An Early Function of Polycystin-2 for Left-Right Organizer Induction in Xenopus

HIGHLIGHTS

- Loss of Polycystin-2 in Xenopus results in LR asymmetry defects upstream of leftward flow.
- LR defects are caused by lack of LR organizer induction.
- Polycystin-2 is required upstream of foxj1 for specification of superficial mesoderm.
- Polycystin-2 and Xnr3 synergistically induce foxj1 in the superficial mesoderm.

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An Early Function of Polycystin-2 for Left-Right Organizer Induction in Xenopus

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SUMMARY
Nodal signaling controls asymmetric organ placement during vertebrate embryogenesis. Nodal is induced by a leftward fluid flow at the ciliated left-right organizer (LRO). The mechanism of flow sensing, however, has remained elusive. pkd2 encodes the calcium channel Polycystin-2, which is required for kidney development and laterality, and may act in flow perception. Here, we have studied the role of Polycystin-2 in Xenopus and show that pkd2 is indispensable for left-right (LR) asymmetry. Knockdown of pkd2 prevented left-asymmetric nodal cascade induction in the lateral plate mesoderm. Defects were due to failure of LRO specification, morphogenesis, and, consequently, absence of leftward flow. Polycystin-2 synergizes with the unconventional nodal-type signaling molecule Xnr3 to induce the LRO precursor tissue before gastrulation, upstream of symmetry breakage. Our data uncover an unknown function of pkd2 in LR axis formation, which we propose represents an ancient role of Polycystin-2 during LRO induction in lower vertebrates.

INTRODUCTION
Asymmetries along the left-right (LR) body axis are a common feature of most animals. Vertebrates, for example, show asymmetric arrangements of most visceral organs (Grimes and Burdine, 2017). In all deuterostomes examined, as well as in several protostomes, asymmetries are induced by unilateral activation of the Nodal signaling cascade, consisting of nodal, lefty, and pitx2, before onset of asymmetric organogenesis (Namigai et al., 2014; Shiratori and Hamada, 2014). How the Nodal cascade is activated unilaterally is a matter of intense research. The most common symmetry-breaking mechanism in vertebrates is a transient extracellular leftward fluid flow at the archenteron of gastrula/neurula embryos (Blum et al., 2009). Although this flow lost in birds, it is absolutely required for symmetry breakage in rabbit, mouse, frog, and fish (Essner et al., 2005; Nonaka et al., 1998; Schweickert et al., 2007). Flow is generated at the left-right organizer (LRO), a transient ciliated epithelium. Owing to the clockwise rotation of monolocia and their posterior tilting, they generate a leftward flow of extracellular fluids (Blum et al., 2011b; Yoshida and Hamada, 2014). Putative LROs have been pinpointed in further vertebrates (axolotl, additional frog species, sturgeon), the ancient chordate amphioxus, and the sea urchin Paracentrotus (Blum et al., 2009; Säenz-Ponce et al., 2011; Tisler et al., 2016).

How flow is sensed on the left side of the LRO and how it activates the Nodal cascade in the lateral plate mesoderm (LPM) is still largely unknown. The Nodal inhibitor Dan5 is one decisive factor in chordates. dan5 and nodal are expressed bilaterally on both sides of the LRO, just before flow becomes established. As a result of flow, dan5 becomes down-regulated on the left (Schweickert et al., 2010; Shinohara et al., 2012). In Xenopus, knockdown of dan5 rescues flow-deficient embryos, establishing dan5 mRNA asymmetry as the first detectable molecular feature of LR asymmetry. How this inhibition is realized at the cellular level is not known. One mechanism that has been proposed is a calcium-dependent down-regulation of dan5 during flow (Yoshida et al., 2012). Left-sided calcium fluxes during flow stages have been described and implicated in flow sensing, but the molecular consequences have remained elusive (Sarmah et al., 2005; Yuan et al., 2015). According to the two-cilia model of symmetry breakage, motile cilia at the center of the LRO create a leftward flow that bends immotile mechanosensory cilia at the left margin. This bending results in left-sided calcium fluxes, which supposedly activate downstream calcium-dependent events that break the bilateral symmetry (McGrath et al., 2003; Tabin and Vogan, 2003). Genetic experiments in the mouse have unequivocally demonstrated that the calcium channel Polycystin-2 is specifically required in lateral cells of the mouse LRO to break symmetry (Yoshida et al., 2012).

We and others have previously shown that pkd2, the gene encoding Polycystin-2, is necessary for kidney and LR development in mouse and for kidney development in Xenopus, as it is in other vertebrates (Futel et al., 2012).
et al., 2015; Pennekamp et al., 2002; Sullivan-Brown et al., 2008; Tran et al., 2010). Here, we revisited the role of Polycystin-2 in LR development in *Xenopus*, in which the sequential steps of laterality development are known in great detail and can be experimentally manipulated in a sided manner. *pkd2* knockdown resulted in failure of Nodal cascade activation, as previously shown for fish and mouse. LRO morphogenesis and function were lost in morphants, as revealed by altered marker gene expression and a lack of ciliation and leftward flow. This phenotype was due to a lack of *pkd2*-dependent induction of the LRO precursor tissue, the superficial mesoderm (SM), during early gastrulation. Polycystin-2 and the FGFR-binding ligand Xnr3 synergize to induce *foxj1* in the SM, establishing an unknown function of Polycystin-2.

**RESULTS AND DISCUSSION**

**pkd2 Is Required for LR Axis Development in Xenopus**

As a reference point for the functional assessment of *pkd2* during symmetry breakage, we analyzed mRNA expression during embryogenesis. Signals at the animal pole of the zygote and during early cleavage stages represented maternally deposited transcripts (Figures 1A and 1B). Sense probe control specimens lacked staining (Figure 1C; data not shown). During early gastrulation, *pkd2* was restricted to the deep mesoderm, excluding the dorsal lip and the superficial epithelial layer, i.e., the SM, from which the frog LRO, the gastrocoel roof plate (GRP), develops during gastrulation (Figure 1D; Blum et al., 2014a). Later expression sites included the notochord, trunk organizer, deep neuroectoderm, intermediate mesoderm, and developing pronephric kidney (Figures 1E–1G and S1A–S1E; cf. Tran et al., 2010).

![Figure 1. Expression of pkd2 mRNA during Xenopus Embryogenesis](image-url)

(A–C) Maternally deposited mRNA localized to the animal hemisphere of the zygote (A, A’) and four-cell stage embryos (B). Note animal-vegetal shift of expression as indicated in the zygote. (C) Lack of a specific signal in specimen hybridized with a sense probe. (D–F) *pkd2* transcripts in deep mesodermal layers of the early gastrula (D, D’, D”; black arrowheads; plane of section indicated in D by dotted line), in dorsal notochordal mesoderm at mid gastrula (E), and in tail organizer (white arrow) and posterior notochord at late gastrula stages (F, F’). Please note the lack of transcripts in the dorsal lip and the SM (white arrowheads). Border of inner and outer layer of marginal zone indicated by dotted line. Yolk plug and archenteron roof indicated by dashed lines. (G) Deep neuroectodermal, tail organizer, and posterior notochordal expression at neurula stages; no expression of GRP detected (G4).

See also Figure S1.
Remarkably, zygotic pkd2 mRNA was not detected in the SM or in the GRP (Figures 1D and 1G), i.e., the early LR-relevant tissues. In the mouse, Polycystin-2 acts downstream of flow but upstream of flow-dependent dand5 repression and LPM Nodal induction (Yoshiba et al., 2012). To test whether this function was conserved in Xenopus, we targeted a previously characterized antisense morpholino oligomer (MO) that blocks translation of pkd2 (Pkd2-MO; Tran et al., 2010) to the future GRP (cf. Figure 2A). Morphant embryos lacked induction of the Nodal cascade in the left LPM in a dose-dependent manner, in agreement with this notion (Figures 2B–2D). As expected for a flow-sensing function, pkd2 was required on the left side of the GRP, as revealed by unilateral injections (Figures S2A and S2B; cf. Vick et al., 2009 for left-sided flow knockdown). Knockdown in the LPM itself did not cause LR defects, suggesting that pkd2 was not relevant for Nodal induction in the LPM.

Figure 2. pkd2 Is Required for LR Asymmetry in Xenopus, Independently of dand5
(A) Putative sensory function of Polycystin-2 during symmetry breakage downstream of GRP-flow.
(B–D) Dose-dependent loss of left-sided pitx2c expression following Pkd2-MO targeting to the GRP. Black arrowhead indicates approximate location of present or absent pitx2c expression on both sides of the embryo. White arrow highlights expression of pitx2c around the eye as proof of working ISH.
(E) Model of hierarchy of the flow sensing module and possible role of pkd2 on the lateral side of the LRO downstream of flow and upstream of Dand5.
(F) Epistatic single and double knockdown experiments, performed on the left or right side, as indicated. Differences in expected and experimental outcome highlighted by red significance. See text for details.
See also Figure S2.
**p < 0.01, ***p < 0.001 in all Figures.
Numbers in parentheses indicate the number of embryos analyzed for each condition.

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propagation (Figure S2B). In summary, these results showed that \textit{pkd2} was required for the activation of the Nodal cascade in the left LPM, suggesting a conserved role during flow-dependent symmetry breakage.

**\textit{Pkd2} Functions Independently of \textit{dand5}**

\textit{Dand5} plays a key role at the interface between flow and Nodal induction (Figure 2E). To test whether \textit{pkd2} acted upstream of \textit{dand5} in the process of flow sensing, we performed combinatory knockdown experiments. When \textit{Pkd2-MO} was injected on the left side of the GRP, embryos failed to induce \textit{pitx2c} in the left LPM, similar to a GRP-specific \textit{nodal1} knockdown. Inhibition of \textit{dand5} alone had no effect, as described (Figure 2F; cf. Schweickert et al., 2010), because flow down-regulates \textit{dand5} as a physiological target. When \textit{Pkd2-MO} and \textit{Dand5-MO} were co-injected, most specimens still did not induce \textit{pitx2c} (Figure 2F). \textit{Dand5} knockdown thus failed to rescue loss of Polycystin-2, as was expected from the sensor function described in mouse (Figure 2F). \textit{Pkd2} therefore could act upstream of \textit{nodal} induction in the LPM. Knockdown of \textit{pkd2} in the LPM, however, did not affect \textit{pitx2} expression (Figure S2B). Alternatively, \textit{pkd2} could be required for \textit{nodal} function at the lateral cells (sensory part) of the GRP. The possibility of sided injections in \textit{Xenopus} afforded the opportunity of investigating this notion on the right side of the GRP, independently of flow. Here, \textit{dand5} knockdown induces right-sided Nodal cascade induction (Figure 2F; cf. Schweickert et al., 2010). Parallel knockdown of \textit{Dand5-MO} and \textit{Pkd2-MO} to the right GRP margin counteracted this effect, as loss of the Nodal inhibitor \textit{Dand5} can only be effective in the presence of Nodal. Simultaneous targeting of \textit{Dand5-MO} and \textit{Pkd2-MO} to the right GRP margin still prevented right-sided Nodal cascade induction (Figure 2F), suggesting that \textit{pkd2} was required for Nodal function at the GRP or GRP function in general.

**Leftward Flow and GRP Morphogenesis Are Compromised in \textit{pkd2} Morphants**

The high efficiency of \textit{Pkd2-MO}, comparable with a \textit{nodal1} knockdown in the left GRP, asked for an in-depth analysis of GRP function itself. Leftward flow was analyzed in dorsal explants prepared from flow stage embryos that were injected with control (co-)MO or \textit{Pkd2-MO} as described (Vick et al., 2009). Time-lapse videography of fluorescent beads added to the explants demonstrated that flow was absent at the targeted area in morphants (Figures 3A, 3C, and Video S1), whereas co-MO injected specimens displayed the previously reported directionality and velocity of bead transport (Figures 3B and 3D; cf. Schweickert et al., 2007). This result suggested that GRP function was lost in \textit{pkd2} morphants. A morphological analysis of GRP tissue using immunofluorescence (IF) to highlight cilia and cell boundaries confirmed this notion, as cilia were literally absent (Figures 3E–3G). To gain further insights into the nature of GRP defects, scanning electron microscopy revealed an increase in cell surface area and thus resembled endodermal cells rather than the small GRP cells (Figure S3B), indicating a potential change of fate. GRP marker genes, such as the tektin isofrom \textit{tekt2} as well as the axonemal dynein motor protein \textit{dnah9}, were absent from morphant explants, whereas control samples exhibited wild-type expression patterns (Figures 3H–3K, S3C, and S3D). Importantly, GRP ciliation and gene expression were rescued by co-injection of a full-length \textit{pkd2} mRNA construct that was not targeted by the \textit{Pkd2-MO} (Figures 3G, 3J, and 3K).

To investigate the GRP fate in depth, we analyzed marker genes that highlight the lateral sensory cells, namely, \textit{nodal1} and \textit{dand5}. Both were absent from morphant specimens, in which \textit{Pkd2-MO} was targeted to these cells (Figures 3L, 3M and S3E–S3H). A further SEM analysis revealed that lateral GRP cells were missing completely and that remaining ciliated central GRP cells directly bordered non-ciliated endodermal cells (Figure S3I). In the absence of \textit{nodal1}, the \textit{Dand5-MO} thus remained without effect, providing a stringent explanation for the nearly complete lack of \textit{pitx2c} induction upon \textit{dand5} knockdown in \textit{pkd2} morphants (cf. Figure 2F).

Polycystin-2 is mainly part of a cilia- or endoplasmic reticulum (ER)-located calcium channel complex, which can modulate cellular calcium levels, and itself can be regulated by intracellular calcium (Busch et al., 2017). To test if manipulations of intracellular calcium levels also affected \textit{nodal1} expression, we treated embryos with Thapsigargin (Tg), a well-known antagonist of ER-located SERCA pumps (Thastrup et al., 1990). Embryos treated at early gastrulation showed blastopore closure defects, caused by lack of necessary intracellular calcium waves, as previously reported (Wallingford et al., 2001; data not shown). Treatment from late gastrulation was ineffective (Figure S2B). In summary, these results suggested that \textit{pkd2} was required for the activation of the Nodal cascade in the left LPM, suggesting a conserved role during flow-dependent symmetry breakage.
gastrula stages onward, however, did not affect gastrulation and resulted in reduced or absent 
nodal1 expression at neurula stages, i.e., when nodal1 is initiated in lateral GRP cells before flow occurs (Figures S3J–S3L). This outcome was supported by another set of experiments. When embryos were treated in the same way with BAPTA-AM, an intracellular calcium chelator, they also showed reduced expression of nodal1 at neurula stages (Figures S3M–S3P). These results support the notion that Polycystin-2 is necessary to induce the lateral cell fate of the LRO in Xenopus, and this required intracellular calcium changes. Yet, these findings were unexpected, as altered LRO nodal expression was not reported from pkd2-knockout mice or zebrafish morphants (Bisgrove et al., 2005; Pennekamp et al., 2002). Mouse embryos treated with Tg, however, showed loss of nodal1 in the LRO and LPM as well, arguing for conservation of a calcium-dependent induction of lateral LRO fates (Takao et al., 2013). Taken together, these analyses demonstrated that LR defects in Pkd2 morphants were caused by impaired GRP morphogenesis and function and, specifically, the absence of lateral LRO cells expressing nodal1 and dand5.

**pkd2 Is Required for SM Specification**

Next, we asked whether the GRP precursor tissue, the SM, was correctly specified during gastrulation. To that end, we analyzed SM marker genes xnr3 and foxj1. Both genes are known targets of canonical Wnt signaling; foxj1 is instrumental for SM and GRP function downstream of Wnt signaling (Glinka et al., 1996; Smith et al., 1995; Stubbs et al., 2008; Walentek et al., 2013). Remarkably, these genes responded differently to pkd2 knockdown: xnr3 was slightly upregulated, whereas foxj1 expression was reduced or absent (Figures 4A–4F). This effect was specific, as foxj1 expression was rescued (or even super-induced) upon...
Figure 4. Polycystin-2 and Xnr3 Synergize to Induce the LRO Precursor Tissue of the Superficial Mesoderm

(A–F) Increased xnr3 (A–C) and reduced foxj1 expression (D–F) in stage 10.5 pkd2 morphants (B and E) as compared with control specimens (A and D). Dotted lines in A and B indicate plane of histological sections shown in A', and B', respectively. Note that foxj1 expression was rescued upon co-injection of a full-length pkd2 mRNA (F).

(G–I) Reduced foxj1 expression in early gastrula stages (st. 10.5) following Tg treatment (G, DMSO control embryo; H, Tg treated specimen; I, quantification of results).
co-injection of full-length *pkd2* mRNA (Figures 4F and S4A–S4C). As reported, calcium spikes during gastrulation were dependent on intracellular calcium (Wallingford et al., 2001), and we treated embryos again with Tg. To test whether *foxj1* expression was dependent on calcium dynamics as well, specimens were treated with brief pulses (maximum 20 min) at stage 9, when *foxj1* expression is initiated before the start of gastrulation. Treated embryos developed without gastrulation defects but showed a lack of *pitx2c* expression in more than 60% of cases, reminiscent of *pkd2* morphants (Figures S4D–S4F). When such specimens were analyzed at the onset of gastrulation (st. 10.5), *foxj1* was reduced or absent as well, confirming a requirement of both calcium-dependent signals and Polycystin-2 for *foxj1* induction (Figures 4G–4I). Surprisingly, in our hands, similar incubations during the initiation of *foxj1* expression (late blastula st. 9 until early gastrula st. 10.5) using the calcium-chelator BAPTA-AM did not alter *foxj1* expression (Figures S4G–S4I). Accordingly, such treated embryos did not show LR axis alterations at tail bud stages, as shown by wild-type *pitx2c* expression (Figure S4J). However, embryos incubated in BAPTA-AM from late blastula (st. 9) to late gastrula stages (st. 12.5), i.e., additionally covering the later sensitive time window of *nodal* initiation in the lateral GRP (cf. Figures S3J–S3P), again developed laterality defects by misexpression of *pitx2c* (Figure S4J). These results suggested that Polycystin-2/Tg and BAPTA-AM have different effects on the process of *foxj1* induction. Yet, in sum, these experiments are in line with a function for *pkd2* and specific calcium changes in the specification of the SM as the LRO precursor tissue.

**pkd2 Synergizes with xnr3 to Induce foxj1 in the Superficial Mesoderm**

Little is known about early signaling pathways that set up the SM. We and others previously demonstrated that canonical Wnt signaling, which depends on type 3 serotonin receptor signaling (Htr3) in the SM, was required for the induction of both *xnr3* and *foxj1* (Beyer et al., 2012). In that light, the above-mentioned up-regulation of *xnr3* in *pkd2* morphants (Figures 4A–4C) argued against the participation of Polycystin-2 as part of the Wnt signaling module upstream of *xnr3* and *foxj1*. To verify this, we analyzed whether *pkd2* was required for Wnt-dependent secondary axis induction. Twinning was induced by ventral injection of *wnt8* mRNA, in the presence or absence of *pkd2*-MO. Inhibition of Polycystin-2 did not affect the frequency of secondary axis formation (Figures S5A–S5C), nor did it affect endogenous organizer gene expression (not shown). Polycystin-2, thus, should not be part of upstream canonical Wnt signaling but control *foxj1* induction in the SM independently.

The opposing effects of *pkd2* knockdown on *foxj1* and *xnr3* made us wonder whether the effect on *foxj1* was mediated through Xnr3. This nodal-related gene is unusual, as it does not interact with TGF-β-type receptors but interacts with Fgf receptor 1 (Fgfr1); MO-mediated *xnr3* knockdown specifically inhibits *brachyury* expression (Yokota et al., 2003). Besides serving as an SM marker gene, this Xenopus-specific factor has not been investigated for a possible function in the context of LR asymmetry. To test whether Xnr3 played a role in laterality determination, we targeted the Xnr3-MO to the dorsal midline (organizer and SM). Knockdown resulted in loss of dorsal *brachyury* expression, as described previously (Figures S5D–S5F; Yokota et al., 2003). Strikingly, when such specimens were analyzed for *foxj1*, expression in the SM was also lost or strongly reduced as compared with control embryos (Figures 4J, 4K, and 4O). This phenotype was specific to loss of *xnr3* function, as reintroduction of full-length *xnr3*-mRNA, which is insensitive to the Xnr3-MO, was able to rescue the observed reduction of *foxj1* very efficiently (Figures 4L and 4O). Morphants that were raised further revealed the previously reported convergent extension defects (Yokota et al., 2003) and altered *pitx2c* expression (data not shown).

Conversely, when *xnr3* was overexpressed in the dorsal SM, an increase of *foxj1* expression was observed, specifically in the more lateral part of the expression domain, the future lateral GRP, demonstrating that Xnr3 was able to enhance *foxj1* expression (Figures 4M and 4O). Interestingly, injecting *xnr3* mRNA into the ventral side was sufficient to induce *foxj1* expression ectopically, demonstrating its role in SM fate.
specification (Figures 4N and 4O). This could indicate a potential role of the well-characterized ventral Bmp signaling pathway in preventing the activation of foxj1 in the ventral superficial layer, as it has been shown that Xnr3 itself has a dorsalizing effect by inhibiting Bmp signaling (Hansen et al., 1997; Haramoto et al., 2004). Thus, to finally test whether xnr3 and pkd2 synergized in SM foxj1 induction, we performed epistasis experiments. MO doses for both genes were reduced such that individually they did not result in a strong reduction of foxj1 expression. Combined injection of Pkd2-MO and Xnr3-MO, however, resulted in a strong reduction or loss of foxj1, establishing a genetic interaction of these genes in SM foxj1 induction and, thus, in setting up a functional LRO (Figures 4O–4S). Interestingly, although exogenously introduced pkd2 mRNA caused an increase of foxj1 expression (Figure S4C), it was not sufficient to rescue foxj1 expression in xnr3 morphant specimens (data not shown).

In summary, our data demonstrate that in general terms, pkd2 is a conserved determinant of LR symmetry breakage in Xenopus. Surprisingly, however, pkd2 is strictly required upstream of leftward flow to set up the LRO during gastrulation, together with xnr3 (Figure 4T). Polycystin2 in the future SM cells could provide a crucial signal for specification. However, the protein of such cell-autonomous function should be of maternal origin, particularly because zygotic mRNA was not detected in the LR-relevant tissues, SM and GRP. Alternatively, Pkd2 could act non-cell autonomously in the deep tissue to induce foxj1 in the superficial layer, i.e., indirectly. It remains to be seen which type of activating signal could mediate this induction. Based on our timed Tg treatments at the blastula or late gastrula stages (Figures 4G–4I and S3J–S3L), and the corresponding central or lateral Pkd2-MO injections (Figures 3Ma and4E, respectively), which resulted in either loss of foxj1 or loss of nodal1, respectively, the following conclusion can be drawn. These differentiable results from two different time points of LR symmetry breakage imply that both Pkd2 function and intracellular calcium changes are necessary at two separate steps of LRO induction and sub-functionalization in Xenopus (Figure 4T). It remains to be seen which other signaling pathways could be required for each of these steps.

In any way, we propose that in evolutionary terms, this may represent an ancestral Polycystin-2 function in amphibian embryos, which undergo a basal mode of vertebrate gastrulation and thus LRO development (Blum et al., 2009; Cooper and Virta, 2007). Interestingly, zebrafish morphant but not mutant embryos display early mesendodermal defects after maternal pkd2 knockdown, reminiscent of SM loss in Xenopus (Schottenfeld et al., 2007). It is tempting to speculate that Fgf signaling represents the common denominator in this context, as Xnr3 binds and signals via the Fgfr1 (Yokota et al., 2003). In zebrafish, Fgf1 signaling affects foxj1 expression, LRO ciliogenesis, and flow (Neugebauer et al., 2009). In mouse embryos, early loss of Fgfr1-mediated signaling results in gastrulation defects, but pkd2-knockout mice do not display impaired LRO formation or flow (Yamaguchi et al., 1994; Yoshiba et al., 2012). Treatment with an Fgfr1 inhibitor, however, blocked nodal1 expression in lateral LRO cells (Oki et al., 2010) in much the same way as observed upon loss of pkd2 in Xenopus. Thus, LRO induction, although still dependent on Fgfr1, may have become independent of (maternal) pkd2 in modern bony fish and mammals. The later role of Polycystin-2 in flow sensing at the lateral LRO cells may very well be conserved in amphibians as well. The loss of LRO and flow in pkd2 morphants precluded the analysis in the context of the present study. The novel role ascribed to Polycystin-2 in Xnr3-/FGF-dependent LRO morphogenesis adds to the long list of functions of this factor that is conserved in animals from nematodes to humans.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Transparent Methods, five figures, and one video and can be found with this article online at https://doi.org/10.1016/j.isci.2018.03.011.

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AUTHOR CONTRIBUTIONS
P.V., M.B., and A.S. conceived, designed, and supervised the experiments; P.V., J.K., I.S., M.T., M.G., T.T., and T.B. performed and analyzed the experiments; P.V. and A.S. interpreted results; and P.V. wrote the original and the revised manuscript with the help of M.B. and final input of all authors.

DECLARATION OF INTERESTS
The authors declare no competing interest.

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Supplemental Information

An Early Function of Polycystin-2
for Left-Right Organizer Induction in *Xenopus*

Philipp Vick, Jennifer Kreis, Isabelle Schneider, Melanie Tingler, Maike Getwan, Thomas Thumberger, Tina Beyer, Axel Schweickert, and Martin Blum
Figure S1: Related to Figure 1. Late expression pattern of pkd2.
Expression in the neuroectoderm (A-C), intermediate mesoderm (B, C), pronephric system (D), branchial arches (D’) and tailbud (E) in early to late tailbud stages.
a, anterior; ba, branchial arches; d, dorsal; e, endoderm; fp, floorplate; im, intermediate mesoderm; l, left; lpm, lateral plate mesoderm; mhb, mid hindbrain boundary; n, notochord; ne, neuroectoderm; pd, pronephric duct; pn, pronephros; r, right; s, somites; v, ventral
Figure S2: Related to Figure 2. Lineage-specific knockdown of pkd2 reveals a left-sided dorsal requirement of Polycystin-2 in the LRO

(A) Schematic depiction of the differentially targeted areas of the gastrocoel roof in a dorsal explant of a stage 17 neurula embryo with the central, flow-generating population of the LRO (1. red), the lateral, sensory population of the LRO (2. green), and the surrounding endodermal cells (3. yellow) that cover the lateral plate mesoderm, which will express nodal and pitx2c on the left side after flow-dependent symmetry breakage to govern asymmetric organogenesis.

(B) Unilateral left-sided pkd2 knockdown experiment showing dose-dependent loss of pitx2c when injected into the left dorsal marginal zone (DMZ) to target the central, flow-generating GRP. Injection into the dorso-lateral marginal zone (D-LMZ) to target the sensory part of the GRP (i.e. the lateral LRO cells) caused fewer, yet significant LR defects. Injecting the ventral marginal zone (VMZ) to target the left lateral plate mesoderm did not result in miss-expression of pitx2c.

*p<0.05, **p<0.01, ***p<0.001 for all panels.
Figure S3: Related to Figure 3. GRP defects in pkd2 morphants

(A, B) SEM analysis of co-MO (A) and Pkd2-MO (B) injected specimens reveal loss of ciliation and altered GRP cell morphology in morphants. Examples are representative for 6 specimens analyzed each. Scale bars represent 20 micrometers.

(C-H) Unilateral absence of dna9 (D), nodal1 (F) and dand5 (H) in left-injected embryos in comparison to internal control sides at stage 17. Control embryos (C, E, G) show wildtype expression levels.

(I) SEM analysis of ciliation and cell morphology in dorsal explant of stage 17 specimen unilaterally injected with Pkd2-MO targeted exclusively to the lateral, sensory part of the GRP. Shown is a ventral view of the GRP broken transversally and revealing the deep tissue arrangements in the top half of the picture. Right (I’) and left (I”) magnifications show both the sensory areas of the GRP. Note that the lateral GRP cells were absent on the MO-injected side, such that central GRP cells, which are characterized by posteriorly polarized cilia, directly bordered non-ciliated endodermal cells. Sensory GRP cilia highlighted with red arrowheads, central GRP cilia with green arrowheads.

(J-L) Expression of nodal1 in lateral GRP cells of flow stage (st. 17) specimens following Tp (K, L) or 1% DMSO (J) treatment during mid to late gastrula stage (st. 11.5). nodal1 was lost (K’) or strongly reduced (L) after Tp treatment, without causing gastrulation defects.

(M-P) Expression of nodal1 in lateral GRP cells of flow stage (st. 16-19) specimens following 25-200µM BAPTA-AM (N-P) or 0.05-1% DMSO (M, P) treatment during mid and late gastrula stages (st. 11.5-13/14). nodal1 was lost (O) or reduced (N) after BAPTA-AM treatment. *p<0.05, **p<0.01, ***p<0.001 Black arrowheads indicate reduction or lack of expression.

a, anterior; e, endoderm; l, left; n, notochord; p, posterior; r, right; s, somites
Calcium manipulating agents Thapsigargin or BAPTA-AM impacted differently on LR development

(A-C) *foxj1* expression (A) was reduced in *pkd2* morphants (B) and rescued upon co-injection of Pkd2-MO and a full-length *pkd2* rescue mRNA not targeted by the MO (C).

(D-F) A 20-minute thapsigargin treatment before gastrulation resulted in loss of *pitx2c* expression in >50% of embryos without concomitant gastrulation defects, reminiscent of Pkd2-MO injected specimens (cf. Figure 2D).

(G-J) Treatment of embryos between blastula (st. 9) and early gastrula stages (st. 10.5) with different concentrations of the calcium chelator BAPTA-AM did not reduce *foxj1* expression (H, I), in comparison to 0.05-1.00% DMSO treatment (G, I). (J) Embryos analyzed for *pitx2c* expression at tailbud stages after short (left side, until stage 11) or long (right side, until stage 12.5) treatment with BAPTA-AM. Longer (st. 9-12.5), but not short treatment (st. 9-11) resulted in significant LR defects. Please note the high proportion of embryos with general axis malformations when treated until late gastrulation (right side) as compared to shorter treatment (left side). Significances in (J) were calculated for LR expression patterns of *pitx2c* only, not including malformed embryos, which are also shown in the graph.

*p<0.05, **p<0.01, ***p<0.001 for all panels.
Figure S5: Related to Figure 4. Polycystin-2 function in LR axis formation is independent of canonical Wnt signaling

(A-C) Wnt-dependent double axis formation. Injection of \textit{wnt8a} mRNA resulted in >75% of conjoined twinning (A, C). Co-injection of Pkd2-MO resulted in a very moderate drop of twinning rates to approx. 70% (B, C). Quantification of results (C). Arrowhead indicates induced secondary axis.

(D-F) Wildtype expression of \textit{brachyury} during gastrulation in control specimens (D) was reduced (E) or lost (F) after injection of Xnr3-MO into the dorsal marginal zone (DMZ), demonstrating that Xnr3 was required for dorsal \textit{bra} expression. Bisected embryos in D', E' and F' highlight these effects.
TRANSPARENT METHODS

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, Philipp Vick (philipp.vick@uni-hohenheim.de).

EXPERIMENTAL MODEL AND SUBJECT DETAIL
For in vivo studies, Xenopus laevis was used as model organism. Frogs were obtained from Nasco (901 Janesville Avenue P.O. Box 901 Fort Atkinson). Handling, care and experimental manipulations of animals was approved by the Regional Government Stuttgart, Germany (Vorhaben A379/12 ZO „Molekulare Embryologie”), according to German regulations and laws (§6, article 1, sentence 2, nr. 4 of the animal protection act). Animals were kept at the appropriate condition (pH=7.7, 20°C) at a 12 h light cycle in the animal facility of the Institute of Zoology of the University of Hohenheim. Female frogs (4-20 years old) were stimulated with 25-75 units of human chorionic gonadotropin (hCG; Sigma), depending on weight and age, that was injected subcutaneously one week prior to oviposition. On the day prior to ovulation, female frogs were injected with 300-700 units of hCG (10-12 h before). Eggs were collected into a petri dish by carefully squeezing of the females and in vitro fertilized. Sperm of male frogs was gained by dissecting of the testes that was stored at 4°C in 1x MBSH (Modified Barth’s saline with HEPES) solution.

METHOD DETAILS

Plasmid construction
For xnr3 mRNA-rescue experiments, the xnr3 coding sequence was isolated from the original plasmid using Pfu DNA polymerase and sub-cloned into the CS2+ plasmid with EcoRI and XhoI restriction sites with the following oligonucleotides for PCR:
Xnr3.1 forward primer: 5’ CCGGAATTCATGGCATTTCTGAACCTG 3’
Xnr3.1 reverse primer: 5’ CCGCTCGAGTTACATGTCCTTGAACCTG 3’
For in vitro synthesis of mRNA using the Ambion sp6 message kit, the plasmid was linearized with NotI.

Microinjections
Embryos were injected at the 4- to 8-cell stage, using a Harvard Apparatus. Drop size was calibrated to 4-6 nl / injection, amounts of injected MOs are indicated in the main text. Lineage tracers used for injection control were fluorescein (70,000 MW) or rhodamine B dextran (10,000 MW; both ThermoFisher). More detailed lineage-specific injections to target GRP and ventro-lateral tissues have been described previously (Blum et al., 2009a).

Treatments with calcium inhibitors
To manipulate intracellular calcium levels and wave patterns, embryos were incubated at two different time points, either with the ER SERCA-pump inhibitor Thapsigargin, or
with BAPTA-AM (cell-permeable form of the calcium-chelator BAPTA), both diluted in 0.1x MBSH.

For foxj1 induction, embryos were incubated at stage 9 (before start of gastrulation) for 10, 15 or 20 min in 2µM Tg, then solution was replaced by 0.1x MBSH and embryos fixed at stage 10.5. Most robust effects were obtained with 15 or 20 min treatments, longer treatments with this concentration started to impact on gastrulation. Alternatively, ca. 120 min treatment with 0.75µM caused similar results (not shown). A similar procedure was applied for BAPTA-AM but solution was not replaced until fixation at stage 10.5 (begin of gastrulation). Tested concentration range was as indicated in Figure S4.

For analysis of nodal1 induction, embryos were incubated in 4µM Tg, or in BAPTA-AM as indicated in Figure S3, both from stage 11.5 on to avoid gastrulation defects, and embryos were fixed at stage 15-18 (during flow-stages) for analysis by ISH.

For complementary pitx2c analyses, some embryos were reared until tailbud stages and processed for ISH after washout of the drug.

**Immunofluorescence staining**

For immunofluorescence staining, embryos were fixed in 4% PFA (Polyoxymethylene) for 1h at RT on a rocking platform, followed by 2 washes in 1x PBS for 15 min each. For staining of GRP explants, embryos were manually dissected transversally using a razor blade. Posterior halves (GRP explants) were collected and transferred to a 24 well plate and washed twice for 15 min in PBST. GRP-explants and whole embryos were blocked for 2h at RT in CAS-Block diluted 1:10 in PBST (0.1% Triton X-100). The blocking reagent was replaced by antibody solution (anti-acetylated tubulin antibody, 1:700 in CAS-Block) and incubated ON at 4°C. Antibody solution was removed and explants washed twice for 15 min in PBS, then the secondary antibody (1:1000 in CAS-Block) was added. Alexa Fluor 488 Phalloidin (1:200) was incubated over-night. Before photo documentation with a Zeiss LSM 700 Axioplan2 Imaging microscope, embryos or explants were shortly washed in PBS and transferred onto a microscope slide.

**SEM and GRP Analysis**

c-15 MO or Pkd2-MO injected specimens were fixed with 4% paraformaldehyde/ 2.5% glutaraldehyde and processed for SEM analysis. SEM photographs were analyzed for ciliation, polarization and cell surface area by individual full GRP analysis using ImageJ and evaluated as described (Beyer et al., 2012; Sbalzarini and Koumoutsakos, 2005).

**Whole-mount in situ hybridization (ISH)**

Embryos were fixed in MEMFA for 2h and processed following standard protocols (Sive et al., 2000). RNA in situ probes were transcribed using SP6 or T7 polymerases. In situ hybridization was modified from (Belo et al., 1997).

**Flow-analysis**

For analysis of leftward flow, dorsal posterior GRP-explants were dissected from stage 16/17 embryos injected with Pkd2-MO/co-MO and the lineage tracer rhodamine-B dextran (0.5 mg/ml) into one or two dorsal blastomeres. GRP-explants were placed in a petri-dish containing fluorescent microbeads (diameter 0.5 µm; diluted 1:2500 in
1xMBSH) and incubated for a few seconds. Explants were transferred to a microscope slide which was prepared with vacuum grease to create a small chamber that contained fluorescent microbeads solution; a cover slip carefully pressed on to seal the chamber. Time lapse movies of leftward flow were recorded using a AxioCam HSm video camera (Zeiss) at 2 frames per second using an Axioplan2 imaging microscope (Zeiss). For flow analysis, ImageJ and statistical-R, were used. Using the Particle-Tracker plug-in from ImageJ, leftward flow was analyzed and particle movement was measured, and data processed as described previously to create corresponding GTTs (Gradient Time Trails) as shown in Figure 3 (Vick et al., 2009).

**Axis induction Assay**

Double axis induction was performed by single injection of 40-80 pg wnt8a mRNA with or without 1pmol Pkd2-MO into one ventral mesodermal blastomere at the 4-8 cell stage. Embryos were cultured until late tailbud stage and scored for double axis induction.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistical analysis**

Statistical calculations of marker gene expression patterns and cilia distribution were performed using Pearson’s chi-square test (Bonferroni corrected). For statistical calculation of ciliation, cilia length, cell size, flow velocity and directionality Wilcoxon-Match-Pair test was used (statistical R).

*=p<0.05, **=p<0.01, ***=p<0.001 were used for all statistical analyses.
# KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti acetylated α-tubulin | Sigma | AB_477585 |
| Anti-mouse IgG (whole molecule) F(ab’)2 fragment-Cy3 | Sigma | AB_258785 |
| Alexa Fluor 488 Phalloidin | Invitrogen | AB_2315147 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Pfu DNA Polymerase | Promega | M7745 |
| FluoSpheres™ Carboxylate-Modified Microspheres, 0.5 µm, yellow-green fluorescent (505/515) | Invitrogen | F8813 |
| Thapsigargin (3S,3aR,4S,6S,6aR,7S,8S,9bS)-6-(Acetyloxy)-4-(butyryloxy)-3,3a-dihydroxy-3,6,9-trimethyl-8-[(2Z)-2-methylbut-2-enoyl(oxy)-2-oxo-2,3,3a,4,5,6,6a,7,8,9b-decahydroazulenol[4,5-b]furan-7-yl octanoate] | Tocris | 1138 |
| BAPTA-AM 1,2-Bis(2-aminophenoxy)ethane-N,N',N,N'-tetraacetic acid tetrakis(acetoxyethyl ester) | abcam | Ab120503 |
| Human chorionic gonadotropin (hCG) | Sigma | C0809-1VL |
| **Critical Commercial Assays** | | |
| mMessage mMachine™ SP6 Transcription Kit | Thermo Fisher Scientific | AM1340 |
| **Experimental Models: Organisms/Strains** | | |
| Xenopus laevis (female, male) | Nasco | https://www.enasco.com/xenopus/ |
| **Oligonucleotides** | | |
| Xnr3.1 forward primer [5’ CCGGAATTCCATGGCATTTCTGAACCTG 3’] | Sigma | |
| Xnr3.1 reverse primer [5’ CCGCTCGAGTTACATGTCCTTGAATCC 3’] | Sigma | |
| **Software and Algorithms** | | |
| Adobe Suite CS6: Photoshop and Illustrator | Adobe | |
| ImageJ/Fiji | | https://fiji.sc/ |
| AxioVision 4.6 | Zeiss | |
| Zen 2012 Blue edition | Zeiss | https://www.zeiss.com/ |
| Statistical R-Gui | | https://www.r-project.org/ |
| **Other** | | |
| Pkd2-MO: 5’ GGTGGATTTCGCTGGGATTCATCG 3’ | Gene Tools, Philomath, USA | (Tran et al., 2010) |
| Xnr3-MO: 5’ TCTCTGGGTAGATTTTGGTGTAACCTC 3’ | Gene Tools, Philomath, USA | (Vonica and Brivanlou, 2007) |
| Nodal1-MO: 5’ GCTGTCAGAAATGCCATGCTTGCAC 3’ | Gene Tools, Philomath, USA | (Vonica and Brivanlou, 2007) |
| Dand5-MO1: 5’ CTGGTGCGCTGGAACAACAGCATGT 3’ | Gene Tools, Philomath, USA | (Vonica and Brivanlou, 2007) |
| Dand5-MO2: 5’ TGTTGGCGCTGGAACAACAGCATGTC 3’ | Gene Tools, Philomath, USA | |
| standard control-MO | Gene Tools, Philomath, USA | |
| Axioplan2 Imaging microscope | Zeiss | |
| Zeiss LSM 700 | Zeiss | |
| AxioCam HSm video camera | Zeiss | |
| Xenbase | | https://xenbase.org |
| PubMed | | https://www.ncbi.nlm.nih.gov/pubmed/ |
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