined

* Ref.18

** Ref4

The relation between ciliary localization, Ca2+ channel activity, ability of L-R rescue, and interaction with Pkd1l1 is summarized for various Pkd2 mutants.