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4 **Roles of cilia, fluid flow, and Ca²⁺ signaling in breaking of left-right**
5 **symmetry**
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Abstract

The emergence of left-right (L-R) asymmetry during embryogenesis is a classic problem in developmental biology. It is only since the 1990s, however, that substantial insight into this problem has been achieved by molecular and genetic approaches.

Various genes required for L-R asymmetric morphogenesis in vertebrates have now been identified, and many of these genes are required for the formation and motility of cilia. Breaking of L-R symmetry in the mouse embryo occurs in the ventral node, where two types of cilia are present. Whereas centrally located motile cilia generate a leftward fluid flow, peripherally located immotile cilia sense a flow-dependent signal, which is either chemical or mechanical in nature. Although Ca^{2+} signaling is implicated in flow sensing, the precise mechanism remains unknown. Here we summarize current knowledge of L-R symmetry breaking in vertebrates (focusing on the mouse), with a special emphasis on the roles of cilia, fluid flow, and Ca^{2+} signaling.

An introduction to left-right asymmetry

Visceral organs of vertebrates exhibit left-right (L-R) asymmetry with regard to their position and morphology. Recent molecular and genetic studies, which began in the 1990s, have uncovered mechanisms responsible for the generation of such L-R asymmetry. These mechanisms are largely conserved among vertebrates, although substantial diversity has been identified¹. Four steps are required to establish L-R asymmetric patterning in the mouse embryo (Figure 1)²: (1) symmetry breaking by a leftward fluid flow (nodal flow) generated by the rotational movement of primary cilia at the node; (2) transmission of an asymmetric signal (or signals) produced in or around the node to the lateral plate mesoderm (LPM); (3) asymmetric expression of *Nodal* and the gene for its feedback inhibitor *Lefty2* in the left LPM; and (4) situs-specific morphogenesis as a result of asymmetric expression of *Pitx2*, which encodes a transcription factor activated by Nodal signaling. Despite recent progress, a key issue that remains unresolved is how the asymmetric signal (or signals) is transferred from the node to the LPM. Here we describe current understanding of the mechanism of L-R patterning, which is based largely on genetic evidence, with special emphasis on cilia, fluid flow, and Ca^{2+} signaling. Observations discussed hereafter were made with mouse embryos, unless indicated otherwise.

Symmetry breaking by cilia

Unidirectional fluid flow generated by rotational movement of cilia

There are 200 to 300 monociliated pit cells in the node cavity of the mouse embryo at embryonic day (E) 8.0 (early somite stage), and the cilia of these cells rotate in a clockwise direction at a speed of ~600 rpm. This rotational movement generates a leftward fluid flow, rather than a vortical flow, because the cilia protrude in a posteriorly tilted manner^{3,4}. Asymmetry in the rotational stroke may also contribute to generation of the unidirectional flow⁵.

An essentially similar mechanism is adopted by many, if not all, vertebrates. The gastrocoel roof plate in *Xenopus*⁶ and Kupffer's vesicle (KV) in zebrafish, which are equivalent to the mouse node, also possess motile cilia whose movement generates a unidirectional flow. The architecture of KV in zebrafish differs from that of the mouse node, however. The motile cilia are present at the ventral and dorsal surface inside KV,

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2 but they are preferentially localized in the anterior region. In contrast to the cilia in the
3 anterior region, the basal bodies of the cilia are not markedly shifted toward the
4 posterior side. Nonetheless, a unidirectional flow is generated in the anterior-dorsal
5 region of KV^{7,8}. In the chick embryo, however, there is no cavity on the ventral
6 surface of Hensen's node in which a flow of fluid could be established, and motile cilia
7 equivalent to those in the mouse node are not present. It has been suggested^{9,10} that the
8 chick embryo adopts a different symmetry-breaking mechanism involving L-R
9 asymmetric cell migration around Hensen's node. Interestingly, in the urochordate
10 *Ciona intestinalis*, which also manifests L-R asymmetric expression of *Nodal* and *Pitx2*,
11 cilia appear immediately before the onset of asymmetric *Nodal* expression. These cilia
12 are immotile, however, and thus can only act as sensors¹¹. Flow may be generated by
13 motile cilia yet to be discovered in *C. intestinalis* or by an unknown mechanism not
14 involving motile cilia.
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17 ***Node cilia are tilted as a result of planar cell polarity***

18 Each cilium protrudes from a basal body, and the location of the basal body within
19 individual pit cells of the mouse node is posteriorly shifted (Figure 2). Time-lapse
20 observations of the node in live mouse embryos have revealed that the position of the
21 basal body changes during development¹². At the early bud stage, when unidirectional
22 flow is not yet apparent, the basal body is localized in a relatively central region of each
23 pit cell. The position of the basal body gradually shifts toward the posterior side,
24 however, so that at the three-somite stage, when the leftward flow is maximal, the basal
25 body is found on the posterior side of most pit cells. The position of the basal body thus
26 closely correlates with the strength of the leftward flow.
27

28 The basal body is positioned by a mechanism known as planar cell polarity
29 (PCP). Deficiency of PCP core proteins such as Dvl¹² and Vangl2¹³ results in failure
30 of the basal body of the pit cells to shift posteriorly. Furthermore, some PCP core
31 proteins are asymmetrically distributed in the node cells: Dvl2 and Dvl3 are localized to
32 the posterior side of these cells¹², whereas Prickle2¹⁴ and Vangl1¹³ are localized to the
33 anterior side (Figure 2). Posterior positioning of the basal body is thus determined by
34 planar polarization of node cells along the anterior-posterior (A-P) axis. Dynamic
35 rearrangement of the actin cytoskeleton is important for the polarized localization of
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2 PCP core proteins, given that polarization of node cells is disrupted in the absence of
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4 cofilin1, a regulator of the actin cytoskeleton ¹⁵. Both the posterior tilt of node cilia and
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6 leftward flow are also impaired in mutant mice lacking Bicaudal C ¹⁶. This putative
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8 RNA-binding protein uncouples Dvl2 signaling from the canonical Wnt signaling
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10 pathway ¹⁶ and regulates Pkd2 (polycystin 2) expression in the kidney by antagonizing
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12 the activity of the microRNA miR-17 ¹⁷, but its exact role in positioning of the basal
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14 body remains unclear. In ciliated cells of developing *Xenopus* skin ¹⁸, flow refines the
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16 orientation of the cilia (and possibly the position of the basal body as well), giving rise
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18 to a flow-mediated self-organizing system. Such a mechanism does not seem to operate
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20 in the mouse embryo, however, given that the basal body of pit cells in the node is
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22 positioned normally in the *iv/iv* mutant ⁴, which completely lacks nodal flow ¹⁹.

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24 The identity of the initial A-P cue (or cues) responsible for such polarization
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26 remains unknown. Noncanonical Wnt ligands such as Wnt5a and Wnt5b, which are
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28 expressed asymmetrically with respect to the position of the node, are good candidates
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30 for the initial A-P cue, as are secreted antagonists of noncanonical Wnt signaling such
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32 as Sfrp, which is expressed in the region anterior to the node ²⁰.

33 34 ***Genesis and motility of node cilia***

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36 Ciliated cells in the node possess a single primary cilium. Impairment of the formation
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38 or motility of the node cilia results in a loss of unidirectional flow and consequent L-R
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40 patterning defects. Since the first report of a mouse mutant with impaired ciliogenesis
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42 (*Kif3b* mutant mouse) ²¹, numerous genes related to ciliogenesis have been identified in
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44 the mouse and other vertebrates, some of which are essential for formation of node cilia.
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46 Knowledge obtained from the study of these mutants has contributed to our
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48 understanding of how the primary cilia of the pit cells in the node are formed under
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50 normal conditions.

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52 Unlike other primary cilia present in various organs of the body, the primary
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54 cilia of pit cells in the node are motile (as we will describe below, there are also
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56 immotile cilia in the node). Motile cilia in the node rotate in a clockwise direction
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58 (when observed from the ventral side of the embryo) and at an average speed of ~600
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60 rpm. In general, motile cilia possess the 9+2 arrangement of microtubules having a ring
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62 of nine peripheral doublets of microtubules plus a pair of central microtubules. Motile
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2 cilia in the node, however, appear to possess the 9+0 arrangement lacking the central
3 pair of microtubules. The existence of motile cilia without the central pair of
4 microtubules is not necessarily surprising, given that studies with *Chlamydomonas*^{22, 23}
5 and human²⁴ suggest that the central microtubules affect the pattern and speed of
6 flagellar movement but are not essential for motility per se. Although the molecular
7 basis for the driving force of rotational movement is not known, ciliary motility appears
8 to depend on the sliding of dynein arms, as has been suggested for the movement of
9 *Chlamydomonas* flagella²⁵. Dynein arms, which are composed of the motor protein
10 dynein and many associated proteins, are assembled in the cytoplasm and transported to
11 the cilium. The assembly of these structures requires additional proteins, many of which
12 are cytoplasmic²⁶⁻³⁰. Mutation of any of these proteins might thus be expected to result
13 in a loss of ciliary motility and in disorders known as primary ciliary dyskinesia (PCD).
14 Humans with PCD manifest laterality defects as well as infertility and respiratory
15 disorders. Recessive mutations in *DNAH5* (which encodes a heavy chain of the outer
16 dynein arm) or in *DNAI1* or *DNAI2* (which encode intermediate chains of the outer
17 dynein arm) cause PCD associated with L-R defects in some patients³¹⁻³³. Mutations in
18 *CCDC39* and *CCDC40* (which encode coiled-coil proteins required for assembly of the
19 inner dynein arm) also result in PCD in humans^{34, 35}.

20
21 The molecular phenotype (that is, the pattern of *Nodal* expression in the LPM)
22 associated with the loss of motility of node cilia can be seen from the well-studied *iv/iv*
23 mouse mutant, which harbors a mutation in the *Dnah11(Lrd)* gene for an axonemal
24 dynein protein that renders node cilia immotile³⁶. The loss of ciliary motility in this
25 mutant results in a loss of leftward fluid flow in the node¹⁹ and in consequent
26 randomization of *Nodal* expression in the LPM, with the expression pattern being either
27 left-sided, right-sided, or bilateral^{37, 38}. Although it remains unknown how nodal flow
28 is sensed (as discussed in the next section), the L-R decision becomes randomized in the
29 absence of the flow.

30 ***Immotile cilia at the edge of the node sense the fluid flow***

31 There are two types of ciliated cells at the node^{39, 40} (Figure 3). Cells located at the
32 central region of the node (pit cells) possess motile cilia, which generate the fluid flow.
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2 On the other hand, most cells located at the edge of the node (crown cells) possess
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4 immotile cilia ⁴¹.
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6 Recent evidence indicates that these immotile cilia of the crown cells act as
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8 sensors of the fluid flow ⁴¹. *Kif3a*^{-/-} mouse embryos, which are deficient in the kinesin
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10 motor protein KIF3A, lack all cilia including those at the node, fail to develop nodal
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12 flow, and manifest L-R defects ⁴². They are also unable to respond to flow. However,
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14 restoration of *Kif3a* expression specifically in crown cells resulted in the formation of
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16 cilia in these cells. More importantly, the modified embryos were also able to respond to
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18 flow, suggesting that cilia of the crown cells, most of which are immotile, sense the
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20 fluid flow ⁴¹.
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22 Can motile cilia of pit cells also sense nodal flow? The flagella of
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24 *Chlamydomonas* not only move but also sense mechanical force ⁴³. In Medaka, all of
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26 the cilia in KV are motile ⁴⁴, suggesting that motile cilia may also sense fluid flow.
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28 Motile cilia of the mouse node are unlikely to sense nodal flow, however, based on
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30 studies in *Pkd2* mutant embryos, which possess motile cilia and develop nodal flow but
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32 are unable to sense the flow ⁴⁵. Restoration of the expression of *Pkd2* (a Ca²⁺ channel
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34 required for flow sensation) specifically in pit cells of *Pkd2*^{-/-} mouse embryos did not
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36 prevent the development of L-R defects ⁴¹, supporting the idea that in the mouse, motile
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38 cilia of pit cells are not involved in detecting nodal flow.
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40 ***Mechanosensing or chemosensing?***

41 There are two prevailing models explaining how the embryo senses nodal flow. The
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43 embryo may sense the mechanical force of the flow (two-cilia model or mechanosensor
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45 model), or alternatively, the flow may transport a determinant molecule toward the left
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47 side of the embryo (chemosensor model). Circumstantial evidence, including the recent
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49 observation that as few as two rotating cilia are sufficient for the breaking of L-R
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51 symmetry ⁴⁶, favors the latter model. Because the flow generated by two rotating cilia is
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53 highly local and the flow velocity would be attenuated over a distance from the rotating
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55 cilia by a factor of 2-3, it would take a long time to for a molecule or a particle to travel
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57 from one side of the node to the other side. On the other hand, even a weak, local
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59 mechanical force can be transmitted instantly from the rotating cilia to the edge of the
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node⁴⁷ because the node cavity is a semi-closed space. However, it is still not clear what exactly the cilia sense during the symmetry-breaking process.

Ca²⁺ signaling in flow sensing

The requirement for a Ca²⁺ channel composed of Pkd2⁴⁵ and Pkd111^{44, 48} in L-R patterning as well as the direct detection of L-R asymmetric Ca²⁺ signaling at the node³⁹ have suggested that Ca²⁺ signaling plays a role in the sensing of nodal flow. Given that the L-R defects of *Pkd2*^{-/-} mutant mice can be rescued by crown cell-specific expression of a *Pkd2* transgene, Pkd2-mediated Ca²⁺ signaling in crown cells appears to be sufficient for flow sensing. Indeed, several blockers of Ca²⁺ signaling have been shown to disrupt asymmetric gene expression in crown cells⁴¹. In particular, the observed effects of GdCl₃ [an inhibitor of stretch-sensitive transient receptor potential (TRP) channels], 2-ABP [an inhibitor of the inositol 1,4,5-trisphosphate (IP₃) receptor], and thapsigargin (an inhibitor of Ca²⁺-dependent ATPase activity in the endoplasmic reticulum) implicate Ca²⁺ signaling by a TRP-type channel such as Pkd2 as well as that by the IP₃ receptor in the sensing of nodal flow. Pkd2, together with Pkd111, likely functions in the ciliary compartment of crown cells, given that a mutation in *Pkd2* that disrupts the ciliary localization of the encoded protein results in L-R defects similar to those of *Pkd2*^{-/-} embryos^{41, 48}. Whereas *Pkd2* encodes a Ca²⁺ channel with a short extracellular domain located between two transmembrane domains, Pkd111 possesses a much larger extracellular domain at its amino terminus, suggesting that Pkd111 is responsible for sensing the flow signal, be it mechanical or chemical.

However, Ca²⁺ signaling with obvious L-R asymmetry has not been detected in crown cells. Examination of transgenic mice that express the Ca²⁺ indicator GCaMP2 specifically in crown cells revealed that Ca²⁺ signaling was operative in these cells, but that it was present bilaterally and was retained in *Pkd2*^{-/-} embryos⁴¹. More recently⁴⁹, oscillations of Ca²⁺ signaling were detected in the node (oscillatory Ca²⁺ signals are found in both pit cells and crown cells, but those in crown cells may be functionally more relevant). The frequency of the oscillations was higher on the left side than on the right side, and it was reduced on both sides in *Pkd2*^{-/-} embryos. Dynamic oscillatory (rather than static) Ca²⁺ signaling may thus be responsible for symmetry breaking.

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2 L-R asymmetry of Ca^{2+} signaling was first described at the node ³⁹, with
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4 similar observations being subsequently reported by others. However, it is likely that
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6 this asymmetric Ca^{2+} signaling occurs in endoderm cells near the node, rather than in
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8 crown cells. As described below, Ca^{2+} signaling in endoderm may have a different role:
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10 signal transfer from the node to LPM.

11 12 13 ***Readout of the flow-mediated signal***

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15 What happens to crown cells after they have received the flow-mediated signal?
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17 Expression of *Cerl2* appears to be the most immediate readout of the flow signal
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19 because it exhibits the earliest L-R asymmetry at the node ^{46, 50} (Figure 3). *Cerl2*
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21 encodes a Nodal antagonist, although its precise action is not clear. It is asymmetrically
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23 = expressed in crown cells (i.e., the expression on the right side is higher than that on
24
25 the left side), and its absence results in randomization of L-R decision making ⁵¹.
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27 Whereas expression of *Nodal* is bilateral in crown cells, the $R > L$ expression of *Cerl2*
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29 renders Nodal activity in crown cells higher on the left side (Figure 4). The
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31 *Cerl2*-generated asymmetry ($R < L$) of Nodal activity at the node closely correlates with
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33 the asymmetric pattern of *Nodal* expression in the LPM ⁵². Expression of *Cerl2* is
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35 initially symmetric ($R = L$) at the early headfold stage, but it becomes $R > L$ as the
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37 velocity of nodal flow increases, with expression on the left side being down-regulated
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39 ^{46, 52}. Recent genetic evidence suggests that *Cerl2* is the major target of the flow signal.
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41 *Pkd2*^{-/-}*Cerl2*^{-/-} double-mutant embryos thus manifest randomized *Nodal* expression in
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43 LPM, resembling the *Cerl2* single mutant (but unlike the *Pkd2* single mutant, which
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45 loses *Nodal* expression in LPM)⁴¹.

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47 L-R asymmetry of *Cerl2* expression is determined not at the transcriptional
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49 level but rather at the posttranscriptional level ⁵³, specifically by the decay of *Cerl2*
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51 mRNA in a manner dependent on its 3' untranslated region. Preferential decay of *Cerl2*
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53 mRNA on the left is initiated by the leftward fluid flow and further enhanced by the
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55 operation of *Wnt*-*Cerl2* interlinked feedback loops, in which *Wnt3* up-regulates *Wnt3*
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57 expression and promotes *Cerl2* mRNA decay whereas *Cerl2* promotes *Wnt3*
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59 degradation. Mathematical modeling and experimental data ⁵³ suggest that these
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61 feedback loops behave as a bistable switch that is able to amplify in a noise-resistant
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2 manner a small L-R bias conferred by the leftward fluid flow, which is weak at the stage
3 when asymmetry of *Cerl2* mRNA is established at the node⁴⁶.

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5 Although *Cerl2* mRNA exhibits R>L asymmetry at the node, Cerl2 protein
6 displays a dynamic behavior⁵⁴. Cerl2 protein initially localizes to crown cells on the
7 right side, but later it will be translocated to the left side. This right-to-left translocation
8 is dependent on the flow, suggesting that Cerl2 protein may be transported from the
9 right side to the left side of the node.
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16 17 **Signal transfer from the node to the LPM**

18 19 ***Identity of the signal transferred from the node to the LPM: Is it Nodal protein?***

20 The asymmetric signal (or signals) generated in or near the node, whether it be a
21 mechanical stress or a molecular determinant, must be transferred to the LPM, where it
22 induces the asymmetric expression of *Nodal*. Several lines of evidence suggest that an
23 active form of Nodal protein, perhaps the Nodal-GDF1 heterodimer, is the signal that is
24 transferred from the node to the lateral plate (Figure 4).
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30 Among several signaling molecules expressed at the node, Nodal-GDF1 is the
31 best candidate for the determinant that is transferred from the node to the left LPM.
32 First, *Nodal* is absolutely essential for asymmetric gene expression in the lateral plate.
33 Specific ablation of *Nodal* expression in the perinodal crown cells thus prevents *Nodal*
34 expression in the left LPM^{55, 56}. Second, asymmetric *Nodal* expression in the LPM is
35 conferred by two enhancers, both of which are Nodal responsive and dependent on the
36 transcription factor FoxH1⁵⁷⁻⁶¹. Third, introduction of a Nodal expression vector into
37 the right LPM can induce ectopic expression of endogenous *Nodal* on the right side⁶².
38 Fourth, *Gdf1* is coexpressed with *Nodal* bilaterally in the perinodal crown cells, and
39 *Gdf1* mutant mice do not manifest asymmetric *Nodal* expression in LPM⁶³. GDF1
40 alone does not activate signaling under physiological conditions, but it interacts with
41 Nodal and thereby increases Nodal activity⁶⁴.
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53 54 ***The route by which the Nodal signal is transferred***

55 If Nodal protein is transferred from the node to the LPM, how is it transferred? *Nodal* is
56 expressed in only two regions, the node (crown cells) and left LPM, at this stage of
57 mouse embryogenesis, with its expression in the node turning on slightly earlier than
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2 that in left LPM. It is easy to envisage that Nodal secreted from crown cells into the
3 node cavity would be transported by the leftward flow to the left side of the embryo (an
4 extra-embryonic route; Fig. 4). Nodal protein that reaches the left side could then be
5 incorporated into the embryo via the endoderm and be transported to the left LPM,
6 where it would induce endogenous *Nodal* expression. This is unlikely to be the case,
7 however, because cultured mouse embryos in the presence of recombinant Nodal does
8 not induce *Nodal* expression in the right LPM, whereas injection of Nodal into the right
9 side of the embryo (near the paraxial mesoderm and LPM) does induce endogenous
10 *Nodal* expression on the right side^{52, 65}. Instead, several lines of evidence suggest that
11 Nodal-GDF1 secreted into the embryo is transported from the node to the LPM via an
12 intra-embryonic route (Fig. 4). First, Cryptic, an essential component of the Nodal
13 signaling pathway, is required only in the LPM for correct L-R patterning: It is not
14 required in the region between the node and the LPM⁶⁵. Second, Nodal interacts with
15 sulfated glycosaminoglycans, which are specifically localized to the basement
16 membrane between the node and the lateral plate. Moreover, inhibition of sulfated
17 glycosaminoglycan synthesis in *Xenopus*⁶⁶ and mouse⁶⁵ embryos prevented *Nodal*
18 expression in the LPM.

19 These results collectively suggest that Nodal protein produced at the node and
20 secreted inside the embryo is transported from the node to the lateral plate via the
21 intra-embryonic route (Fig. 4), where it activates *Nodal* expression. Secreted
22 Nodal-GDF1 protein has not been directly detected in the mouse embryo, but secreted
23 Nodal protein undergoing transport has been detected in the frog embryo⁶⁶.

24 ***The role of the endoderm in node-LPM signal transfer***

25 Two recent studies of *Sox17* mutant mice^{67, 68} suggest that the endoderm plays a role in
26 signal transfer from the node to the LPM. These studies found that embryos deficient in
27 *Sox17*, a transcription factor required for definitive endoderm formation, manifest L-R
28 patterning defects. Whereas asymmetric gene expression including that of *Cer12* was
29 found to be maintained in the node (specifically in the perinodal crown cells), *Nodal*
30 expression in the LPM was lost. The LPM retained the ability to respond to Nodal
31 signaling, however. *Sox17* is expressed in endoderm near the node, but it is not
32 expressed in the node itself, including the perinodal crown cells. These observations

strongly suggest that signal transfer from the node to the LPM depends on Sox17 in the endoderm.

The endoderm of the *Sox17* mutant mice is defective in multiple respects^{67, 68}. First, the gap-junction component Connexin43 is absent or mislocalized, and gap-junctional transport (examined by injection of the diffusible dye Lucifer yellow) is impaired in the mutant endoderm. Second, epithelial polarity and cellular adhesion are disorganized in the mutant endoderm. Third, the distribution of extracellular matrix (ECM) components such as chondroitin sulfate and laminin is partially disorganized in the mutant embryos.

It is not clear how these defects in the endoderm are linked to the L-R defects of *Sox17* mutant mice, but several mechanisms are possible. For instance, gap junctional communication in the endoderm is essential for transfer of signal to the LPM in *Xenopus*⁶⁹. Gap junction-mediated propagation of signaling (e.g., Ca^{2+} signaling) may influence the underlying ECM and facilitate ECM-mediated transport of Nodal protein from the node to the LPM (Figure 4), as proposed previously⁷⁰. This scenario is consistent with most, if not all, of the observations described above.

Concluding remarks

L-R symmetry breaking takes place in a transient structure called the “node” of the mouse embryos, or in an equivalent structure of other vertebrates. In most vertebrates including fish, frog and mouse, cilia play an essential role in symmetry breaking. They generate uni-directional fluid flow by rotating, as well as sense the flow via ciliary-localized Ca^{2+} channels. Sensing the flow at the node will eventually lead to L-R asymmetric gene expression and situs-specific formation of visceral organs. By contrast, other animals seem to make use of a completely different mechanism for symmetry breaking. In *Drosophila* and snail, for example, L-R symmetry breaking does not appear to depend on cilia⁷¹. It will thus be of interest to determine the precise mechanisms of symmetry breaking in diverse organisms. See box 2 for other open questions in this field.

As cilia and fluid flow are involved in a wide range of physiological processes, studying the role of cilia and flow in the mouse node will not only deepen our understanding of symmetry-breaking mechanisms but also provide valuable insights

1
2 into general function of cilia in human physiology. Further advances will require
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4 development of various new approaches including genetic, cellular, biophysical, and
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6 mathematical methods.
7

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Box1. Glossary

Crown cells: Cells located at the periphery of the ventral node. Most possess an immotile cilium and express specific genes required for normal L-R patterning including *Nodal*, *Gdf1*, and *Cerl2*.

Endoderm: A group of cells that line digestive and respiratory tubes within the embryo or body. At E8 during mouse development, when breaking of L-R symmetry takes place, the surface layer of cells near the node is also endoderm.

Gastrocoel roof plate: a ciliated epithelium located at the posterior end of the notochord in amphibian embryos, where flow develops shortly before the onset of L-R asymmetric gene expression.

Headfold stage: a stage about 7.5-8.0 days after fertilization in the mouse embryo, when a ventral fold is formed by rapid growth of the head of the embryo.

Kupffer's vesicle: a transient spherical structure that arises in the tailbud of teleost embryos. It is filled with fluid and its inside is covered with cells with motile cilia.

Lateral plate mesoderm (LPM): A group of mesoderm cells located in the lateral region of the embryo that will contribute to the mesenchyme of various organs. Although LPM is present on both sides of the embryo, that on the left side expresses *Nodal* and will be patterned differently from that on the right side.

Nodal: A transforming growth factor β (TGF β)–related factor that regulates various aspects of early embryogenesis including mesoderm formation and L-R patterning.

Node: a ventral indentation with a ciliated epithelium located posterior to the notochord and anterior to the primitive streak in the mouse embryo. Although “the posterior notochord” may be an embryologically more precise term⁷², “node” is used in this review.

Pit cells: Cells located at the central region of the ventral node. Most possess a motile cilium that generates nodal flow.

Planar cell polarity (PCP): A conserved mechanism responsible for the polarization of cells along specific axes in a tissue. Its core components include Dvl (Dishevelled), Fz (Frizzled), Vangl, and Prickle, some of which are localized to one side of a cell while others are localized to the opposite side.

Primary ciliary dyskinesia (PCD): a group of genetic disorders caused by a defect of

1
2 motile cilia. Its main symptoms include chronic respiratory infections, infertility and
3 laterality defects. A number of PCD-causing genes have been identified in human^{73, 74}.
4
5 Many of these genes encode an axonemal dynein component, while others are required
6 for assembly of dynein complex, transport of axonemal dyneins to the cilium, or
7 regulation of dynein activity.
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11 **Situs-specific morphogenesis:** formation of visceral organs according to left-right
12 positional information. Almost all visceral organs are left-right asymmetric in their
13 position or shape.
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17 **Box2. Outstanding questions**

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19 Substantial progress has been achieved in our understanding of L-R symmetry breaking,
20 but many important questions remain unanswered. For instance,
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- 22 (1) How is A-P information translated into the posterior tilt of node cilia?
- 23 (2) How is the rotational direction of nodes cilia determined?
- 24 (3) How is the fluid flow sensed by immotile cilia, and what is the precise role of Ca^{2+}
25 signaling?
- 26 (4) How is *Cerl2* mRNA degraded in response to the flow?
- 27 (5) To what extent is the mechanism for breaking of L-R symmetry conserved among
28 species?
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Figure Legends

Figure 1: Four steps for generating left-right asymmetry

Four steps in the generation of L-R asymmetry are shown. The black arrow on the left represents a time course during development, from earlier embryonic stages (E7.5-E8.5) to later ones (E10-E11.5). For the first step (symmetry breaking), an embryo exhibiting asymmetric gene expression at the node is shown (top photo). For patterning of the lateral plate mesoderm (LPM) (third step), an embryo exhibiting left-sided expression of *Nodal* is shown (middle photo). For the last step, three different mechanisms for the generation of morphological asymmetries are shown (bottom illustration): differential branching for the lung, directional looping for the stomach, and one-sided regression for the blood vessels.

Figure 2: Polarization of node cells

Polarized localization within node cells is shown for the basal body of the cilium and for PCP core proteins. A, anterior; D, dorsal; P, posterior; V, ventral. Note that the basal body is positioned at the posterior side of node cells, which results in tilting of the motile cilium toward the posterior side. *Prickle2* and *Vangl1* proteins are localized to the anterior side of the cells, whereas the *Dvl* protein on the posterior side. Putative anterior-posterior information responsible for polarization of these proteins is indicated by the yellow gradient on the top.

Figure 3: Model for the sensing of nodal flow by immotile cilia

Two types of ciliated cell are present in the node: Those located centrally (green) have motile cilia that generate nodal flow, whereas those located peripherally (pink) possess immotile cilia that sense the flow. Sensing of the flow requires ciliary localization of a *Pkd2-Pkd111* complex with Ca^{2+} channel activity. The flow-mediated signal results in degradation of *Cerl2* mRNA in crown cells on the left side. In this model, an immotile cilium located on the left side of the node is bent in response to the flow. However, such bending has not been verified by in vivo observations.

Figure 4: Generation of molecular asymmetries at the node

(A) Whereas *Nodal* mRNA and *Gdf1* mRNA are present at similar levels on both sides

1
2 of the mouse embryo, *Cerl2* mRNA shows a $R > L$ distribution. **(B)** Nodal (yellow) and
3
4 GDF1 (purple) proteins form a heterodimer that constitutes an active form of Nodal.
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6 Given that *Cerl2* (blue) is an inhibitor of Nodal, the effective level of active Nodal,
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8 which is reflected by the level of phosphorylated Smad2/3 (pSmad2), shows a $R \ll L$
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10 pattern [the right most panel of **(A)**]. **(C)** The Nodal-GDF1 heterodimer produced by
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12 crown cells may be transported to the left LPM via an extra-embryonic (black dotted
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14 arrow) or intra-embryonic route (red dotted arrow). According to the former route, the
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16 Nodal-GDF1 heterodimer would be secreted into the node cavity, transported to the left
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18 side by the leftward flow, absorbed by the endoderm and transported to the LPM on the
19
20 left side. Alternatively, the Nodal-GDF1 heterodimer may be secreted within the
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22 embryo, and transported along ECM to left LPM (intra-embryonic route). In either route,
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24 Nodal-GDF1 that has reached the LPM will activate expression of *Nodal*, which is
25
26 responsive to Nodal signaling. Note that the level of Nodal-Gdf1 heterodimer is much
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28 lower on the right side than that on the left side. Nodal signaling in crown cells is not
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30 essential for L-R asymmetry, as inhibition of Nodal signaling in crown cells does not
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32 interfere normal L-R patterning⁵².
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Fig.1

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