Wnt11b Is Involved in Cilia-Mediated Symmetry Breakage during Xenopus Left-Right Development

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Abstract

Breakage of bilateral symmetry in amphibian embryos depends on the development of a ciliated epithelium at the gastrocoel roof during early neurulation. Motile cilia at the gastrocoel roof plate (GRP) give rise to leftward flow of extracellular fluids. Flow is required for asymmetric gene expression and organ morphogenesis. Wnt signaling has previously been involved in two steps, Wnt/β-catenin mediated induction of Foxj1, a regulator of motile cilia, and Wnt/planar cell polarity (PCP) dependent cilia polarization to the posterior pole of cells. We have studied Wnt11b in the context of laterality determination, as this ligand was reported to activate canonical and non-canonical Wnt signaling. Wnt11b was found to be expressed in the so-called superficial mesoderm (SM), from which the GRP derives. Surprisingly, Foxj1 was only marginally affected in loss-of-function experiments, indicating that another ligand acts in this early step of laterality specification. Wnt11b was required, however, for polarization of GRP cilia and GRP morphogenesis, in line with the known function of Wnt/PCP in cilia-driven leftward flow. In addition Xnr1 and Coco expression in the lateral-most GRP cells, which sense flow and generate the first asymmetric signal, was attenuated in morphants, involving Wnt signaling in yet another process related to symmetry breakage in Xenopus.

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Introduction

In vertebrates many inner organs of the chest and abdomen, such as heart and stomach, are asymmetrically localized along the left-right (LR) body axis [1]. Initiation of LR asymmetry in fish, amphibians and mammals is achieved by a cilia-driven leftward flow of extracellular fluids during neurulation [2–4]. Ciliated epithelia exist only transiently and are represented by the amphibian GRP [5], Kupffer’s vesicle in fish [6] and posterior notochord (“node”) in mammals [7,8]. The lateral margins of these epithelia are characterized by cells which co-express the Wnt signaling pathway, which plays a plethora of important roles during animal development, tissue homeostasis and in human disease [13–15]. During LR axis development the canonical Wnt/β-catenin pathway initiates the expression of the transcription factor Foxj1, a master regulator of motile cilia, in the SM [16–18]. The SM represents a part of the epithelial outer layer of the gastrula embryo. It neighbors the organizer caudally and involutes during gastrulation to give rise to the GRP [19]. Foxj1 expression in Kupffer’s vesicle of zebrafish embryos is also regulated by Wnt/β-catenin [20], indicating conserved Wnt-dependency of Foxj1 expression during LR axis development. The non-canonical Wnt/PCP pathway was shown to be necessary for cilia polarization to the posterior pole of GRP cells [21], as a prerequisite for the generation of a directed laminar flow from right to left [16,22]. This role of Wnt/PCP for LR axis specification was also described in mouse [23,24], arguing for evolutionary conservation of this Wnt-dependent step in LR development as well.

In zebrafish the ligands Wnt3a, Wnt8 and Wnt11 were shown to be required for LR development [20,25,26]. In Xenopus, Wnt8a is not expressed in the SM or GRP [27,28]. Wnt8a expression only starts at stages when Foxj1 is already expressed in the SM [29,30]. Wnt11b, in contrast, is present in the oocyte and zygotic expression persists in dorsal regions before and after the onset of gastrulation [29–31]. Wnt11b can activate both canonical and non-canonical signaling branches during Xenopus development [32–34]. Maternally deposited Wnt11b mRNA is enriched on the dorsal side during cleavage stages and contributes to organizer formation by activation of Wnt/β-catenin signaling [31,35]. During gastrulation and later development, Wnt11b and Wnt11r regulate convergent extension [36–38], neural crest cell induction and migration [39–41] as well as heart [42] and pronephric development [43] by activation of non-canonical Wnt signaling branches, i.e. Wnt/PCP and Wnt/calcium signaling [44,45]. Wnt11b was therefore analyzed for a potential role in Wnt/β-catenin dependent Foxj1 expression and Wnt/PCP dependent cilia polarization during Xenopus LR development.
Results

Wnt11b is Expressed in the Superficial Mesoderm

As a first step to elucidate the role of Wnt11b during LR axis development we analyzed mRNA expression patterns at LR relevant sites (Figure 1A–D). With the onset of gastrulation (stage 9.5) zygotic Wnt11b expression started in the dorsal region of the prospective mesoderm (Figure 1A). Manual bisection of embryos revealed expression in the SM, but not in deeper layers of the organizer (Figure 1A’), reminiscent of Foxj1 expression (Figure S1 A, A’). At stage 10.5 the domain expanded laterally (Figure 1B), eventually forming a ring of expression around the blastopore by stage 11.5 (Figure 1C). In dorsal regions the expression remained restricted to the SM, while mRNA in more lateral and ventral regions was detected in deep mesodermal cells as well (Figure 1B’). By stage 13, when the blastopore is closing, Wnt11b was expressed within the circumblastoporal collar (Figure 1D–D’), i.e. a ring of cells which involute into the gastrocoel. These expression patterns support a possible role of Wnt11b during GRP formation and LR development.

Wnt11b-manipulated Embryos show Loss of Asymmetric Pitx2c Expression

Wnt11b function in LR development was addressed by morpholino oligonucleotide (MO) mediated knockdown [50,51] of Wnt11b translation and in gain-of-function experiments using a full length Wnt11b DNA expression construct. Short of a Xenopus Wnt11b-specific antibody, knockdown efficiencies could not be addressed directly. SM and GRP were targeted by injecting 4-cell embryos into the dorsal marginal zone [52]. Specimens were cultured until they reached stage 31 and analyzed for Pitx2c gene expression by whole mount in situ hybridization [2,53–55]. Remarkably, both gain and loss of Wnt11b function resulted predominantly in loss of asymmetric Pitx2c expression in the left LPM (Figure 1E, F). In Wnt11b morphants Pitx2c expression was mostly absent, while bilateral expression of Pitx2c represented the most frequently encountered phenotype following Wnt11b DNA injection (Figure 1E, F). Ectopic expression of Wnt11b in addition resulted in severely shortened anterior-posterior axes (Figure 1E), indicative of convergent extension defects [56].

Next we asked whether changes in Foxj1 expression might correlate with absence of Pitx2c expression in morphants. Unexpectedly, differences from the wildtype pattern were recorded only in a minority of cases (Figure S1B, C). As both organizer function and notochord formation are required for normal LR development, we analyzed Xnr3 and Not mRNA expression in Wnt11b morphants [18,57,58]. Both were expressed in wildtype fashion (Figure S1D, E), demonstrating that organizer and notochord were not affected.

In order to test whether ligand-mediated Wnt signaling was indeed required for Foxj1 induction in the SM, we used a previously characterized antisense MO to interfere with translation of the canonical Wnt receptor Frizzled 8 (Fz8), which was shown to be active on the dorsal side of the Xenopus gastrula [59,60]. As shown in Figure S1 (F, G), Foxj1 expression in the SM was severely affected in Fz8 morphants, and down-regulation was rescued by co-injection of a β-catenin DNA expression construct. These experiments confirm our previous findings that ligand-mediated canonical Wnt signaling is required for Foxj1 independent manner in Wnt-dependent LR axis development.

Manipulation of Wnt11b Perturbs Leftward Flow at the GRP

In an attempt to systematically dissect the LR pathway in Wnt11b manipulated embryos, we turned to leftward flow as the next step downstream from SM specification and Foxj1 induction. Directionality and velocity of flow were analyzed in dorsal explants of Wnt11b morphants and Wnt11b DNA injected specimens (Figure 2 and Movie S1). Robust leftward flow was detected in un.injected control explants (Figure 2A), while flow directionality was compromised in Wnt11b morphants (Figure 2B) as well as following Wnt11b DNA injection (Figure 2C). To evaluate flow in groups of manipulated specimens we used the dimensionless number rho (ρ), which provides a qualitative measure (Figure 2D). Rho was calculated from time-lapse movies and represents the mean resultant directionality of particle trails (Rayleigh’s test of uniformity) [5]. Rho values range from 1, when all trajectories point in the same direction, to 0, when particles move randomly. Explants from un injected control embryos showed a mean ρ-value of 0.82±0.12, while flow in Wnt11b morphants and Wnt11b DNA injected specimens reached ρ-value of 0.51±0.17 and 0.6±0.28, respectively. While these data clearly demonstrate the impact of Wnt11b knockout on flow directionality, the residual flow has not lost directionality altogether. Flow velocities were calculated from the same set of time-lapse movies (Figure 2E). Velocities in Wnt11b morphants and following Wnt11b DNA injection were found at 1.37μm/s±0.32 and 1.19μm/s±0.23, respectively, compared to un injected controls which displayed a mean velocity of 2.54μm/ s±0.94 (Figure 2E). Velocity thus was affected in a more pronounced manner than flow directionality. These data pinpoint flow as a decisive step for Wnt11b function during LR development.

Wnt11b Regulates Wnt/PCP Dependent Cilia Polarization and Morphogenesis of the GRP

Next we analyzed cilia polarization, a process known to depend on Wnt/PCP [21–24]. GRP explants from Wnt11b manipulated embryos were stained for cilia and cell borders using an antibody against acetylated tubulin and phalloidin (Figure 3A–D). In un injected control GRPs most cells were ciliated (79%) and cilia were localized to the posterior pole (Figure 3A’, E). Analysis of Wnt11b morphants and embryos injected with DNA encoding either a dominant negative Wnt11b construct (dnWnt11b; [37]), which is specific for Wnt5/11-type ligands without affecting the canonical pathway, or wildtype Wnt11b revealed disturbed cilia polarization (Figure 3B–D’ and E). Remarkably, clear differences were seen between loss-of-function scenarios and ectopic expression of Wnt11b. The ciliation rate was reduced to 50% and 66% in Wnt11b morphants and following injection of dnWnt11b DNA, respectively. Overexpression of Wnt11b did not alter the wildtype ciliation rate of about 80%, but cilia were predominantly unpolarized, i.e. arose in a central position (Figure 3D’, E). In addition we observed that the apical surface of GRP cells was enlarged upon Wnt11b manipulation (Figure 3F). Average surface areas measured 193.71μm²±143.00, 195.79μm²±92.33 and 187.64μm²±95.73 in Wnt11bMO, dnWnt11b DNA and Wnt11b DNA injected specimens, respectively, compared to 123.88μm²±74.28 in control specimens, indicating an effect on GRP cell morphogenesis (Figure 3F). Taken together, balanced levels of Wnt11b seem to be required for Wnt/PCP dependent cilia polarization and GRP morphogenesis.
Loss of Wnt11b Disrupts Xnr1 and Coco Expression in Lateral Sensory GRP Cells

GRP analyses implemented Wnt11b in the LR cascade at the level of flow or events downstream. They do, however, not provide an explanation as to the opposing effects of Wnt11b manipulation on Pitx2c expression, namely absence in morphants and bilateral induction upon ectopic expression (cf. Figure 1E, F). Our previous analysis of ATP4α has shown that a turbulent and attenuated cilia-driven flow is sufficient to induce the nodal-cascade in a bilateral fashion [16], in line with the characterization of Wnt11b DNA injected specimens presented here (Figure 1E, F and 2C). In order to elucidate the opposing effect in morphants, we analyzed the lateral GRP cells which express both Xnr1 and its inhibitor Coco (Figure 4A, F), and which are required for LPM Xnr1 induction [9]. Wnt11b morphants and specimens injected with dnWnt11b showed significantly reduced expression levels of both genes (Figure 4B, C, G, H). Ectopic expression of Wnt11b, in contrast, showed comparable signal strength to wildtype specimens (Figure 4D, I), although domains were not aligned in parallel due to more pronounced convergent-extension phenotypes encountered in these experiments (cf. Figure 1E). Specificity of treatments was confirmed by co-injection of Wnt11b DNA in Wnt11b morphants, which partially restored Xnr1 expression (Figure 4E). These differential effects on Xnr1/Coco provide an explanation for LPM Pitx2c induction in the various experiments.
Taken together, our data involve Wnt11b in the setup of the GRP and leftward flow.

Discussion

A role of Wnt signaling in LR axis development has been previously demonstrated in vertebrate model organisms including *Xenopus* [16,20–26,49,61–70]. The sequential activity of two Wnt pathway branches is required for cilia-driven leftward flow: (1) canonical Wnt/β-catenin signaling regulates Foxj1 expression during gastrulation in the *Xenopus* SM and in zebrafish Kupffer’s vesicle [16,20]; (2) non-canonical Wnt/PCP signaling is required for the posterior alignment of motile cilia at the frog GRP and the posterior notochord in mouse [16,21,23,24]. The present work confirmed the Fz8-mediated Wnt/β-catenin dependent activation of Foxj1 expression in the SM [16]. Wnt11b, however, contributes only marginally, if at all, to this process. Two additional canonical Wnt ligands are expressed during *Xenopus* gastrulation, Wnt3a and Wnt8a [27–30]. Wnt3a expression starts only after the onset of Foxj1 transcription in the SM. It is therefore tempting to speculate that Wnt8a represents the main canonical activator of Foxj1 expression during gastrulation. This notion may seem contradictory at first glance, as Wnt8a is expressed in ventral and lateral portions of the prospective mesoderm but not in the SM itself [27–28]. We have, however, previously shown that ventral and lateral portions of the mesodermal ring are competent to express Foxj1 upon activation of canonical Wnt signaling [16]. Restriction of Foxj1 expression to the SM thus might be mediated by expression of the receptor Fz8.

Our study clearly demonstrates a role of Wnt11b in Wnt/PCP dependent cilia polarization at the GRP. Cilia alignment was altered by both gain and loss of non-canonical Wnt11b signaling, in agreement with findings in other systems in which non-canonical Wnt signaling was manipulated [16,21,23,24]. Although the role of Wnt/PCP in Vangl2-dependent cilia polarization is well established [21–24], the initial global cue(s) for posterior orientation of cilia in vertebrate flow-generating epithelia has not been identified as yet. Wnt11b is expressed in the circumblastoporal collar, i.e. in cells en route to involute into the gastrocoel. Cells expressing Wnt11b mRNA therefore likely start secreting the protein following involution, i.e. when they are localized at the posterior margin of the GRP. Such a localization might establish a posterior to anterior gradient of Wnt11b protein, which, together with the co-expressed non-canonical ligand Wnt5a [32,37,38] might serve as instructive cue for cilia polarization at the GRP.

Figure 2. **Wnt11b is required for leftward flow at the GRP.** Flow analysis in wildtype and manipulated specimens. (A–C) Frequency distribution of trajectory angles in representative explants of uninjected control embryos (uninj., A), Wnt11bMO (B) and Wnt11b DNA (C) injected specimens. Dashed circles indicate maximum frequency (in %), n represents the number of tracked particles above threshold. a = anterior, l = left, p = posterior, r = right, v = average velocity of particles, p = quality of flow. (D, E) Compiled results of all embryos analyzed for flow directionality (D) and velocity of fluorescent beads added to GRP explants at stage 17 (E). Note that both parameters were significantly reduced in Wnt11b morphants or Wnt11b DNA injected embryos. In (D, E), n represents number of analyzed explants. * Significant (p<0.05), **Very highly significant (p<0.001), n = number of analyzed explants.

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Wnt gradients might mediate asymmetric phosphorylation of Vangl2, leading to anterior-posterior asymmetric localization of motile cilia, similar to the polarization mechanism proposed in the mouse limb bud [71].

Remarkably, we found another Wnt-dependent process during LR axis formation in *Xenopus*, namely *Xnr1/Coco* expression at the lateral-most aspects of the GRP. Previous reports have implicated canonical Wnt/β-catenin signaling in the expression of the *Xnr1/Coco* homologs *spaw/charon* in zebrafish [25,62] and in the homologous nodal expression domain in mouse [63]. Canonical Wnt signaling is important for organizer formation and function, which in turn is required for correct LR axis development [57].

Figure 3. *Wnt11b* is required for cilia polarization and GRP morphogenesis. Embryos were injected at the 4-cell stage into the prospective dorsal marginal zone and dorsal explants were prepared at stage 17. Specimens were processed for immunohistochemistry (IHC) to assess cilia polarization, ciliation rate and cell surface area. (A–D) Presence and polarization of cilia, as shown by acetylated tubulin IHC to stain cilia (red) and phalloidin to stain actin (green) in order to outline cell boundaries. (A) Control uninjected (uninj.) specimen. (B) *Wnt11b* morphant. (C) Specimen injected with dominant-negative *Wnt11b* DNA (*dnWnt11b*). (D) Specimen injected with wild-type *Wnt11b* DNA. (A’–D’). Evaluation of ciliation and polarization. Green = posterior localization of cilia, yellow = other localization, red = cells without cilia. (E, F) Evaluation of results. (E) Cilia polarization. (F) Apical cell surface area. ***Very highly significant (p<0.001). a = anterior, l = left, p = posterior, r = right.

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effects on nodal expression therefore might be indirect. The unaltered Xnr3 and Not expression patterns in our Wnt11b morphants and the effectiveness of a dnWnt11b DNA construct, which is only activated post MBT [72], however, argue for a specific impact of Wnt signaling on lateral cells of the GRP, unrelated to organizer function and notochord formation.

We have recently shown that ATP4a is required for Wnt/β-catenin and Wnt/PCP signaling during Xenopus LR development [16], similar to ATP6 [73–75]. It seems unlikely that Wnt11b acts on Xnr1/Coco expression via the Wnt/β-catenin or Wnt/PCP pathways, because morpholino-mediated loss of ATP4a function did not affect Xnr1 or Coco expression. Furthermore, pharmacological inhibition of ATP6 during frog, chick and zebrafish development did not lead to a loss, but randomized Xnr1, nodal or spaw expression, respectively [76].

Which signaling branch might Wnt11b act on in the context of LR development? We like to propose an involvement of Wnt/calcium signaling. Wnt11b is known to interact with the Wnt/calcium pathway during Xenopus development, especially during gastrulation [43,45,77]. Manipulation of calcium signaling during gastrulation alters LR development in zebrafish, Xenopus and mouse [63,78–81], in line with a possible role of Wnt/calcium signaling in the regulation of Xnr1 and Coco expression. Further experiments are required in the various model organisms to

**Figure 4. Altered Xnr1 and Coco expression in Wnt11b manipulated embryos.** Embryos were injected at the 4-cell stage into the DMZ and analyzed for Xnr1 (A–E) or Coco expression (F–J) by whole mount in situ hybridization. Wildtype expression patterns (A, F) were reduced (B, G) or absent/strongly reduced (C, H) in Wnt11b morphants and in embryos injected with a dnWnt11b DNA construct, while signal intensities were unaltered upon ectopic wildtype Wnt11b expression from a DNA construct (D, I). (E, J) Quantification of results. Note that co-injection of wild-type Wnt11b DNA was sufficient to partially rescue Xnr1 expression at the GRP of Wnt11b morphants. ***Very highly significant (p = 0.001). a = anterior, I = left, p = posterior, r = right.

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resolve the precise mechanism of Wnt-dependent expression of nodal and its respective inhibitor in the lateral flow-sensing cells of the ciliated organs of laterality.

Materials and Methods

Ethics Statement

All animals were treated according to the German regulations and laws for care and handling of research animals, and experimental manipulations according to §6, article 1, sentence 2, nr. 4 of the animal protection act were approved by the Regional Government Stuttgart, Germany (Vorhaben A 365/10 ZO “Molekulaire Embryologie”).

Statistical Evaluation of Results

Statistical evaluation of experiments represented by bar graphs was performed using chi-square tests (http://www.physics.csbsju.edu/stats/contingency.html). In Figure 1F, the number of manipulated embryos (Wnt11bMO or Wnt11b DNA) displaying wt, inverse, bilateral or absent Pitx2c expression was compared to the numbers of embryos expressing these patterns in uninjected control specimens. In Figure S1C, F, the number of morphant embryos with wt, reduced and absent FoxJ1 expression was compared to the number of embryos with wt, reduced or absent expression in uninjected controls and embryos co-injected with β-catenin DNA. In Figure 4E, J, the number of Wnt11bMO or dnWnt11b DNA injected embryos with wt, reduced or absent strongly reduced expression of Xmt1 or Caoa, respectively, was compared to the number of embryos with these characteristics in uninjected control specimens and Wnt11b morphants co-injected with wt Wnt11b DNA. Statistics of experiments represented by box plots were calculated by Wilcoxon sum of ranks (Mann-Whitney) tests (http://www.fon.hum.uva.nl/Service/Statistics/Wilcoxon_Test.html).

Manipulation of Embryos

Embryos were injected at the two- to four-cell stage using a Harvard Apparatus setup in 1 x modified Barth’s solution (MBSH) with 4% Ficoll (BioChemica) and transferred to 0.1 x MBSH 15 min after injection. Drop size was calibrated to about 7-8 nl per injection. Rhodamine-B or Cascade blue dextran (0.5–15 min after injection. Drop size was calibrated to about 7–8 nl with 4% Ficoll (BioChemica) and transferred to 0.1 x MBSH) was used at 1–2 pmol per embryo, Wnt11b MO or Wnt11b DNA. In situ hybridization was performed as described [16,48,49]. The whiskers of the box plots extend to maximal 1.5 x IQR, and outliers are displayed as circles.

Immunohistochemistry and GRP Analysis

Immunohistochemistry was performed as described [48] using Anti-Tubulin Acetylated (mouse, 1:700; Sigma) and anti-mouse Cy3 (sheep, 1:250; Sigma). Cell boundaries were visualized by Alexa 488-conjugated phalloidin (Invitrogen), which stained the actin cytoskeleton. Imaging was performed on a Zeiss LSM700. To determine GRP cell parameters, an area of 320 x 320 μm at the center of the GRP was selected for manual analysis of cilia number/polarization and GRP cell size using ImageJ [16,49]. The whiskers of the box plots extend to maximal 1.5 x IQR, outliers are displayed as circles.

Supporting Information

Figure S1 FoxJ1 expression requires Wnt signaling through Fzβ, but is largely independent of Wnt11b. (A, A’) FoxJ1 expression in the superficial mesoderm at stage (st.) 10.5. in whole mount (A) and bisected specimens (A’). (B, C) Marginal effects on FoxJ1 mRNA expression levels and localization in Wnt11b morphants (quantification in C). (D, E) Wildtype expression of Xmt3 (D) and Not (E) in Wnt11b morphant embryos. (F, G) FoxJ1 expression requires Fzβ. (F) Summary of results. (G) Altered FoxJ1 expression in Fzβ morphants is partially rescued by co-injection of β-catenin (β-cat). Green arrowhead, wild-type expression; red arrowhead, reduced expression; gray arrowhead, absent expression. Dashed line in (A) indicates plane of bisection. ** Highly significant (p<0.01), *** Very highly significant (p<0.001), a = anterior, d = dorsal, l = left, n = number, p = posterior, r = right, v = ventral, veg = vegetal.

Movie S1 Flow defects in GRP explants from Wnt11b manipulated embryos. Movie shows time-lapse sequences of dorsal explants to which fluorescent beads were added (cf. Figure 2A-C). Specimens were mounted dorsal side down and viewed from the ventral side, anterior to the top. Movie represents a total length of 500 frames taken at a rate of 2 frames/sec and runs at 40 x real time. Opening frame displays bright field images and indicates orientation of GRP (dashed lines). Videos were processed to yield gradient time trails (GTTs), i.e. color-coded tracks of beads which revealed direction of transport and velocity of particles (from green to red; 25 s). Note that robust leftward flow (uninjected controls) was impaired in Wnt11b morphant and upon injection of wild-type Wnt11b DNA.

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Author Contributions

Conceived and designed the experiments: PW IS AS MB. Performed the experiments: PW IS. Analyzed the data: PW IS AS MB. Wrote the paper: MB PW.
References

1. Basu B, Brueckner M (2008) Cilia: multifunctional organelles at the center of vertebrate left-right asymmetry. 1st ed. Elsevier Inc. doi:10.1016/S0070-2153(08)00099-6.

2. Schwickert A, Valevskt P, Thumberg T, Danilchik M (2011) Linking early determinants and cilia-driven leftward flow in left-right axis specification of Xenopus laevis. A theoretical approach. Differentiation; research in biological diversity 75: 133–146. doi:10.1016/j.ydbio.2009.09.039.

3. Schwickert A, Valevskt P, Thumberg T, Danilchik M (2011) Ciliation and gene expression distinguish between node and posterior notochord in the mammalian embryo. Differentiation; research in biological diversity 75: 133–146. doi:10.1016/j.ydbio.2009.09.039.

4. Nonaka S, Tanaka Y, Okada Y, Takeda S, Harada a, et al. (1998) Left-right asymmetry. 1st ed. Elesvier Inc. doi:10.1016/S0070-2153(08)00099-6.

5. Macdonald RT, Tamkai K, He X (2009) Wnt/beta-catenin signalling: components, mechanisms, and diseases. Developmental cell 17: 9–26. doi:10.1016/j.devcel.2008.10.009.

6. Emmerich JJ, Amack JD, Nyholm MK, Harris EB, Yost HJ (2005) Kupffer's vesicle is a ciliated organ of asymmetry in the zebrafish embryo that initiates left-right development of the brain, heart, and gut. Development, Cambridge (England) 132: 1247–1260. doi:10.1242/dev.01660.

7. Blum M, Andre P, Moders K, Schwickert A, Fischer A, et al. (2007) Cilia-driven leftward flow determines laterality in Xenopus. Current biology? CB 17: 60–66. doi:10.1016/j.cub.2006.10.067.

8. Emmerich JJ, Amack JD, Nyholm MK, Harris EB, Yost HJ (2005) Kupffer's vesicle is a ciliated organ of asymmetry in the zebrafish embryo that initiates left-right development of the brain, heart, and gut. Development, Cambridge (England) 132: 1247–1260. doi:10.1242/dev.01660.

9. Schweickert A, Walentek P, Thumberger T, Danilchik M (2011) Linking early development and Nodal ciliogenesis in the mouse node. Nature cell biology 13: 514–524. doi:10.1038/ncc2857.

10. Shook DR, Majer C, Keller R (2004) Pattern and morphogenesis of presumptive node and gonad in Xenopus. Developmental biology 270: 163–185. doi:10.1016/j.ydbio.2009.05.547.

11. Hojo M, Takashima S, Kobayashi D, Sumeragi A, Shimada A, et al. (2007) Loss of the Dkk1 antagonist Cerl-2 in the mouse node is required for correct leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. Cell 95: 829–837.

12. Blum M, Andre P, Moders K, Schwickert A, Fischer A, et al. (2007) Cilia-driven leftward flow determines laterality in Xenopus. Current biology? CB 17: 60–66. doi:10.1016/j.cub.2006.10.067.

13. Enser JJ, Anack JD, Nyholm MK, Harris EB, Yost HJ (2005) Kupffer's vesicle is a ciliated organ of asymmetry in the zebrafish embryo that initiates left-right development of the brain, heart, and gut. Development, Cambridge (England) 132: 1247–1260. doi:10.1242/dev.01660.

14. Hirokawa N, Tanaka Y, Okada Y, Takeda S, Harada a, et al. (1998) Planar cell polarity signalling regulates cell adhesion properties in progenitors of the zebrafish lateral lobe. Development, Cambridge (England) 137: 3459–3468. doi:10.1242/dev.00991.

15. Caron A, Xu X, Lin X (2009) Distinct functions of Wnt/beta-catenin signaling in KV development and cardiac asymmetry. Development (Cambridge, England) 136: 2157–2167. doi:10.1242/dev.021946.

16. Nonaka S, Tanaka Y, Okada Y, Takeda S, Harada a, et al. (1998) Planar cell polarity signalling regulates cell adhesion properties in progenitors of the zebrafish lateral lobe. Development, Cambridge (England) 137: 3459–3468. doi:10.1242/dev.00991.

17. Stubbs JL, Oishi I, Izpisua Belmonte JC, Kintner C (2008) The forkhead protein Foxj1 regulates anterior and posterior neural tube morphogenesis in Xenopus. Developmental biology 319: 279–291. doi:10.1016/j.ydbio.2008.04.016.

18. Alten L, Schuster-Gossler K, Beckers A, Groos S, Ulmer B, et al. (2012) ATP4a Is a Critical Target of Leftward Flow in Xenopus. Current Biology. doi:10.1016/j.celrep.2012.03.005.

19. Shook DR, Majer C, Keller R (2004) Pattern and morphogenesis of presumptive node and gonad in Xenopus. Developmental biology 270: 163–185. doi:10.1016/j.ydbio.2009.05.547.

20. Basu B, Brueckner M (2008) Cilia: multifunctional organelles at the center of vertebrate left-right asymmetry. 1st ed. Elsevier Inc. doi:10.1016/S0070-2153(08)00099-6.
66. Zhang Y, Levin M (2009) Left-right asymmetry in the chick embryo requires Plxna1 and Plxna2. Development (Cambridge, England) 136: 389–400.

67. Mahaffey JP, Grego-Bessa J, Liem KF, Anderson KV (2013) Coflin and Vangl2 cooperate in the initiation of planar cell polarity in the mouse embryo. Development (Cambridge, England) 140: 1262–1271. doi:10.1242/dev.085316.

68. Bajoghli B, Aghaizahi N, Norodiad S, Czerny T (2007) The roles of Groucho/Tle in left-right asymmetry and Kupffer’s vesicle organogenesis. Developmental biology 303: 347–361. doi:10.1016/j.ydbio.2006.11.020.

69. Rodriguez-Esteban C, Capdevila J, Kawakami Y, Iripiusa Belmonte JC (2001) Wnt signaling and PKA control Nodal expression and left-right determination in the chick embryo. Development (Cambridge, England) 128: 3189–3195.

70. Nascone N, Mosela M (1997) Organizer induction determines left-right asymmetry in Xenopus. Developmental biology 189: 68–78. doi:10.1006/dbio.1997.0633.

71. Gao B, Song H, Bishop K, Elliot G, Garrett L, et al. (2011) Wnt Signaling Gradients Establish Planar Cell Polarity by Inducing Vangl2 Phosphorylation through Ror2. Developmental cell 20: 163–176. doi:10.1016/j.devcel.2011.01.001.

72. Newport J, Kirschner M (1982) A major developmental transition in early Xenopus embryos: I. characterization and timing of cellular changes at the midblastula stage. Cell 30: 673–686.

73. Buschling T, Bartscherer K, Ohikawara B, Chaudhary V, Spirohn K, et al. (2010) Wnt/Frizzled signaling requires dPrr, the Drosophila homolog of the protein receptor. Current Biology 20: 1263–1268. doi:10.1016/j.cub.2010.05.028.

74. Cruciat C, Mediated VH, Ohikawara B, Acebron SP, Karaulanov E (2011) Requirement of Prorenin Receptor and Vacular H+–ATPase – Mediated Acidification for Wnt Signaling. Science. 439. doi:10.1126/science.1179602.

75. Niehrs C, Boutros M (2010) Trafficking, acidification, and growth factor signaling. Science signaling 3: pe26. doi:10.1126/sciadv.3134pe26.

76. Adams DS, Robinson KR, Fukumoto T, Yuan S, Albertson RC, et al. (2006) Early, H+–VATPase-dependent proton flux is necessary for consistent left-right patterning of non-mammalian vertebrates. Development (Cambridge, England) 133: 1657–1671. doi:10.1242/dev.02941.

77. Kohl M, Sheldahl LC, Mallon CC, Moon RT (2006) Ca(2+)/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in Xenopus. The Journal of biological chemistry 275: 12701–12711.

78. Hatayama M, Mikoshiba K, Aruga J (2011) IP(3) signaling is required for cilia formation and left-right body axis determination in Xenopus embryos. Biochemical and biophysical research communications 410: 520–524. doi:10.1016/j.bbrc.2011.06.014.

79. Keeling J a, Balantac ZL, Crawford AR, Ren Y, Toure J, et al. (2008) Suppression of the endoplasmic reticulum calcium pump during zebrafish gastrulation affects left-right asymmetry of the heart and brain. Mechanisms of development 125: 396–410. doi:10.1002/dvdy.21855.

80. Nakaya M, Biris K, Tsukiyama T, Jaime S, Rawls JA, et al. (2005) Wnt3a links left-right determination with segmentation and anteroposterior axis elongation. Development (Cambridge, England) 132: 5425–5436. doi:10.1242/dev.02149.

81. Zhang Y, Levin M (2009) Left-right asymmetry in the chick embryo requires core planar cell polarity protein Vangl2. Genesis (New York, NY) 2000: 47: 719–726. doi:10.1002/dvdy.20551.