Left–right patterning from the inside out: widespread evidence for intracellular control

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Summary

The field of left–right (LR) patterning—the study of molecular mechanisms that yield directed morphological asymmetries in otherwise symmetrical organisms—is in disarray. On one hand is the undeniably elegant hypothesis that rotary beating of inclined cilia is the primary symmetry-breaking step: they create an asymmetric extracellular flow across the embryonic midline. On the other hand lurk many early symmetry-breaking steps that, even in some vertebrates, precede the onset of ciliary flow. We highlight an intracellular model of LR patterning where gene expression is initiated by physiological asymmetries that arise from subcellular asymmetries (e.g. motor-protein function along oriented cytoskeletal tracks). A survey of symmetry breaking in eukaryotes ranging from protists to vertebrates suggests that intracellular cytoskeletal elements are ancient and primary LR cues. Evolutionarily, quirky effectors like ciliary motion were likely added later in vertebrates. In some species (like mice), developmentally earlier cues may have been abandoned entirely. Late-developing asymmetries pose a challenge to the intracellular model, but early mid-plane determination in many groups increases its plausibility. Multiple experimental tests are possible.

***BioEssays* 29:271–287, 2007.**

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Funding agency: This work was supported by NIH grant GM-06227, March of Dimes grant #6-FY04-65, American Heart Association Established Investigator Grant #0740088N, and NHTSA grant DTNH22-06-G-00001 to M.L. This manuscript was written in part in a Forsyth Institute facility renovated with support from Research Facilities Improvement Grant Number CO6RR11244 from the National Center for Research Resources, National Institutes of Health. ARP was funded by NSERC Canada Discovery Grant A7245.

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DOI 10.1002/bies.20545

Published online in Wiley InterScience (www.interscience.wiley.com).

Introduction

The comfortably familiar bilateral symmetry of so many organisms belies a surprising observation: very few are fully symmetrical. Visceral organs like the vertebrate brain, heart and gut[(1)] or the nematode nervous system,[(2)] appendages like the claws of many crustaceans,[(3)] and even the genitalia of seemingly bilaterally symmetrical flowers,[(4)] may be conspicuously asymmetrical. To become asymmetrical, symmetry must somehow be broken during development. How this symmetry-breaking occurs remains one of the most fascinating and provocative questions in developmental biology because it touches on so many key issues: pattern formation, signaling mechanisms, the evolution of developmental pathways, and, most fundamentally, how 'right' and 'left' can possibly be distinguished at the molecular level in the first place.

A deep-seated theoretical problem fuels the sustained interest in mechanisms of left–right (LR) asymmetry determination: 'right' and 'left' can only be identified unambiguously by comparison to some pre-existing asymmetrical reference.[(5)] Although right and left clearly lie on opposite sides of a midplane, the side that we call right is arbitrary. In addition, although the anteroposterior and dorsoventral axes may be defined independent of one another, right and left—which are best considered separate axes in most systems[(6)]—are defined only in relation to these other axes.[(7)] As Gardner charmingly recounts,[(5)] should humans make contact with an alien race, we could not communicate unambiguously the side of the body on which our heart lies without reference to some other identifiable handed asymmetry shared between our two worlds. In Gardner's example the asymmetric reference was spin direction of cobalt 60 nuclei aligned in a magnetic field (the first published demonstration, in 1957 by Madame Chien-Shiung Wu of Columbia University, that parity of the universe was not conserved), where excess emission of electrons from the south end of the spinning nucleus allowed unambiguous identification of 'south' and therefore the direction of spin.

Cells in developing embryos face the same problem: to what asymmetrical reference do they resort to decide whether they are on the right or left side? Brown and Wolpert,[(7)] like Harrison nearly 70 years earlier,[(8)] suggested that an asymmetry at the molecular level could provide a useful

reference. Brown and Wolpert proposed a chiral ''F-molecule'' (see Box 1 and Fig. 2c below) that, when aligned with the antero-posterior and dorsoventral axes, transports intracellular constituents to create an intracellular gradient. By virtue of the ''F-molecule's'' chirality and its alignment with the other body axes, the intracellular gradient created by the ''F-molecule'' will parallel the medio-lateral gradient on one side of the body but run opposite to it on the other. The concordance or discordance of these two gradients within a cell—the ''F-molecule'' gradient and the mediolateral gradient—would allow a cell to determine its position on the right or left side.

Box 1. Glossary

Anatomical polarity: a structural difference between one end of a cell, tissue or organism, and the opposite end. Apical–basal polarity is a well-known example of polarization in epithelial cells. Anterior–posterior polarity with respect to genes such as *bicoid* is an example of embryo-wide polarity in *Drosophila*.

Bioelectrical polarity: a voltage gradient aligned with an anatomical axis exerted across a cell or tissue, or a difference in ion flow at one end of a structure versus its other end. Many epithelia pump specific ions from the apical end, resulting in an electrical polarization across the layer.

GJC: gap junctional communication occurs when cells are connected via membrane structures known as gap junctions. These highly regulated aqueous pores allow small signaling molecules to pass directly from the cytosol of one cell into that of its neighbor. Such signals have been implicated in many aspects of normal development and disease.

5HT: serotonin, a small-molecule neurotransmitter also implicated in left–right patterning.

H^+ and K^+ transporters: proteins or protein complexes that form passive channels and active pumps, and which, in turn, produce a charge separation across cell membranes.

''F-molecule'': a two-dimensional version of a three-dimensional structure that occurs in living forms in only one enantiomer, originally proposed by Brown and Wolpert.[7] Aligning such a molecule with respect to the anteroposterior and dorsoventral axes will allow the cell containing it to unambiguously determine which direction is left or right (once the major axis of the ''F'' is fixed in a plane, the prongs always point in the same direction).

Vertebrate LR asymmetry: the most thoroughly studied system

Although the ''F-molecule'' remains elusive, some remarkable progress has been made over the last 10 years unveiling the molecular mechanisms that control the leftward asymmetry of the vertebrate heart and viscera.[6,9–12] Three surprising conclusions emerge from this body of work. First, even for something as simple as the binary-switch developmental decision—'go right' versus 'go left'[6]—multiple signaling and receptor molecules are involved. Common to all vertebrates studied so far is a core and apparently conserved signaling cascade (the *Nodal* cascade) involving three TGF-β family member genes, *Nodal*, *Lefty1* and *Lefty2*, and the homeobox transcription factor gene *Pitx2*. An excess of nodal protein on the left side of the node/organizer, which lies on the midline in the primitive streak region of the vertebrate embryo, activates both *Lefty* genes. But the *Lefty* genes play different roles. *Lefty1* erects a midline barrier to inhibit bleeding of nodal signal to the right side and *Lefty2* inhibits *Nodal* overexpression on the left side (nodal activates its own expression). The nodal protein also activates *Pitx2* gene expression on the left side, which by some as yet unknown mechanism triggers a heart-specific signaling cascade involving *Nkx, GATA*, and *Hand*.

Second, the discovery that beating cilia in or near the node may play a critical role in initiating left-sided *Nodal* expression in both zebrafish and mice, generated considerable excitement[13] (see next section for details). Rotary beating of inclined cilia (Fig. 1B), which generates a leftward flow across the embryonic midline (at least in mice),[12] seemed to provide the much sought-after symmetry-breaking step whereby an asymmetry at the molecular level—the chiral arrangement of dynein motor proteins between microtubules around the cilium that make a cilium beat in a particular direction (Fig. 1A)—promoted the initial left-sided *Nodal* expression.

Third, despite strong evidence for conservation of (a) a left-sided heart in vertebrates, (b) node monocilia in vertebrate embryos, and (c) the *Nodal* signaling cascade (*Nodal, Lefty1, Lefty2, Pitx2*), expression data revealed a startling variety of other genes that were or were not expressed asymmetrically upstream of the *Nodal* cascade in different vertebrates.[6] In the extremes, at least 15 genes are expressed asymmetrically before the *Nodal* cascade is initiated in chick embryos, whereas 13 of those same genes are clearly expressed *symmetrically* upstream of the *Nodal* cascade in mouse embryos.[6] Therefore, contrary to all prior expectations, although some components of the signaling pathway are conserved (e.g. the *Nodal* cascade and possibly a dependence on cytoskeletal asymmetries), the entire pathway guiding development of a conserved anatomical feature—the left-sided heart in vertebrates—has more divergent than conserved elements. Interestingly, asymmetric expression of the early LR pathway genes upstream of *Nodal* has not been reported in any animals other than birds, suggesting perhaps

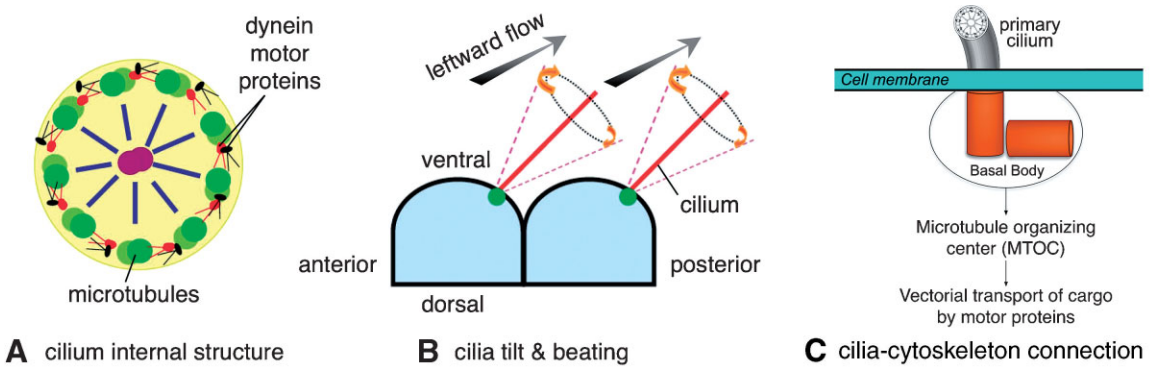


Figure 1. Chiral cilium structure, leftward nodal flow, and cilia-cytoskeletal connections. **A:** The microtubules in a ring around the margin of an individual cilium are connected to one another by oriented dynein motor proteins that cause a cilium to beat by sliding the microtubules relative to one another. The oriented motor proteins give cilia a consistent chirality that causes them to beat clockwise when viewed from the tip (modified from Ref. 17). **B:** In mice, node cilia do not extend perpendicular to the epithelial surface but, rather, tilt posteriorly. Because of this posterior tilt, the leftward-moving part of the stroke extends further from the cell surface than the rightward return stroke, which generates a leftward flow of extraembryonic fluid across the surface (modified from Ref. 113). **C:** The basal body–centrosome complex is upstream of both the construction of primary cilia and cytoplasmic organization, which in turn determines transport of ion channel/pump proteins and other cargo by motor proteins.[60,61]

that the planar form and ready accessibility of the early chick embryo may permit subtle asymmetries to be visualized with greater confidence than in more three-dimensional embryos. Thus, lack of reported asymmetric gene expression in pre-node mouse embryos may not be definitive.

This unexpected variation among species in genes that direct the left-sided development of the vertebrate heart has prompted a wholesale re-evaluation of how development evolves[6] and also a re-assessment of the primacy of node cilia as the determinant of directed LR asymmetry in vertebrates.[13–15] When combined with emerging observations about how conspicuous morphological asymmetries develop in other eukaryotes, the central question of how symmetry is broken in the first place clearly needs to be revisited.

Lots of evidence now suggests that, even in vertebrates, the real excitement lies well before node cilia function, and that these early events may be shared much more widely than generally appreciated. Here, we examine some key evolutionary aspects of LR asymmetry, and ask whether similar molecular mechanisms guide symmetry-breaking across all eukaryotes and, if so, whether these involve cilia or not.

The siren song of cilia: universal effector or seductive red herring?

Some biologists might be tempted to think that the essential factors controlling LR asymmetry have largely been worked out (at least for vertebrate visceral asymmetry), and all that's left to do is some fine-tuning. But even in vertebrates many puzzling questions remain, particularly regarding the primacy of node cilia as the symmetry-breaking step in directed LR asymmetry.

Ciliary rotation was thought to be the critical symmetry-breaking step determining the direction of vertebrate visceral asymmetry.[11] This proposal is conceptually satisfying because cilia are theoretically an ideal chiral component whose orientation relative to the other two body axes can unambiguously define a LR identity (Fig. 1). Studies of mouse mutants suggest two basic models by which leftward ciliary flow triggers the *Nodal* cascade on the left side: (a) leftward flow wafts an extracellular morphogen (possibly nodal itself) to the left, or (b) leftward flow somehow activates Ca^{2+} signaling in sensory cilia on the left.[16,17] Because cilia and the ciliary motor protein gene *left–right dynein* occur in all four vertebrate model organisms (zebrafish, *Xenopus*, chicken and mouse), cilia motion during late gastrulation emerged as a possible universal mechanism determining the direction of LR asymmetry.[18]

The seemingly compelling data for mice, and the appealing logical parsimony of the cilia model, have led many biologists outside the field to believe—and some cell biology textbooks and reviews to state explicitly[19,20]—that cilia produce the initial symmetry-breaking event of LR asymmetry and hence the problem is solved. This premature conclusion unfortunately deters additional research where many important questions remain. Although cilia appear to be a key effector of mouse[12] and fish[1] visceral asymmetry, they may not be in other organisms,[14,15] and their role in other conspicuous vertebrate asymmetries remains unclear (see below). Even in the mouse system, questions about cilia-driven nodal flow remain. For example, what happens under true "no flow"

condition, such as in a viscous extracellular medium? In vitro culture of rodent embryos, by itself, randomizes visceral *situs*[21] which complicates interpretations of laboratory results. But even if cilia do play an essential role directing heart asymmetry in mice and fish, an important question remains: do they actually generate LR information de novo, or mainly transmit as yet unknown upstream LR signals?

Doubts about the primary role of cilia in symmetry breaking arise from an underappreciated observation: most ciliary proteins have additional subcellular roles, like motor protein transport, anatomical cell polarity determination, and transcription control.[22] Cilia motion itself also has physical (traction) effects on cytoplasmic components via the ciliary rootlet.[23] Because rodent embryos with mutated ciliary motors (Fig. 1C) would also have impaired intracellular transport (which may be important for asymmetry[24]), ciliary functions of the LR-relevant motors cannot readily be separated from other cytoplasmic transport roles. True, ciliary protein deletion mutants tend to give laterality defects, but they also implicate other roles. For example, the OFD1 knockout has altered HoxA and HoxD expression in the limb.[25] Interestingly, OFD1 is a centrosomal/basal body protein, and another possible candidate for the intracellular ''F-molecule'' that allows right to be distinguished from left. Similarly, although cilia defects are associated with laterality disruptions in humans,[26] patients with classical primary ciliary dyskinesia do not exhibit reversed brain asymmetry.[27] So, some aspects of human laterality are unaffected by mutations affecting ciliary function. Finally, the presence of consistent asymmetry in organisms that do not have cilia at all or establish laterality long before cilia appear (Table 1) is difficult to reconcile with a model in which cilia is a conserved initiator of asymmetry.

Elsewhere, the main concerns regarding popular views of cilia in asymmetry are discussed in detail.[9,13,28] The molecular components of ciliary structure and function are clearly present, and in several cases, asymmetrically localized, in very early chick and frog embryos.[24] Even in those species in which cilia may impinge on asymmetry, they may merely function in the middle of the pathway, mediating LR signals between earlier upstream events and later downstream gene expression cascades (e.g. the *Nodal* cascade).

The intracellular model: initiation of LR asymmetry at the subcellular level

A qualitatively different intracellular model[14,15] that does not depend on cilia (Fig. 2) may better account for directional control of LR asymmetry in a wider range of organisms. Growing evidence supports many features of this model, and this evidence now comes from organisms as diverse as plants, protists, invertebrates and vertebrates (see *Common pathways and evolutionary trends* for details). Significantly, in the cilia model, asymmetry arises as an extracellular event, at a fairly late developmental stage in a multicellular embryo, and the cilium functions as the ''F-molecule''. In contrast, the intracellular model proposes that asymmetry arises at very early stages inside cells, and oriented cytoskeletal elements assume the ''F-molecule'' function. In addition, the intracellular model predicts a connection between subcellular polarity and ion flux.

In the intracellular model (Fig. 2), an early component of the cell-polarity system (likely involving the cytoskeleton) orients the transport of key molecules within cells that ultimately creates an LR difference in the embryo. Randomly distributed maternal mRNAs and ion-transporter proteins in the egg (e.g. H^+ and K^+ transporters) become asymmetrically distributed by the activity of intracellular motor proteins (Fig. 2A–C). Ion transporter proteins are an attractive feature of this model because they have the potential to allow subcellular asymmetries to be imposed on a large field of cells. The motor proteins that move these ion transporters move in one direction along asymmetric cytoskeletal elements that derive their chirality from a basal body or other oriented ''F-molecule'' (Fig. 2C). As ion channels and pumps become asymmetrically distributed, they create a LR gradient in membrane voltage (Figs. 2D,E) that, in turn, moves small molecule determinants such as serotonin through a system of gap junctions by an electrophoretic mechanism (Figs. 2F,G). The eventual buildup of these small molecule determinants on one side of the embryo eventually induces unilateral gene expression of a key signaling molecule such as nodal (Figs. 2H, I).

This intracellular model shows how physiological mechanisms integrate to produce large-scale LR gradients from initial subcellular polarities. Although the orienting cytoskeletal element is not yet known, this model provides a comprehensive, quantitative[29] synthesis of all the molecular and biophysical steps leading from LR orientation within single cells to asymmetric gene expression in the early embryo, and it does not depend on ciliary motion. This scheme also parallels a model of dorsoventral patterning, where an embryo-wide pattern arises from early intracellular movement of Wnt-containing particles by kinesin motor proteins along oriented microtubule tracks.[30] A model involving the subcellular targeting of ion transporters by molecular motors is further supported by mammalian data, because the LR-implicated kinesin motor protein Kif3A is known to interact directly with the ion channel Polycystin-2, which is also required for normal asymmetry.[31] Finally, although the model presumes that the cytoskeleton provides true (reflective) left–right directionality—a presumption supported by evidence of LR asymmetries in tubulin[24]—the initial cue might also be an east–west (rotational) chirality of intracellular structures,[32] which determines the asymmetry of cilia forms.[33]

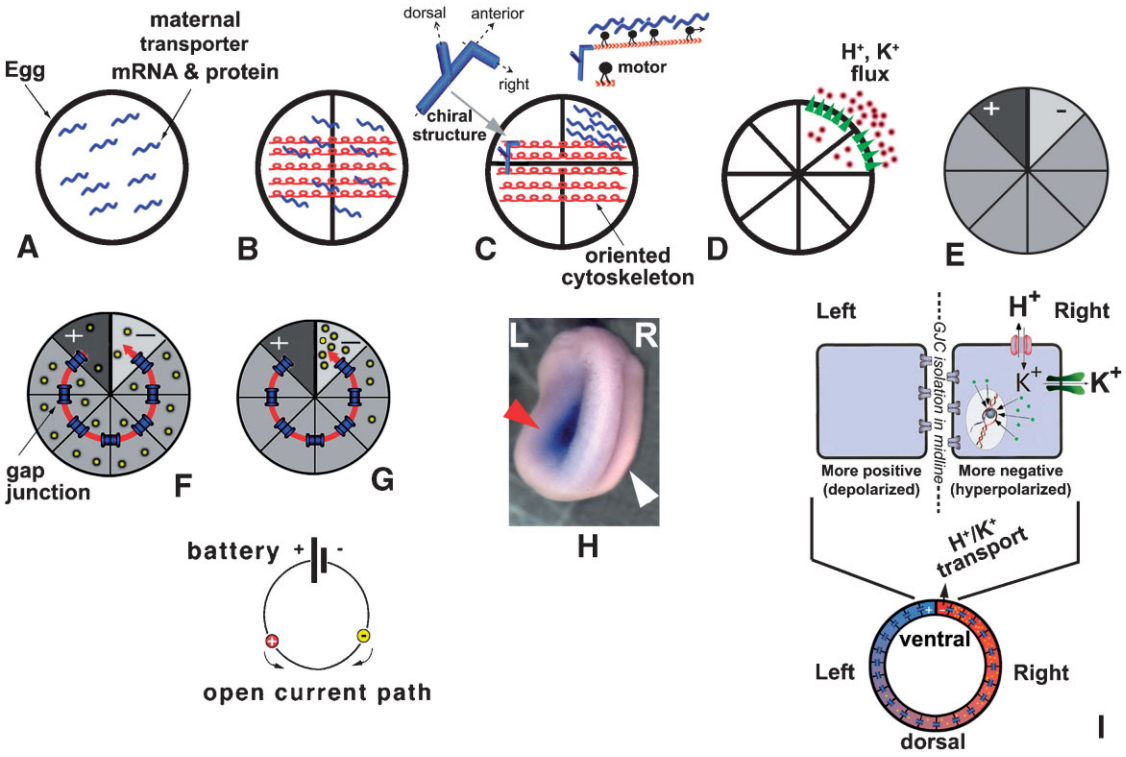


Figure 2. The intracellular model of early LR asymmetry determination. A temporal sequence of events that ultimately yields a large-scale LR gradient in early embryos may actually start in the egg (developmental time moves from **A** to **G**). **A:** Maternal mRNAs and proteins encoding H^+ and K^+ transporters (blue wavy lines) are randomly distributed within the egg. **B:** Cytoskeletal tracts (red corkscrew lines) that derive their chirality from a basal body or other oriented "F-molecule"[7] (chiral structure inset) become oriented relative to early cleavage planes. These cleavage planes may define the embryonic midplane. **C**: Maternal mRNAs and ion-channel proteins become asymmetrically distributed due to the action of motor proteins that ride the oriented cytoskeleton in a directional manner. **D:** The resulting asymmetrically distributed ion channels and pumps insert into the cell membrane and expel cations (red dots) selectively from one side of the embryo. **E:** The deficiency of cations on one side yields a consistent asymmetry in membrane voltage between L- and R-side cells. **F:** When the gap junction system becomes functional, it provides an open circuit around the zone of isolation, and the battery formed by the differentially charged ventral cells exerts a consistently biased voltage gradient across the embryo. **G:** The voltage gradient propels small charged molecules (e.g. serotonin; yellow dots) to become net-asymmetrically localized by an electrophoretic mechanism. **H:** Excess serotonin (and perhaps other long-distance signaling molecules) initiate asymmetric gene expression, ultimately feeding into key players like the *Nodal* cascade (red arrow indicates left-sided expression of *XNR-1* in a frog embryo, white arrow indicates lack of expression on the right side). **I:** This close-up schematic view of the battery cells shows how physiological mechanisms produce large-scale LR gradients from initial subcellular mechanisms that ultimately initiate asymmetric gene expression. Figure 2 is modified from Levin M. 2006 Birth Defects Res C Embryo Today 78:191–223.

Evidence for the intracellular model: subcellular polarity

The earliest evidence for the intracellular model (Fig. 2) came from studies of *Xenopus* (reviewed in Ref. 14), where asymmetry was shown to be imposed on cell fields via the steps outlined in Figs. 2A–G. Although details of this novel pathway have only been worked out in *Xenopus* (and less fully in chick,[34,35] zebrafish,[36,37] and sea urchin[38]), two components appear to be evolutionarily ancient. Intriguingly, both relate to a common theme of subcellular polarity.

First, intracellular tubulin structures control chirality in plants[39] and ciliates[40] likely via a role similar to that in vertebrates.[24,41] They may therefore play the role of the hypothetical "F-molecule". In *Arabidopsis*, right- or left-handed helical arrays of microtubules within root epidermal cells drive clockwise or counterclockwise helical growth and twisting in elongating organs.[39] As in vertebrates,[24] pharmacological or genetic modulation of tubulin can reverse the direction of asymmetry.[39] Ciliated protists have intrinsically asymmetrical ciliary structures arranged asymmetrically over the cell surface. These structures take two enantiomorphic configurations. One is universal in nature, but mirror-image and reverse forms have been generated.[40] In all these, the internal asymmetry of individual ciliary structures remains

normal even when the chiral orientation of these structures is reversed. This arrangement determines overall chirality of the organism and appears to be driven by the basal body,[42] an intracellular microtubule-organizing center. Importantly, ciliate asymmetry derives from east–west chirality of the cellular cortex,[33] as recently discovered in *Xenopus*.[32]

Second, PAR proteins that control cellular polarity also play a role in embryo axial polarity. So the PAR machinery may play an important role in the primary events that allow LR orientation of the cytoskeletal-organizing centers to be linked with the other two orthogonal axes. For example, a 14-3-3 protein (PAR-5) is required for cellular asymmetry in early *C. elegans*[43] and *Drosophila*[44] embryos, and also cell polarity in mammalian skin.[45] PAR-5 in *C. elegans* is thought to bind to and block recruitment of one or more PAR proteins (notably PAR-2 and PAR-3) to the cell cortex,[43] thus controlling the subcellular localization of other factors that ultimately dictate polarity of the whole embryo. In *C. elegans*, LR asymmetry is known to be determined during the very first cleavage stages.[46] The PAR homologue 14-3-3E also localizes LR

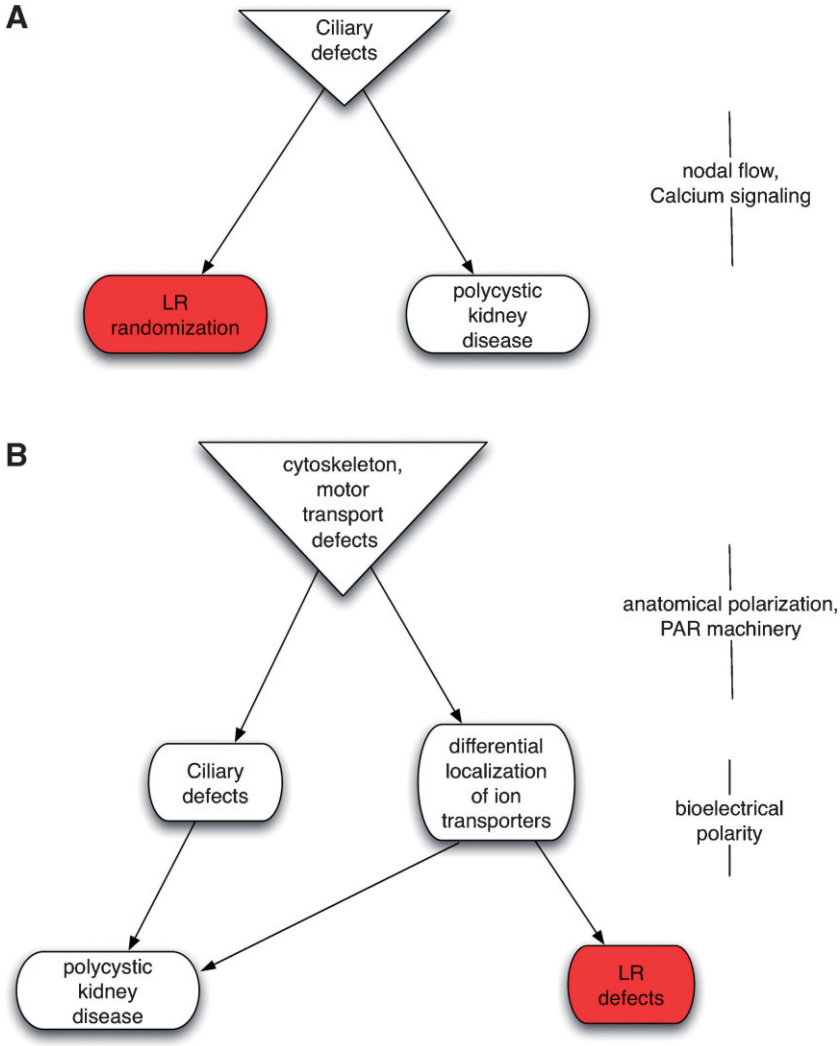


Figure 3. Two alternate origins of defective LR asymmetry. **A:** The popular cilia model, where cilia defects cause both LR malformations (by eliminating cilia-driven nodal flow, which randomizes the side on which the *Nodal* cascade is initiated) and cilia-related diseases such as polycystic kidney disease. **B:** An alternative model where cytoskeletal, cell polarity and motor protein activities are primary. Disruption of these activities have two independent effects: cilia defects (and therefore diseases such as polycystic kidney disease; left pathway) and altered ion flux that in turn causes defective LR patterning (right pathway). To distinguish between these two models, ciliary function would have to be disrupted, in an experimental setting that allows a true negative control "no flow" condition, and crucially, does not alter cytoskeletal and motor protein activities. Figure 3 is modified from Levin M. 2006 Birth Defects Res C Embryo Today 78:191–223.

asymmetrically during the first two cleavages in frog embryos and functions upstream of *Nodal* in the vertebrate visceral LR pathway.[47] Moreover, the interaction of 14-3-3E with a H^+ pump can be perturbed by fusicoccin—a fungal compound that randomizes asymmetry in frog embryos, but was previously thought to interact only with plant cells.[47]

The involvement of 14-3-3 proteins in cellular asymmetry in early cleavages of nematodes (*C. elegans*) and flies (*Drosophila*), and embryonic asymmetry in frogs (*Xenopus*) therefore suggests a potentially ancient mechanism whereby both cellular and organismal polarity are established by similar mechanisms. Consistent with our proposed placement of cilia events after intracellular events in the LR pathway (see next section), PAR proteins also regulate ciliogenesis.[48] Bardet-Biedl genes function in vertebrate planar cell polarity and affect basal body structure.[49] Basal bodies/centrioles[33] are an excellent candidate for an intracellular ''F-molecule'', because they function as a microtubule-organizing center[50] to direct asymmetric localization of maternal components (see Fig. 1C, 2) More broadly, PAR protein roles offer a fundamentally new perspective from which to investigate large-scale morphogenetic control in vertebrates, and highlights possible connections between widely shared subcellular polarity machinery (cytoskeleton and motor proteins) and morphological asymmetry.

Finally, subcellular components, such as motor proteins and the cytoskeletal tracks that guide their localization, are connected to LR asymmetry in other systems. These components control anatomical asymmetry in snails[51] and plants.[39] In addition to the kinesins and dyneins implicated in rodent asymmetry,[52,53] recent data in *Drosophila* also implicate myosin motors.[54] Surprisingly, like the *inversin* deletion in mice,[55] loss of symmetrically expressed myosin I yields mirror-image flies and not randomization.[54] Indeed, even in zebrafish, non-canonical Wnt signaling (often interpreted in the context of ciliary defects) is intimately tied with intracellular actin organization,[56] which is known to be required for proper targeting of LR-relevant ion pumps.[36]

Evidence for the intracellular model: ion flux

The intracellular model (Fig. 2) also proposes an intimate connection between subcellular polarity and ion flux, at least for early-developing asymmetries. Recent work confirms this connection in three different phenomena.

First, some surprising physiological parallels exist between the mechanism of asymmetry determination in frogs and those regulating ion flux in vertebrate neurons and gut cells. For example, early *Xenopus* embryos utilize an H^+/K^+-ATPase exchanger in combination with a K^+ channel to generate LR voltage differences that in turn direct embryo asymmetry.[34] Details in the chick and fish embryo are less clear, but the same components have been implicated.[34,36,57] Normal neurons use exactly the same system to build up a voltage potential: a closely related P-type pump, the Na^+/K^+-ATPase, in combination with a K^+ channel provides the negative membrane voltage as the excess K^+ escapes (although *Xenopus* uses an additional hyperpolarizing pump—the V-ATPase).[36] Gut cells also generate a voltage potential using the H^+/K^+-ATPase pump and the same KCNQ1 channel implicated in frog embryo asymmetry.[58]

Second, some fascinating parallels exist between serotonin and auxin–morphogens that determine LR asymmetry in vertebrates and plants respectively and that have strikingly similar molecular structures.[59] Both participate in similar physiological mechanisms involving ion flows and amplification feedback loops. In frog and chick embryos, LR differences in voltage gradients across cell membranes result in asymmetric distribution of serotonin that ultimately triggers asymmetric gene expression (as in Fig. 2I). Similarly, in plants, asymmetries of auxin across different tissues of both embryos and adult organs are generated by the same molecular components: H^+ pumps, K^+ channels, plasma-membrane transporters, etc. Moreover, not only is the molecular machinery very similar to that of the intracellular model (Fig. 2), but so is the logic of the control pathways, including an electrophoretic system for generating serotonin/auxin asymmetries, and ''canalizing'' loops whereby differences in serotonin/auxin are amplified via positive feedback on the transport machinery (for details see Ref. 59).

Finally, the most-profound analogy involving ion flux is between kidney function and LR asymmetry in vertebrates. Here, the intracellular model yields a very different interpretation of the interconnection between LR defects and cilia-related diseases than the cilia model. On the one hand, in the cilia model, ciliary defects are the direct cause of both LR randomization and cilia-related diseases like polycystic kidney disease (Fig. 3A). In the intracellular model, on the other hand, ciliary defects have no direct effect on LR asymmetry, but both are disrupted by shared upstream cell polarity defects (Fig. 3B). Cilia are an apical specialization, and any defects in apical–basal cell polarity are likely to cause errors in cilia length or position. In addition, the Wnt-signaling pathway provides a mechanistic link between cell polarity and ciliogenesis.[60,61] Likewise, ion-transport disruption caused by cytoskeletal or motor-protein defects may disrupt both kidney function and LR asymmetry (Fig. 3B).

Several mouse knockout studies identified targets whose abrogation causes both kidney and left–right defects.[62–65] According to the cilia theory, a sensory cilium is necessary for both kidney function and visceral asymmetry.[66] However, the salient connection between these seemingly disparate phenomena may actually be the tight linkage of geometrical cell polarity to localized ion transport (and thus physiological or bioelectrical polarization). Kidney cells (and epithelial cells in general) are highly polarized both anatomically and bioelectrically. They control ion flux by using cytoskeletal and

Table 1. Factors implicated to effect LR patterning in eukaryotes

Embryonic time: →

Evolutionary Relationships	Model system	Cytoskeleton/ Motor protein	Ion flux	GJC	5HT	Cilia
	Ciliates	(40)		X		
	Arabidopsis	(111)	(117)	X		X
	Lymnaea	(51)				X
	C. elegans	(118)				X
	Drosophila	(54)				X
	Sea urchin larvae		(38)			
	Ciona		(119)			
	Zebrafish		(36)			(1)
	Xenopus	(24)	(34)	(120)	(121)	
	Chick		(34)	(35)	(121)	X
	Human					(122)
	Rabbit			(123)		
	Mouse					(124)

Each yellow cell indicates that a given factor (column heading) has been functionally implicated in LR asymmetry or chirality determination in a particular model species (numbers in these cells indicate references to representative studies where involvement was demonstrated, as cited in the bibliography). Empty cells indicate unknown or functionally unproven relationships (not all mechanisms have been experimentally probed in all species, so blank cells do not indicate a definitive lack of involvement). X indicates that the factor does not function in the species indicated. Motile cilia are absent (except sometimes for gametes) in plants, nematodes and insects, and are not involved in asymmetry determination in snail embryos, where chirality is determined in the egg cytoplasm. Amphibia and fish (aqua) lie near the transition to ciliary signaling. Abbreviations: GJC = gap-junctional communication. 5HT = serotonin (or auxin, in plants). Phylogenetic relations among mammals from Ref. 115, among Metazoa from Ref. 115, and among remaining Eukaryota from Ref. 116. Table 1 is modified from Levin M. 2006 Birth Defects Res C Embryo Today 78:191–223.

motor-protein elements to regulate localization of membrane-bound ion channels and pumps. This specifically includes the H^+ and K^+ transporters important in LR asymmetry.[58,67,68] For example, *orpk* is required for the epithelial polarity of ventral node cells and also for embryo LR asymmetry.[65] Other components implicated in LR asymmetry, such as the tight junction components claudin and cadherin, also aid kidney function[69] by shaping the *trans*-epithelial voltage gradients resulting from targeted ion flows. These observations may help explain the striking and puzzling association of hemihypertrophy (Beckwith-Widemann and Proteus syndromes, where many tissues on one side of the body resume growth in adulthood) and Wilms' kidney tumors.[70,71]

Connections to other axes and evolutionary trends in control of LR asymmetry

Connections also exist between determination of LR asymmetry and patterning of other body axes. For example, β-catenin, crucial for dorsoventral patterning,[72] binds both inversin[73] and fly myosin I.[54] In addition, cytoskeletal elements direct the large-scale polarity of other axes from the AP axis in planaria[74] to the DV axis in *Xenopus*.[75] All organisms may potentially rely on such cytoskeletal/motor-protein cues to orient directed asymmetry. However, the use of these cues may have diverged considerably or evolved independently multiple times.

Multiple mechanisms have been functionally implicated to orient LR asymmetry in eukaryotes ranging from protists to mammals (Table 1). While much work remains to unravel the full pathways in all these model systems, several important points emerge. First, in species where multiple components have been implicated, the temporal progression during development is: cytoskeletal → physiological → ciliary systems. Second, the distribution of confirmed occurrences suggests that, evolutionarily, a) the earliest mechanism in eukaryotes involved the cytoskeleton and/or motor proteins, b) ion-flux mechanisms arose independently in plants and deuterostomes (sea urchins through mammals), c) within deuterostomes asymmetry determination shifted (gray arrow) from cytoskeletal mechanisms through physiological signals, towards cilia in the middle of the pathway, and d) mechanisms upstream of cilia may even have been lost in rodents and possibly primates. Some tantalizing evidence suggests the loss of signaling steps upstream of node cilia in mice actually has a major benefit. The incidence of spontaneous situs inversus (inverted visceral asymmetry) is more than two orders of magnitude lower in mice than non-mammalian vertebrates.[6] Mice may have lower vulnerability because they no longer depend on so many upstream steps, each of which represents a point of vulnerability during development. Third, among vertebrates, the pivotal taxa appear to be amphibia and fish, where ciliary signaling first evolved but where older mechanisms were still used. Finally, motile cilia are absent in plants, nematodes, and insects (except for motile gametes in insects and some plants)[76] so they cannot play a role in symmetry breaking.

Developmental pathways may evolve in multiple ways, including addition or deletion of steps at either end, or in the middle.[77] The observations in Table 1 suggest that the pathways controlling LR asymmetry in eukaryotes have evolved in two interesting ways. The first is by addition of developmentally later downstream effects. At the very least, subcellular effects of motor proteins, oriented cytoskeletal molecules and ion flows on LR asymmetry appear to be evolutionarily older than cilia effects. In addition, cytoskeletal asymmetries likely preceded ion-flux asymmetries evolutionarily. The second example of pathway evolution is loss of developmentally earlier upstream effects. The apparent absence in mice of asymmetric gene expression upstream of the conserved *Nodal* signaling cascade may very well represent a loss of multiple upstream steps seen in other vertebrates.

Sex determination, cell size and early midplane definition

Although LR asymmetries appear to arise embryologically at different times in different organisms, some intriguing observations of unilateral developmental anomalies (e.g. gynandromorphy, unilateral pigmentation, and other laterality defects) suggest that the midplane—a frame of reference that presumably must exist before left and right are defined—arises quite early during development, even in embryos where cell size and cell number varies enormously at the time it is defined. Early determination of the midplane greatly increases the likelihood that intracellular models of LR asymmetry (Fig. 2) potentially apply to embryos as wildly different in form as those in birds and mammals.

Bilateral gynandromorphs offer some of the most impressive evidence of early midplane determination (see Figs. 4B–E for examples of lobsters, birds, butterflies and crabs). In Orthoptera (katydids, crickets and relatives), complete bilateral gynandromorphs result when one X chromosome in an XX zygote is eliminated from one daughter cell at first cleavage.[78] Similar examples of whole-body laterality defects of sex or pigmentation are known in ribbon worms,[79] ants,[80] flies[81] (including *Drosophila*[82]), caddisflies[83] and ticks.[84] Furthermore, in one example from birds,[85] asymmetry of both the brain and gonads were in the same direction—right side male—suggesting a tighter coupling of the laterality of these organs when symmetry is broken via early chromosome segregation errors than when controlled later in development by *Nodal* cascade signaling (e.g. disruptions of the *Nodal* cascade or node cilia function often do not yield concordant orientation of brain and visceral asymmetries[27]). So, although not all species' gynandromorphs are entirely male on one side and female on the other, the repeated occurrence

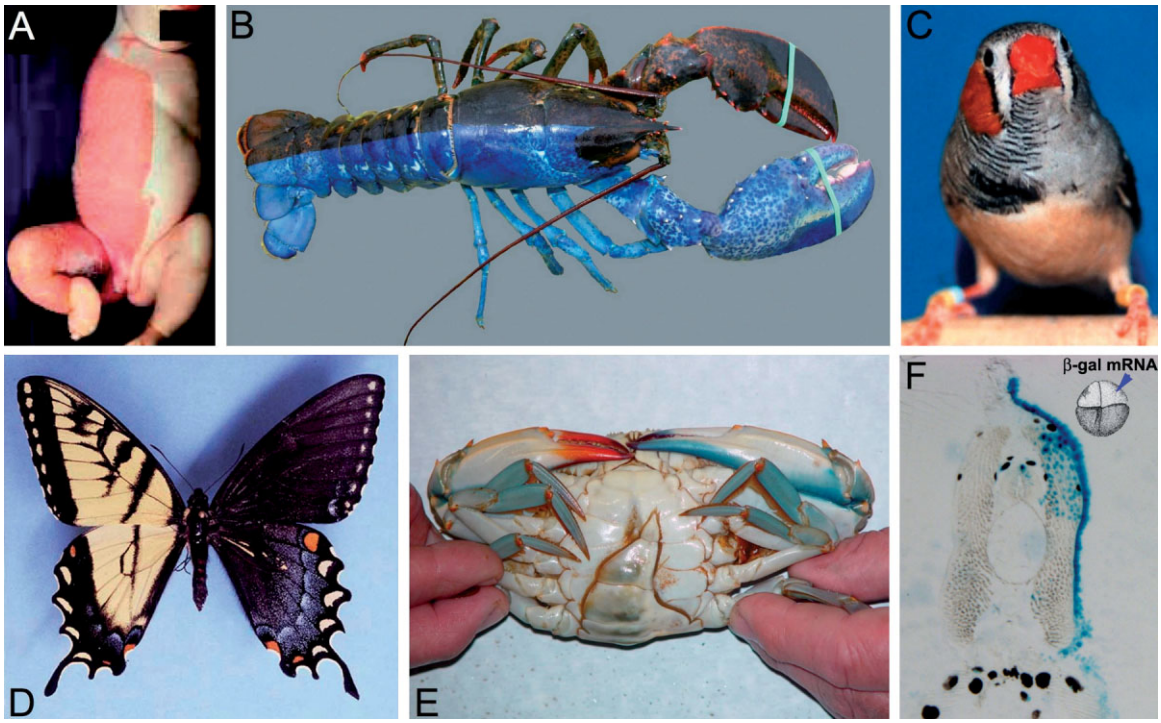


Figure 4. Examples of early midplane determination in various animal groups. In each of these examples, a consideration of the mechanisms underlying dramatic bilateral differences in body form, pigmentation, or cell lineage markers, suggests the midplane is established at very early cleavage stages. **A:** Asymmetric cutaneous pigmentation pattern with a sharp midplane demarcation in the X-linked CHILD syndrome of humans. **B:** A split-color lobster, *Homarus americanus* (likely a gynandromorph, but sex was not examined; original photo courtesy of Ryan Mitchell). **C:** A gynandromorphic zebra finch, *Taeniopygia guttata* (brightly colored right side of body is male).[85] **D:** A gynandromorphic swallowtail butterfly, *Papilio glaucas* (dorsal view; brightly colored left side of body is male). **E:** A gynandromorphic blue crab, *Callinectes sapidus* (ventral view; left side of body bearing the narrower abdomen is male). **F:** Section through a week-old *Xenopus* embryo showing unilateral staining of β-galactosidase mRNA that was injected into one blastomere at the two- or four-cell cleavage stage (inset). Permissions and attributions for Fig. 4 include: **A:** with permission of John Wiley and Sons from the American Journal of Medical Genetics, 2000, 90, p. 340; **C:** with permission of Art Arnold and PNAS (from Fig. 1A of Agate et al. 2003. Neural, not gonadal, origin of brain sex differences in a gynandromorphic finch. PNAS 100:4873–4878, Copyright (2003) National Academy of Sciences, U.S.A.); **D:** with permission from James Adams; and panel E with permission from Rom Lipcius (VIMS).

of nearly perfect bilateral gynandromorphs suggests that the midplane can be determined quite early. Indeed, in crustaceans and *Xenopus* midplane definition clearly does take place at early cleavage stages.[86]

Striking unilateral pigmentation patterns (Fig. 4A) also occur in humans with X-linked diseases such as CHILD syndrome.[87] The required unilateral X-inactivation in such patients also suggests that the midplane is defined quite early in human embryos. Here, too, rodent embryos appear to be atypical. Mouse models of the CHILD syndrome recapitulate all of the important features *except* unilateral pigmentation,[88] and allophenic mice exhibit coat colors that do not respect the midplane. Mice therefore do not appear to set the midplane early enough for X-linked chromosomal defects to produce large-scale asymmetries.

Attempts to apply the *Xenopus* model—where the earliest LR asymmetry appears as cytoplasmic segregation among a few cells at early cleavage stages—to chick or rabbit embryos raises another important question: what happens when early asymmetries arise in a cell field of thousands of small cells as opposed to a few large blastomeres whose cleavage planes are clearly oriented with respect to the final embryonic midplane (as in *Xenopus*)? The alignment of axes in mammalian cleavage is controversial,[89] but the human 3′UTR for *squint* mRNA can drive asymmetric localization in cleaving zebrafish embryos,[90] so future work must determine whether or not mammals use cleavage-based localization mechanisms to define midplane location.

The discordance between cell size/number and midplane definition seems particularly striking in chick embryos, where midplane definition is believed to occur when the primitive streak first forms (i.e. when the blastoderm contains ~50,000 small cells).[91] However, polyspermy sometimes causes a second fertilization event involving a polar body

in birds.[92] Clonal expansion of both diploid "individuals" produces a blastodisc with one side composed largely of male cells and the other largely of female cells, and therefore lacking the radial symmetry of normal chick embryos. While the streak can be re-positioned experimentally during blastoderm stages,[93] the crisp midplane demarcation of pigment differences in bird gynandromorphs (Fig. 4C) strongly suggests that the primitive streak also normally develops in the same plane as first cleavage: otherwise, gynandromorphic birds would exhibit random, patchy orientations of the color difference.

Even if the midplane is established early—as suggested by the many examples above—is asymmetry or its bias towards one side linked to this process? Several correlations suggest it may be. In human hermaphrodites, ovaries tend to develop on the left, while testes appear on the right.[94] This time, rodents are not the 'odd man out'. Mice also exhibit a strong sidedness of organs in hermaphrodites,[95] although laterality of the testes and ovaries is opposite to that in humans.

Is the early timing of midplane determination and LR asymmetry consistent with clinical data in humans? Apparently it is. Non-conjoined monozygotic twins not only exhibit a higher-than-normal incidence of laterality defects[96] they also show many subtler kinds of mirror-image asymmetry ("bookend" or enantiomer twin pairs). Pairs of such twins present discordances in hemihypertrophy,[97] as well as mirroring of asymmetries in hand preference, hair whorl direction, tooth patterns, unilateral eye and ear defects, cleft lip, cleft palate, supernumerary teeth, tumor locations, undescended testicles, and the sidedness of limb abnormalities (reviewed in Ref. 98). The bookending phenomenon may also be intertwined with the timing of the earliest symmetry-breaking steps in mammals. Most healthy, non-conjoined twins presumably result from separation of cleavage, morula, or early blastocyst stage embryos.[99] Thus, some chiral information may be present even in the early mammalian embryo, which later manifests as hair whorl and other "bookend" asymmetries if the cells are separated at an early stage. In contrast, body organ asymmetry still seems to be labile at those stages because it develops correctly for both monozygotic twins.[100]

Why might early splitting of human embryos have consequences for chirality? Mirror-image cytoskeleton patterns and cell migration tracks have been observed following normal cell division in culture.[101] These, too, underscore the importance of cytoskeleton and subcellular structures for large-scale asymmetry and cellular behavior. The human data on asymmetry suggest that two different pathways operate. The primary control of visceral and cardiac asymmetry takes place via the well-characterized *Nodal* cascade[102] and is vulnerable to ciliary dysfunction.[26] But the clinical data reveal another, still elusive, pathway.[103] As discussed elsewhere,[9–98] chirality of hair-whorls, hand-use preference and hemispheric asymmetries in the brain, all appear to be controlled by a separate pathway independent of the mechanisms disrupted in situs inversus and heterotaxia patients. The conservation of asymmetry of unilateral defects in monozygotic twins suggests that mirror-image LR information was already present at the time of splitting.

The conundrum of late-developing asymmetries

Despite the taxonomically wide-ranging evidence above for early definition of both the midplane (previous section) and the many consistently oriented LR asymmetries (Table 1), and despite the connection of directional symmetry-breaking to oriented subcellular asymmetries in early embryos (Fig. 2), late-developing asymmetries[104] pose a particularly vexing problem. Late-developing asymmetries—like style bending in enantiostylous flowers, side of the attached valve in some cemented bivalves, coiling direction of spirorbin tubeworms, claws in many decapod crustaceans, and eye-side of flatfish—do not appear until after birth, hatching, or metamorphosis, when most major body regions and parts have already developed. These asymmetries clearly manifest well after midplane formation and embryogenesis.

What makes directionally oriented, late-developing asymmetries so fascinating is their evolutionary history. Most arose from ancestors that were asymmetrical, but where direction of asymmetry was random[104] (i.e. 'indifferent asymmetry' or 'antisymmetry'[3]). In other words, evolutionarily these conspicuous morphological asymmetries existed with random orientation *before* they became fixed in a particular direction. Remarkably, where direction of asymmetry is random within a species it is also not inherited: with only one compelling exception, crosses between pairs of dextral or sinistral parents yield a 50:50 mixture of dextral and sinistral offspring.[6] So, each time that a descendant species with fixed direction evolved from an ancestor where direction was random, developmental-genetic mechanisms controlling the direction of asymmetry must have arisen evolutionarily *after* the randomly oriented asymmetric phenotypes already existed, a phenomenon sometimes called genetic assimilation.

These directional, late-developing asymmetries prompt a critical question: is their development guided in a particular direction by the same mechanisms as early-developing asymmetries, like oriented asymmetries in the cytoskeleton or ion channels described above (Fig. 2)? Indeed, subcellular cytoskeletal asymmetries are undoubtedly available as LR cues, but whether specific taxa use that information or not is unclear. Cases of "cryptic" asymmetry[105]—those not evident morphologically but revealed by molecular or genetic perturbation in tissues or organs thought to be symmetrical—may offer tractable systems for testing this proposal. Sadly, virtually nothing is known about how direction of asymmetry is determined in species where it arises late in ontogeny.[6]

Two examples from very different organisms highlight the challenge: claw asymmetry in male fiddler crabs and style asymmetry in otherwise bilaterally symmetrical (zygomorphic) flowers. Male fiddler crabs possess a massive major claw (Fig. 5A, inset) used to signal to females, or to signal or combat other males. The vast majority of living species are antisymmetric: right- and left-handed males are equally common. But the ontogeny of this asymmetry is decidedly peculiar: young males lose the claw on one side at random, either before or soon after both claws start to transform into major claws, and the remaining claw develops into the major.[106] Therefore, among a narrow size range (3–5 mm carapace width) this yields many juvenile crabs either missing one claw altogether or possessing two major-type claws (Fig. 5A). Above 5 mm carapace width, half the population is females with two small symmetrical claws, and the remaining half is males with highly asymmetrical claws. Laboratory experiments confirm that removal of one claw in juvenile males induces the major claw to develop on the other side (Fig. 5B), so direction of asymmetry in these antisymmetric species is not determined genetically. This lack of genetic control of asymmetry echoes results for American lobsters, where whichever claw is used most during a narrow sensitive period in juveniles develops into a major claw (i.e. direction of asymmetry is also not inherited).[107]

Significantly, in one fiddler crab group direction of claw asymmetry is not random: all eight species of *Gelasimus* are right-handed.[108] Evolutionarily, therefore, genetic control of asymmetry *direction* in this clade arose well after conspicuously asymmetrical claws appeared (Fig. 5C). How direction of asymmetry is controlled in *Gelasimus* remains unknown. Most likely, differentiation of the major claw on one side inhibits development of a major claw on the other via the central nervous system, as in lobsters and snapping shrimp.[107] So if the direction of this inhibition is somehow influenced by cytoskeletal or ion-flux asymmetries, the central nervous system would be a promising place to search.

Style asymmetry in enantiostylous flowers is an even more fascinating example because the genetics is better known. Overtly bilaterally symmetrical (zygomorphic) flowers have evolved many times in plants, and among these taxa, asymmetrical floral organs (enantiostyly) evolved in no less than 11 groups.[4] However, two quite different types of enantiostyly exist. In one (monomorphic enantiostyly), both right- and left-bending styles occur in flowers of a single plant (Fig. 6A). Clearly the direction of style bending in these species is not determined genetically, as both floral forms occur on the same individual. In the other type (dimorphic enantiostyly), all flowers on an individual plant bend in the same direction, but roughly half the individuals in a population possess right-bending, and half left-bending styles.[4] In one such species (*Heteranthera multiflora*), breeding experiments confirmed that two alleles at a single locus control direction of style bending and the right-bending allele is dominant (Fig. 6B).[109]

As in fiddler crabs, however, genes controlling *direction* of floral asymmetry clearly evolved after such asymmetries already existed. Within one branch of the Haemodoraceae

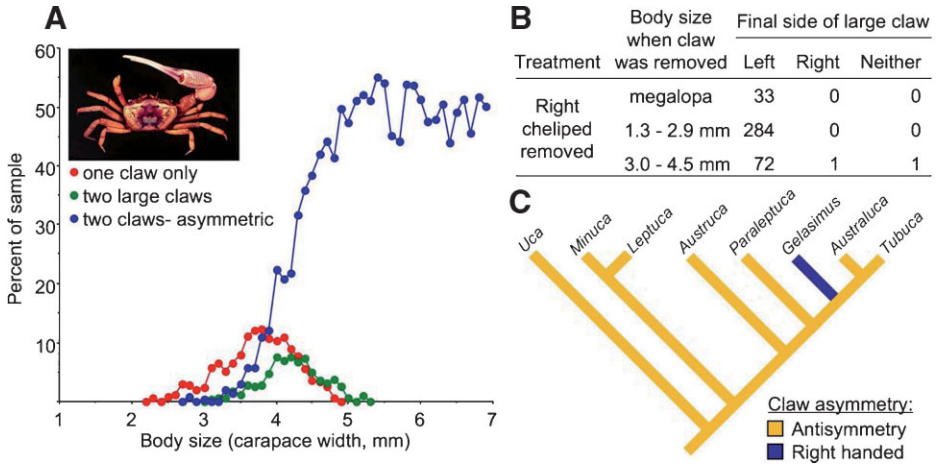
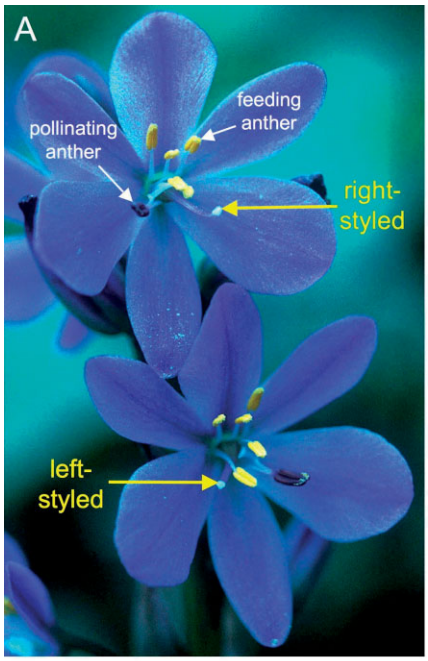


Figure 5. Development and evolution of male fiddler crab claw asymmetry. **A:** Percentage of wild juvenile fiddler crabs (*Uca (Austruca) lactea*; both sexes combined) with different claw configurations as a function of crab size (N = 13,913). Among crabs with asymmetrical claws, 4,071 were right-handed and 4,017 were left-handed,[114] so this species exhibits antisymmetry (indifferent asymmetry). Inset: a right-handed male of *Uca pugilator*. **B:** Numbers of juvenile male fiddler crabs (*U. lactea*) that developed a large claw on the left side, right side or neither side following experimental removal of the right cheliped (the entire leg that bears the claw) at three different initial sizes (carapace width; megalopa refers to the last larval stage before metamorphosis). **C:** Phylogenetic relations among subgenera of fiddler crabs, showing the solitary evolutionary origin of right-handedness in the subgenus *Gelasimus* from an antisymmetric (indifferently asymmetric) ancestor.[108]



B

Direction of style bend in F2	Cross number 1	2	3	4	5	6	total
left	0.26	0.27	0.22	0.22	0.17	0.25	0.24
right	0.74	0.73	0.78	0.78	0.83	0.75	0.76
N	34	62	27	81	18	20	242

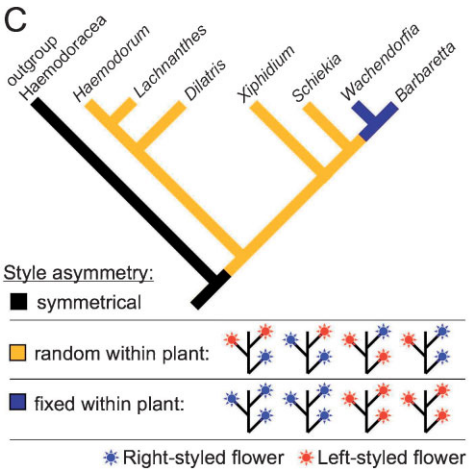


Figure 6. Genetics and evolution of floral enantiostyly (bending of the style to one side). **A:** Two flowers on an individual plant of *Monochoria korsakowii* (Pontederiaceae; original photo courtesy of S.C.H. Barrett). The style, which receives pollen on its tip (stigma), bends to the right of the floral midplane (from the perspective of the observer) in the upper flower and to the left in the lower one. Because both flowers occur on an individual plant and all individual plants bear both style forms (monomorphic enantiostyly), the direction of style bending is not determined genetically. **B:** Inheritance of direction of style bending in *Heteranthera multiflora* (Pontederiaceae), a species where direction of style bending is fixed within an individual plant but varies among plants (dimorphic enantiostyly): the proportions of left-styled and right-styled F_2 offspring from six full-sib crosses where sibs were obtained from selfed parents that yielded only left- or only right-styled F_1 offspring. A single locus with two alleles controls direction of style bending; the right-bending allele is dominant.[109] (**C**) Phylogenetic relations among genera in a subclade of the Haemodoraceae, showing the solitary origin of dimorphic enantiostyly (style orientation fixed within plant; orientation heritable) from a monomorphic enantiostyly (style orientation random within plant; orientation not heritable) ancestor.

that includes both forms of enantiostyly, the clade within which direction of floral asymmetry is genetically determined (dimorphic enantiostyly) evolved from ancestors with no genetic control of asymmetry (monomorphic enantiostyly) (Fig. 6C). The single-locus control of style-bending direction in such species[109] offers an exciting opportunity to identify its molecular basis. If direction of style bending is controlled by the same mechanisms as helical growth in *Arabidopsis*,[39,110,111] then this could become the first example where directed orientation of a late-developing morphological asymmetry has become coupled—both evolutionarily and developmentally—to oriented asymmetries at the intracellular level.

Next Steps

The most-crucial future work, which would specifically test some of the hypotheses outlined here, includes:

1. To determine definitively that cilia control symmetry-breaking in rodents a more sophisticated mutant is needed: a mouse where loss of function of left–right dynein or other ciliary components is restricted to the node, or where function is lost beginning on day 7 of development. If such a mutant has a defective LR phenotype, then ciliary motion—and not other roles of ciliary proteins—is essential for the correct orientation of mouse visceral asymmetry. This question remains open because the key experiment—alteration of nodal flow in the absence of genetic loss of function of proteins with other possible roles in a preparation where a true negative control is possible—has not yet been done.
2. A key test of the intracellular model advanced here is identification and molecular characterization of the oriented cytoskeletal structure responsible for cell polarity and LR asymmetry. The *Xenopus* system holds great promise here, because the known early players such as 14-3-3E and the asymmetrically distributed components[24] may be readily studied.
3. Definitive study of bookending in human twins of known placentation will allow us to understand the true prevalence of this phenomenon and determine which tissues and

structures are involved. A statistically strong analysis of unilateral defects confirming the opposite-sidedness of embryos that split during cleavage stages would provide strong evidence for a parallel, cilia-independent pathway in humans.

4. Twin bovine embryos can be produced by artificial splitting at the 2-cell stage, thus removing the uncertainty in timing. Investigation of hair whorls and subtle asymmetries here would help identify the specific cellular mechanisms that break symmetry near the first cell cleavage in mammals. The intracellular model predicts that, in such fetuses, hair whorls would have opposite handedness.
5. Detailed investigation of lineage and midplane determination in normal and polyspermic chick embryos from the 2-cell stage would test whether the final embryonic axis of symmetry is really set at early cleavage stages. The intracellular model would be refuted (or have to be significantly modified) if the midline in chick embryos is not set until streak initiation.
6. Functional testing of physiological mechanisms (ion transport, serotonergic signaling, syndecan phosphorylation, etc.) in mice and rabbits would test which of these steps persist in different mammals. Evidence of these functional components in mammals would support the intracellular model as a fundamental mechanism of symmetry breaking.
7. Subtractive (differential) mRNA and proteomic analysis of L and R halves of rabbit and mouse embryos may identify markers possessing consistent asymmetry prior to the streak stage. If confirmed, this would conclusively refute the cilia model of LR asymmetry.
8. More data are needed to better complete Table 1. Inexpensive drug screens in other species[112] can reveal which mechanisms are involved. Zebrafish is especially important because it is a pivotal species: some of the same very early physiological mechanisms have been implicated, as have cilia, but cleavage-stage asymmetries have not been described. The zebrafish offers an excellent opportunity to learn how these pathways interact.
9. Identification of the gene or gene product that determines direction of style bending in *Heteranthera multiflora*, where direction of bending is controlled by two alleles at a single locus, would provide the first test of whether late-developing asymmetries can be oriented by subcellular asymmetries as well.

Conclusions

We have highlighted a comprehensive view of symmetry breaking that extends beyond vertebrates and nodal cilia all the way to plants and protists. In this view, ancient cytoskeletal "F-molecules" asymmetrically localize physiological mechanisms that, in turn, exert asymmetries onto cell fields. The linkage between visceral asymmetry and kidney defects suggests a fundamental unity between cell and organismal polarity, because both involve localization of ion transporters, motor protein activity and PAR protein families. In addition, examples of near-perfect bilateral gynandromorphs suggest midplanes may be determined much earlier than normally believed. This increases the attractiveness of intracellular models and strengthens the case that mouse asymmetry is divergent, and thus misleading about general mechanisms of LR determination. Finally, studies of mechanisms controlling the direction of late-developing asymmetries could provide a powerful test of the most wide-reaching hypothesis of all, that morphological asymmetries at the macroscopic level are inexorably tied to molecular asymmetries at the subcellular level—no matter when they arise during ontogeny.

Note

After this manuscript was accepted, two studies reported important results predicted by the intracellular model of symmetry breaking described here. First, Armakolas, A., and Klar, A. J. (2007). Left-right dynein motor implicated in selective chromatid segregation in mouse cells. Science 315, 100-1 demonstrate that LRD—the ciliary motor protein that, when mutated, renders node-monocilia non-functional and randomizes visceral asymmetry in mice—also a) has intracellular roles distinct from ciliary function and b) plays a role in chromosome segregation, errors in which can cause bilateral gynandromorphy (Fig. 4). These results support our proposal that cytoplasmic motor protein activity is fundamentally linked to large-scale morphological asymmetry. Second, Banizs et al. (2006). Altered intracellular pH regulation and $Na+/HCO3^-$ transporter activity in choroid plexus of the cilia defective Tg737orpk mutant mouse. Am J Physiol Cell Physiol show that the Tg373 mutant mouse, which has ciliary defects and altered LR patterning, also has abnormal pH regulation. This supports the view (Fig. 3B) that early movement of H^+ and other ions may be affected by mutations commonly thought mainly to affect laterality determination via disrupted ciliary function. Finally, Schweickert et al. (2007). Cilia-driven leftward flow determines laterality in Xenopus. Curr Biol 17, 60-6 reveals that cilia motion affects visceral laterality in later stage Xenopus embryos. Although these authors could not say whether cilia generate chirality de novo or merely pass on upstream signals, this finding meshes nicely with the evolutionary scenario advanced here (Table 1) that amphibians and fish represent transitional stages where both ciliary and intracellular mechanisms control the orientation of left-right visceral asymmetry.

Acknowledgments

Many thanks are due to Cassandra Extavour, Michael Clinton, Rudolf Happle, the members of the Levin laboratory, and the left–right asymmetry, physiology and neuroscience communities for useful discussions. We apologize to the many authors

whose relevant work could not be cited because of editorial restrictions on length and number of references. We thank Ryan Mitchell and Spencer C.H. Barrett for allowing us to reproduce their original photographs (Figs. 4B and 6A, respectively), and Art Arnold for providing a high-resolution version of Fig. 4C.

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