

Left–right asymmetry in the vertebrate embryo: from early information to higher-level integration

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Abstract | Although vertebrates seem to be essentially bilaterally symmetrical on the exterior, there are numerous interior left–right asymmetries in the disposition and placement of internal organs. These asymmetries are established during embryogenesis by complex epigenetic and genetic cascades. Recent studies in a range of model organisms have made important progress in understanding how this laterality information is generated and conveyed to large regions of the embryo. Both commonalities and divergences are emerging in the mechanisms that different vertebrates use in left–right axis specification. Recent evidence also provides intriguing links between the establishment of left–right asymmetries and the symmetrical elongation of the anterior–posterior axis.

Embryo node

A transient structure located at the anterior tip of the primitive streak in embryos of amniotes (birds, reptiles and mammals); also known as Hensen's node in birds and mammals. The embryo node functions as the gastrula organizer and is therefore functionally equivalent to the dorsal lip of the blastopore (Spemann's organizer) in amphibians and the shield of teleost fishes.

Despite individual random variations, the external bilateral symmetry of the vertebrate body plan is almost perfect. However, on the interior things are not symmetrical¹: the left side contains most of the heart, the stomach, the pancreas and the spleen, whereas the right side contains most of the liver and the gall bladder. In addition, the left lung has fewer lobes than the right lung, and the gut coils anticlockwise. These asymmetries are uniform among individuals within a species, and departures from this situation in humans result in severe medical conditions.

In addition to its relevance to human health, how vertebrate left–right (LR) asymmetry is established is an important question for developmental biologists, and several insights have been gained over the past 10 years from studies of chick, *Xenopus laevis*, mouse and zebrafish embryos^{2–6}. Although they are not necessarily universally applicable, the development of LR asymmetry can be broken down into the following main steps (FIG. 1). First, an initial event breaks the bilateral symmetry of the embryo, probably by converting cues encoded in the already established anterior–posterior and dorsal–ventral axes into LR information⁷. Laterality cues are then transferred to the embryo node (Spemann's organizer in *X. laevis* or Hensen's node in birds and mammals) or its derivatives (Kupffer's vesicle in teleost fish). Subsequently, LR asymmetries are established in and/or around the node, after which LR information is conveyed from the node to the left lateral plate mesoderm (LPM). Side-specific gene-expression domains are then

established and stabilized along wide areas of both the left and right LPM. Finally, LR information is transferred to the organ primordia, so that left- and right-side specific morphogenetic programmes are executed.

It has become clear that the establishment of LR asymmetries is controlled by robust genetic and epigenetic mechanisms, some of which show a remarkable degree of evolutionary conservation, whereas others seem to be species-specific. Although our knowledge of some of the steps involved is still fragmentary, many insights have been gained recently into the molecular and cellular mechanisms that underlie and regulate these processes, especially those that occur at the early stages of LR asymmetry determination. In addition to these advances, exciting new results show how LR asymmetrical patterning is integrated within the context of a bilaterally symmetrical body plan.

Breaking the bilateral symmetry of the embryo

How is the initial bilateral symmetry of the embryo consistently broken in one direction? One way to investigate this is to search for the earliest sign of laterality information, which might provide clues as to how this information is generated. Genetic, pharmacological and microsurgical approaches have identified progressively earlier requirements for the correct establishment of LR asymmetries in frog, chick and zebrafish embryos. Nevertheless, despite years of intensive research, no satisfactory explanation has been provided for the initial symmetry-breaking event in any of these species.

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The mouse embryo might represent an exception to this. Unlike in frog, chick or zebrafish, no LR asymmetrical features, or requirements for LR asymmetry, have been identified in the mouse embryo before node formation. Furthermore, a mechanism that is solely based on the function of the node (the ‘nodal flow’) has been shown to generate laterality information *de novo*

and therefore to be potentially sufficient to break the initial bilateral symmetry of the embryo. The nodal flow refers to the leftward flow of extracellular fluid that is generated in the mouse Hensen’s node by cilia that line the ventral side of this structure. Recent insights into this mechanism, and related hypotheses, are presented in detail below. Here we note that although the nodal flow is crucial for LR patterning in the mouse, whether it represents the initial symmetry-breaking event is a matter of debate, and several alternative possibilities can be proposed^{2,4,6,8–13}.

Recent evidence casts doubt on two extreme hypotheses that have been previously proposed — namely that either nodal cilia provide a general mechanism for breaking the initial symmetry of all vertebrate embryos¹⁴, or nodal cilia are themselves not important for LR asymmetry determination¹⁵. There are currently two equally plausible models. In the first, the nodal flow provides the symmetry-breaking event in the mouse embryo and is likely to represent an innovation of rodents (or, more generally, of mammals) that supersedes more ancient mechanisms of symmetry breaking used by other vertebrates. This is supported by the fact that no evidence of laterality information has been found in the mouse embryo before node function. A second model contends that an unknown symmetry-breaking event is common to all vertebrate embryos. In this case, although the nodal flow is crucial for LR patterning it would not represent an initial symmetry-breaking event, but rather a downstream amplification step. Determining whether either of these hypotheses is correct will require the discovery of the elusive symmetry-breaking event used by species other than the mouse, or the identification of laterality cues or requirements in the mouse before node formation.

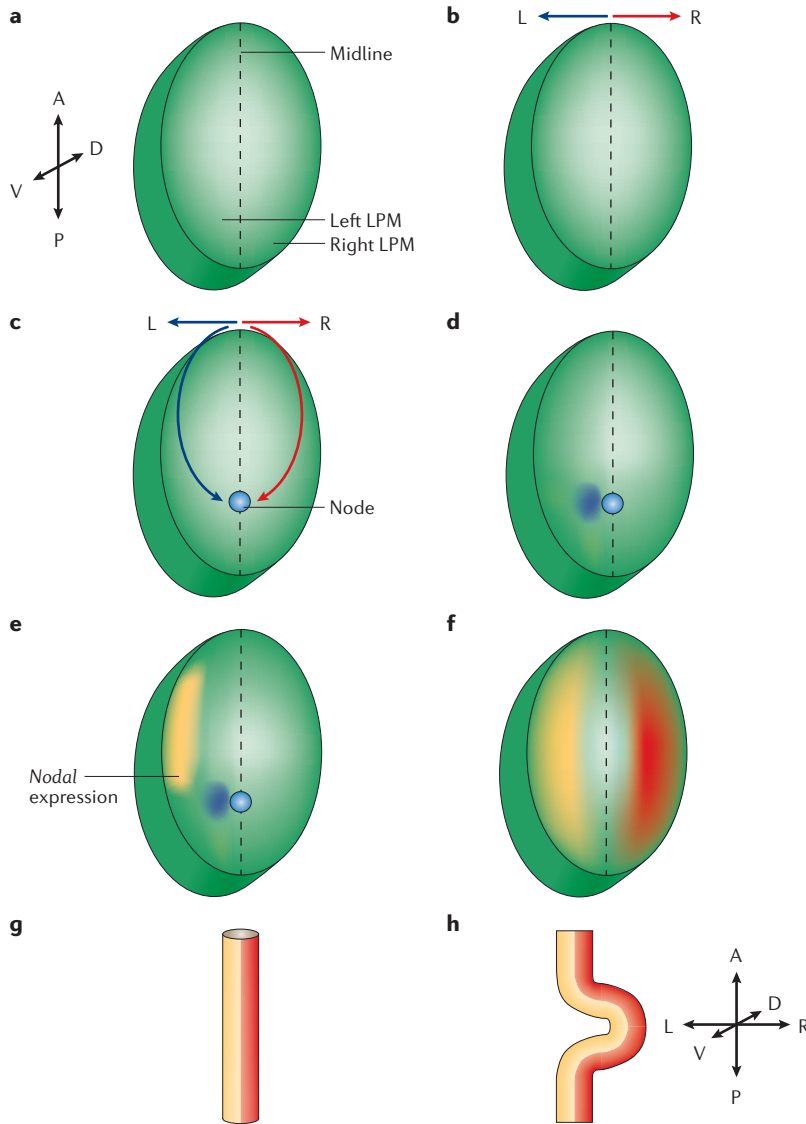


Figure 1 | An overview of left–right axis determination in a generalized vertebrate embryo. Early in embryogenesis, an embryo that is already patterned along the anterior–posterior and dorsal–ventral axes is bilaterally symmetrical (a). A symmetry-breaking step generates initial left–right information (b), although the nature of this event is unknown. The initial left–right information is then transferred to the embryo node (shown as a blue circle)^{91,101–103} (c). The node generates a directional output in the form of a discrete perinodal domain of *Nodal* expression and/or lateralized hedgehog signalling (d), which results in local left–right asymmetries (shown as dark-blue shading). These local asymmetries around the node are conveyed to the left lateral plate mesoderm (LPM) in the form of side-specific *Nodal* expression (e). Broad domains of left- and right-side specific genes (yellow and red, respectively) are then established (f), transferring laterality information to the organ primordia (a structure that represents a single primordium is shown in g), which, in turn, execute left–right asymmetrical morphogenetic programmes (illustrated as the directional looping of the organ primordium in h).

Early laterality information

With the notable exception of the mouse, LR asymmetrical features or signs of laterality information have been identified in vertebrate embryos at developmental stages that predate node formation. In chicks, ground-breaking work from Tabin and colleagues in 1995 identified a regulatory cascade that ultimately locates the expression of transcripts that encode the transforming growth factor- β (TGFB)-like signal NODAL at the left LPM¹⁶. Further studies in chicks refined and completed a molecular cascade that links the activation of an activin-related signal on the right side of Hensen’s node to the local repression of sonic hedgehog (*SHH*) expression. This restricts *SHH* expression to the left side of Hensen’s node, activating *NODAL* expression on the left side of the embryo^{6,17} (FIG. 2). Importantly, most components of this cascade show striking side-specific expression patterns in the early chick embryo that have not been found in other species. Therefore, although the left-sided expression of *NODAL* on the LPM is common to all vertebrates analysed so far (and some invertebrates), as discussed below, its regulation seems to depend on diverse mechanisms in different species.

Ion fluxes during early LR development. The earliest sign of LR asymmetry found so far in the chick embryo is a difference in membrane voltage potential: the left side of the primitive streak and node is more depolarized than the right side. This difference occurs at Hamburger Hamilton (HH) stages 3 to 4 (staging of chick embryo development is according to REF. 18), before asymmetrical *SHH* expression around Hensen's node (HH4⁺) and left-sided expression of *NODAL* in the LPM (HH8) (REF. 19).

The origin of this asymmetry has been attributed to an LR difference in the activity of the H⁺/K⁺-ATPase ion pump¹⁹, pharmacological inhibition of which results in randomization of LR organ placement in chick embryos. Importantly, similar results have recently been obtained in other vertebrates, such as *X. laevis*¹⁹ and zebrafish²⁰, as well as in the distantly related invertebrate taxa Urochordata (*Ciona intestinalis*) (S. Shimeld, personal communication) and Echinodermata (sea urchin) (REF. 21; A. Nishino, personal communication). Ion fluxes that are mediated by H⁺/K⁺-ATPase activity might therefore be an evolutionarily conserved feature in the early steps of LR asymmetry determination among the deuterostomes. However, the mechanisms by which this activity becomes lateralized seem to differ between species. In *X. laevis*, the localization of maternal transcripts that encode the H⁺/K⁺-ATPase α -subunit is asymmetrical with respect to the LR axis as early as the two-cell stage¹⁹. However, no such asymmetries have been found in chick¹⁹ or zebrafish²⁰ embryos, which indicates that the lateralization of H⁺/K⁺-ATPase activity might occur at the translational or post-translational level in these species.

The discovery that H⁺/K⁺-ATPase activity is required early in LR asymmetry determination provided further mechanistic support for a model that involves gap junction communications (GJC), which was previously proposed to explain how initial LR identity is transferred to multicellular fields²². An interesting possibility is that GJC pathways allow the side-specific accumulation of small charged molecules, which could be driven by the electrogenic gradient that is generated by LR differences in H⁺/K⁺-ATPase activity¹⁹. Understanding the role of such a mechanism in the early phases of LR asymmetry determination will require the identification of the molecules that are transferred through the GJC; possible candidates include serotonin²³, inositol polyphosphates²⁴ and Ca²⁺ ions²⁵. Interestingly, extracellular Ca²⁺ levels (BOX 1) in the chick embryo show an LR asymmetrical localization across Hensen's node from stages HH4 to HH6, which depends on H⁺/K⁺-ATPase activity²⁵.

Early requirement of Notch activity. Before the appearance of broad left-sided expression of *Nodal* in the LPM, transcripts of this gene are detected in a discrete domain around the node in all vertebrate species that have been studied (BOX 2). *Nodal* expression around the node and in the LPM is controlled by different mechanisms and is regulated by different enhancers^{26,27}. Experiments in the mouse and chick have uncovered the requirement of this perinodal expression domain for the subsequent

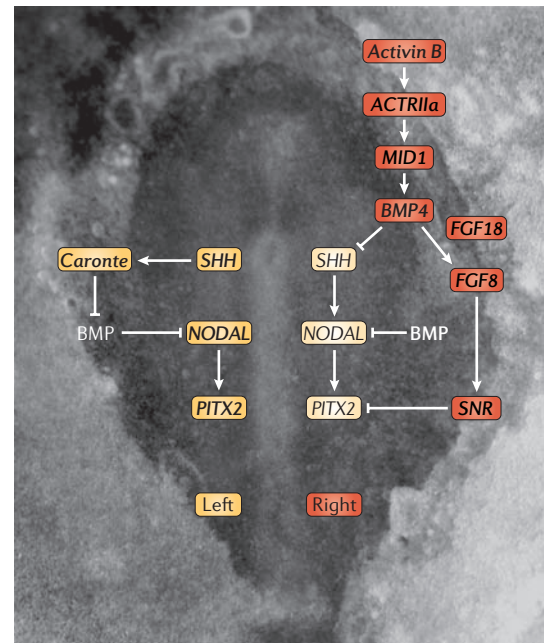


Figure 2 | Early left–right asymmetrical genetic cascades in the chick embryo. A cascade of side-specific transcripts that has been characterized in the chick is shown, and is overlaid on the dorsal view of a Hamburger Hamilton stage 5 chick embryo (anterior to the top). Sonic hedgehog (*SHH*) is expressed on both sides of Hensen's node until the chick embryo is at Hamburger Hamilton stage 4, when its expression is repressed by bone morphogenetic protein 4 (*BMP4*) on the right side, therefore becoming restricted to the left side. The right-sided expression of *BMP4*, in turn, depends on a cascade that is initiated by activin-like activity. Additionally, *BMP4* triggers a downstream cascade of right-sided genes, which also contribute to blocking the expression of left-side determinants (lower expression indicated by paler background and lighter font). By contrast, the persistence of *SHH* expression on the left side of Hensen's node results in the expression of *NODAL* and its downstream target *PITX2* in the left lateral plate mesoderm. The position of the different gene transcripts in the diagram indicates epistatic relationships, rather than specific domains of expression. Right-sided transcripts are depicted in red, whereas genes that are expressed on the left side are indicated in yellow. Note that the expression of the genes depicted in the diagram may extend well before and after the specific stage shown. *ACTR11a*, activin receptor IIa; *MID1*, midline 1; *SNR*; snail related.

establishment of left-sided *Nodal* expression in the LPM, and for proper LR patterning^{25,28–31}.

The Notch signalling pathway has been recently found to direct the perinodal expression of *Nodal*. In the mouse, the establishment of this domain depends on a node-specific enhancer, which lies within a 0.35-kb fragment ~9 kb upstream of *Nodal*^{26–28}. This element contains two functional binding sites for the CSL (CBF1/RBPSUH in vertebrates, Su(H) in *Drosophila melanogaster*, Lag-1 in *Caenorhabditis elegans*) DNA-binding protein, which is the transcriptional mediator of Notch signalling^{30,31}. Furthermore, mouse embryos that are mutant for

Lateral plate mesoderm
The most lateral region of mesoderm in the neurula-stage vertebrate embryo. Among other structures, it gives rise to the heart, blood vessels, blood cells of the circulatory system, the lining of the body cavities, and all the mesodermal components of the limbs other than muscle.

Primitive streak
A transitory embryonic structure, which is present as a strip of cells, that pre-figures the anterior–posterior axis of the embryo. During gastrulation, embryonic cells progress through the streak.

Deuterostomes
A taxon of animals that belong to the Bilateria. They are characterized by having a 'second mouth' (giving them their name) — that is, during embryo development, the blastopore becomes the anus, whereas the mouth forms in a secondary anterior location. Deuterostomes are divided into two major clades: Ambulacraria (which includes echinoderms and hemichordates) and Chordata (which includes vertebrates, urochordates and cephalochordates).

components of the Notch pathway, such as delta-like 1 (*Dll1*) (REFS 30,31), recombining binding protein suppressor of hairless (*Rbpsuh*)³¹, or both *Notch1* and *Notch2* (REF. 30), show LR patterning defects that are consistent with a loss of directional LR asymmetries. Importantly, *Nodal* is not expressed in the perinodal region or the LPM in these mutant embryos^{28,29}. Gain-of-function and loss-of-function analyses of zebrafish^{20,31} and chick²⁵ embryos indicate that the role of Notch signalling in establishing LR asymmetry is likely to be conserved in those species.

A combination of mathematical modelling and experimental approaches has been used to investigate how interactions among components of the Notch signalling pathway might result in the amplification of laterality information in the form of *Nodal* expression²⁵. One model indicates that *Nodal* expression could be induced earlier and more strongly on one side of the node owing to small LR differences in the binding rates of Notch and its ligands DLL1 and serrate 1. Experimental evidence for this prediction was provided by the observation that binding rates of Notch–DLL1 and Notch–serrate 1 complexes depend on the extracellular concentration of Ca²⁺, increased levels of which are transiently but consistently observable in the left side of Hensen's node in the chick²⁵.

A cascade of early laterality information? With the possible exception of the early side-specific cascades of gene expression that have been uncovered in chicks, which seem to be specific to this species (although see below for new evidence relating to this), all the other forms of early laterality information described above are important for LR patterning in two or more model vertebrates. However, no mechanism has been found to be conserved in all of the main vertebrate models, nor has a requirement for all of these mechanisms been shown (or analysed) in any one model (BOX 3). This is particularly

conspicuous in the case of the mouse embryo, for which no requirements before node function have been found (or analysed), with the possible exception of Notch signalling^{30,31}. There are therefore two important questions that future studies will need to address: first, what is the degree of evolutionary conservation of these mechanisms in different vertebrates? Second, could there be a cascade of early laterality information that involves several mechanisms acting sequentially and, if so, what is the hierarchy of steps?

In support of the existence of a cascade of laterality information, the normal asymmetry in extracellular Ca²⁺ levels across the node, and therefore the LR asymmetrical activation of Notch signalling, has been shown to depend on H⁺/K⁺-ATPase activity²⁵. However, studies in the zebrafish challenge the idea of a linear cascade. In this system, H⁺/K⁺-ATPase activity is required for LR patterning earlier than Notch signalling, the function of which, in turn, is required earlier than that of cilia in Kupffer's vesicle. Importantly, however, perturbation of either H⁺/K⁺-ATPase activity or Notch signalling does not significantly impair the formation of cilia or the establishment of the leftward fluid flow in Kupffer's vesicle²⁰. It therefore seems that early laterality information proceeds along parallel pathways that converge at the level of the node (FIG. 3).

The role of the node

The nodal flow hypothesis. Evidence from various experimental approaches and model organisms demonstrates a crucial role of the embryo node or its derivatives for LR asymmetrical patterning in all vertebrate species that have been analysed (reviewed in REFS 1,2,4–6). As discussed above, the role of the node in the establishment of LR asymmetries has been best studied in the mouse embryo, in which it has been proposed to be instrumental for the initial symmetry-breaking event. In the mouse, the ventral side of the node is lined

Box 1 | Calcium and left–right patterning

Left–right (LR) asymmetries in Ca²⁺ levels across the embryo node have been described in the mouse^{45,55}, chick²⁵ and zebrafish²⁴ embryo. In all three species, the left-sided accumulation of Ca²⁺ is transient, precedes the normal left-sided expression of *Nodal* in the lateral plate mesoderm (LPM), and correlates with proper LR patterning. These similarities indicate a conserved biological phenomenon. However, it is important to note that, although the experiments in mouse and zebrafish embryos detected the concentration of free cytoplasmic Ca²⁺ (intracellular Ca²⁺), those in the chick measured extracellular Ca²⁺ levels. It is therefore unclear whether the two phenomena are directly related.

One possibility is that they are unrelated, and that the asymmetries in intracellular Ca²⁺ levels in mouse and zebrafish are established by separate mechanisms to those that establish asymmetries in extracellular Ca²⁺ levels in the chick. The two might also have different downstream effects. Another possibility is that the technical approaches used in the different experiments actually detected asymmetries in Ca²⁺ levels in the same compartments. That is, either that the Ca²⁺ indicators used in the mouse and zebrafish studies detected extracellular instead of intracellular Ca²⁺, or that the indicators used in the chick experiments measured intracellular rather than extracellular Ca²⁺ levels. This possibility is highly unlikely, as stringent controls were carried out in these experiments.

An alternative possibility, which we favour, is that both phenomena (LR asymmetries in extracellular and intracellular Ca²⁺ levels) do take place, but that one of them might be a consequence of the other. There are many examples of increases in extracellular Ca²⁺ levels in response to intracellular Ca²⁺ transients, as well as increments in intracellular Ca²⁺ levels that are a consequence of elevations in extracellular Ca²⁺ concentration (reviewed in REF. 88). If this theory is correct, it will be important to determine the compartment in which the primary change in Ca²⁺ levels occur. The zebrafish embryo could be especially helpful in this respect, as both the nodal flow mechanism (responsible for the asymmetries in mouse intracellular Ca²⁺ levels^{45,55}) and the requirement of H⁺/K⁺-ATPase activity (which is upstream of LR asymmetries in extracellular Ca²⁺ levels in the chick²⁵) have been shown to be important for LR patterning in this system^{20,50,51}.

Box 2 | The perinodal expression of *Nodal*

The appearance of an early domain of *Nodal* expression around the node (or its derivatives), which precedes its left-sided expression on the lateral plate mesoderm, is a conserved feature of all vertebrate species that have been analysed so far, including the chick¹⁶, mouse⁶⁴, *Xenopus laevis*⁶⁶, zebrafish⁶⁸ and rabbit⁷⁰. However, whether this domain of *Nodal* expression is left–right asymmetrical in itself varies greatly between species. Although the perinodal domain of *Nodal* expression in the chick is overtly asymmetrical (left-sided) from the time of its establishment, such asymmetries are subtle in the case of the mouse, and are not detectable in zebrafish or *X. laevis*. Furthermore, the slight left-sided bias of *Nodal* expression around the mouse node is transient and is not necessary for the establishment of the normal left-sided domain of *Nodal* expression in the lateral plate mesoderm⁸⁹.

So, in the case of the mouse, it seems that it is the presence of a perinodal expression of *Nodal*, irrespective of whether this domain is asymmetrical or not, that is crucial for the left-sided transfer of *Nodal* expression to the lateral plate mesoderm^{28,29}. Consistent with this view, the establishment of perinodal *Nodal* expression, but not its asymmetry, has been shown to depend on Notch signalling^{30,31}. Therefore, the significance of the left–right asymmetry of *Nodal* expression around the mouse node is currently unclear. The situation in the case of the chick seems to be more complex, as the activity of the Notch signalling pathway controls both the presence of the perinodal domain of *Nodal* expression and its left–right asymmetry²⁵. However, an evolutionarily conserved role of the Notch pathway (at least in the species that have been analysed so far — the mouse^{30,31}, chick²⁵ and zebrafish²⁰) is the induction of a domain of *Nodal* expression around the node or its derivatives. With the evidence at hand, it is tempting to speculate that the left–right asymmetry of this domain is not important in itself for the specification of left–right organ asymmetries; rather, it might reflect the existence and/or amount of laterality information that converges on the node.

with monociliated cells. In a series of ground-breaking reports, it was shown that these monocilia rotate in a fixed direction (clockwise, when viewed from the ventral side), that the rotation of the cilia is associated with a net leftward flow of extracellular fluid that takes place in the nodal pit, and that both cilia rotation and fluid flow are required for normal LR patterning^{32–34}. In support of this, ten mouse mutations that result in impaired cilia formation and/or function also lead to abnormal LR organ placement^{32–43}. Moreover, LR patterning can be altered in cultured mouse embryos by manipulating the intensity and/or direction of exogenously applied flow⁴⁴. These observations led to the ‘nodal flow’ hypothesis, which proposes that the initial symmetry of the embryo is broken at the level of the node by the left-sided accumulation of a morphogen that is caused by the directional nodal flow.

A role for mechanosensing? The nodal flow hypothesis has been met with several criticisms^{8,15}. Among these was uncertainty about the existence of a morphogen that accumulates on one side of the node in response to the nodal flow. In the light of this criticism, an alternative ‘two-cilia’ model⁸ was put forward that proposes a mechanosensing, rather than a chemosensing, information read-out mechanism. Here the nodal flow would result in increased fluid pressure in the left side of the node, which would be sensed by immotile mechanosensing monocilia, therefore initiating an asymmetrical, Ca²⁺-mediated signal transduction event. Experimental evidence for the existence of these two types of nodal monocilium came from the identification of a population of centrally located, motile cilia that contain left–right dynein (LRD) and polycystin 2 (PKD2; a cation channel), and a population of immotile cilia in the periphery of the node that contain PKD2 but are devoid of LRD⁴⁵. Furthermore, accumulation of intracellular Ca²⁺ was shown to arise in the endoderm at the left margin of the node (BOX 1). Importantly, this left-sided accumulation was perturbed in embryos that were mutant for either *Lrd* or *Pkd2*, which show altered LR patterning⁴⁵.

However, the validity of the mechanosensory model has recently been questioned. Cartwright *et al.*⁴⁶ modelled fluid dynamics in the Hensen’s node of mice and found that that, even in the presence of directional flow, the magnitudes of the shear stresses and flow velocities generated would be symmetrical across the node, and therefore unable to asymmetrically bend monocilia⁴⁶.

Modelling nodal flow. Another criticism to the nodal flow hypothesis was that a limited understanding of fluid biophysics made it difficult to understand how the rotation of the nodal cilia could result in a net leftward flow. Importantly, recent mathematical modelling studies have shed light on this issue. One such study predicted that a net leftward fluid flow would ensue if cilia were angled with respect to the anterior–posterior axis towards the posterior, between 5° to 25° from the vertical⁴⁶. Experimental support for this theoretical prediction has been provided recently^{47,48}. Detailed analyses of the trajectory of the rotating cilia tip⁴⁸ and of scanning electron microscopy images of immotile cilia⁴⁷ revealed a posterior tilting of the cilia axis of 40° ± 10° and 15° to 35° from the vertical, respectively.

Intriguingly, these studies also uncovered a specific rotation behaviour that was not predicted by the model of Cartwright and colleagues. Posteriorly orientated cilia experience a change in their angular velocity while rotating clockwise, and the leftward phase of the rotation cycle (effective stroke) takes place far away from the cell surface and is much faster than the rightward phase (recovery stroke), in which the cilia movement is slowed down by the proximity to the cell surface^{47,48}. The inclusion of this biphasic ‘beating’ behaviour in mathematical models results in a much more robust leftward flow than that predicted by models that only consider a simple rotational movement of cilia⁴⁹. Taken together, these results provide a mechanistically solid model, which is testable at both experimental and theoretical levels, as to how the leftward flow of extracellular fluid is generated in the ventral side of Hensen’s node by the rotational movement of monocilia.

Box 3 | Early mechanisms involved in left–right visceral patterning in vertebrates

Various early requirements for correct left–right (LR) asymmetrical patterning have been identified in different vertebrate species. Currently, none of these mechanisms has been found to be conserved in all the main vertebrate models: *Xenopus laevis*, chicks, zebrafish and mice. Similarly, the requirement for all of these mechanisms has not been shown (or analysed) in any one model.

- Gap junction communications (particularly communication involving the connexin 43 subunit (GJA1)) have been suggested to have an early role in LR patterning in *X. laevis*⁹⁰ and chick⁹¹ embryos. *Gja1* has been mutated in the mouse⁹² and zebrafish⁹³, resulting in cardiac malformations that are probably unrelated to alterations in LR patterning. However, it is possible that other connexin subunits have redundant roles or substitute for GJA1 function during LR patterning in these species.
- H⁺/K⁺-ATPase activity has been shown to be required for proper LR visceral patterning in *X. laevis*¹⁹, chick¹⁹ and zebrafish²⁰ embryos. To our knowledge, the requirement of H⁺/K⁺-ATPase activity for LR patterning in the mouse has not been specifically addressed.
- Notch activity is required for the correct LR asymmetrical development of mouse^{30,31}, chick²⁵ and zebrafish²⁰ embryos. Whether Notch activity has a role in LR patterning in *X. laevis* remains to be investigated.
- Nodal cilia are motile and create a directional fluid flow that is required for LR patterning in mouse^{32,33} and zebrafish^{20,50,51} embryos. Motile nodal cilia have also been identified in rabbit and medaka fish embryos⁴⁸, but whether the establishment of the nodal flow in these species is necessary for proper LR patterning remains to be tested. In this respect, it is worth noting that the nodal flow in the rabbit cannot be detected before the two-somite stage⁴⁸, whereas an earlier role of fibroblast growth factor 8 (FGF8) in LR patterning has been identified in this species⁷⁰. The presence of nodal cilia in *X. laevis* and chick embryos has been reported¹⁴, but whether these cilia are motile or have a role in LR patterning is currently unclear.

Evolutionary conservation of the nodal flow mechanism.

The fact that the generation of the nodal flow seemed only to apply to the mouse embryo raised a further criticism of the nodal flow hypothesis. Although cells in the ventral-node equivalents of chick, zebrafish and frog embryos are also ciliated and express *Lrd*¹⁴, until recently, evidence for evolutionary conservation of the nodal flow mechanism was purely circumstantial.

However, recent studies have changed this. In medaka and zebrafish embryos, Kupffer's vesicle is lined with monociliated cells, and the clockwise rotation of the cilia has been shown to generate a net leftward fluid flow^{20,48,50,51}. In zebrafish, downregulation of *Lrd* function results in a drastic reduction or absence of fluid flow inside Kupffer's vesicle, and also leads to the disruption of normal LR asymmetries — clear evidence that the role of the nodal flow is evolutionarily conserved^{20,50,51}. In fact, these studies in zebrafish strengthen the theory that the nodal flow has an important role in LR patterning in vertebrates. One of the earliest studies that pointed to the importance of nodal flow came from analyses of mouse *inversum viscerum* (*iv*) mutant embryos, in which a deletion of the gene that encodes LRD is associated with immotile nodal cilia, absence of nodal flow and randomized LR asymmetries³³. However, the analysis of these embryos does not allow a clear distinction between ciliary and non-ciliary roles of LRD¹⁵. The feasibility of downregulating specific gene products preferentially in Kupffer's vesicle, by injecting morpholinos into mid-blastula zebrafish embryos⁵², has strengthened the idea that *Lrd* function in Kupffer's vesicle is important for the establishment of LR asymmetries^{20,51}.

The study of rabbit embryos has also been important in this respect, as their architecture resembles that of human embryos much more closely than that of the mouse. In contrast to the situation in mouse, cells in

the ventral side of Hensen's node in the rabbit are devoid of cilia. Instead, monociliated cells are present in the posterior notochordal plate of rabbit embryos, and a net leftward fluid flow can be detected in the posterior groove of this structure, just anterior to Hensen's node⁴⁸. Based on these observations, and on the fact that *NODAL* is expressed in the edges of the posterior notochordal plate, Okada *et al.*⁴⁸ proposed that the groove of this structure could fulfil the role of the mouse nodal pit. These results indicate that a directional nodal fluid flow occurs in vertebrate embryos other than the mouse. Importantly, the generation of nodal flow in embryos from different species does not seem to be constrained by the particular node architecture, cilia length or speed of cilia rotation, which vary between species; rather, it depends on the evolutionarily conserved clockwise rotation of posteriorly tilted cilia⁴⁸. However, whether the establishment of the nodal flow in rabbit embryos is necessary for proper LR patterning remains to be explored.

The identity of morphogen X. Arguably the weakest part of the nodal flow hypothesis, if the mechanosensing theory is incorrect, is the need for a morphogen or morphogens of unknown identity. Several molecules have been suggested as candidates, including SHH³³, fibroblast growth factor 8 (FGF8) (REF. 53), growth differentiation factor 1 (GDF1) (REF. 54) and NODAL^{4,28,29}, but direct evidence has been lacking until recently.

One important advance has been the identification of a novel form of morphogen transport that is based on nodal vesicular parcels (NVPs; REF. 55). NVPs are large (0.3–5 µm in diameter), membrane-sheathed structures that contain multiple lipophilic granules. They are released from nodal cells into the nodal fluid, transported leftward by the nodal flow, and are eventually fragmented when they come into contact with rotating monocilia. The release of NVPs seems to occur when they are

Morpholinos

Morpholino-modified antisense oligonucleotides (generally known as 'morpholinos') are reagents that are widely used to knockdown gene function in zebrafish by pairing to complementary sequences in gene transcripts and blocking their translation or splicing.

Posterior notochordal plate

The posterior part of the notochordal plate that lies adjacent to the node. It is a flattened, grooved plate, which originates as a result of the fusion and subsequent disappearance of the floor of the notochordal process with the underlying endoderm. The notochordal plate eventually folds inwards to give rise to the notochord.

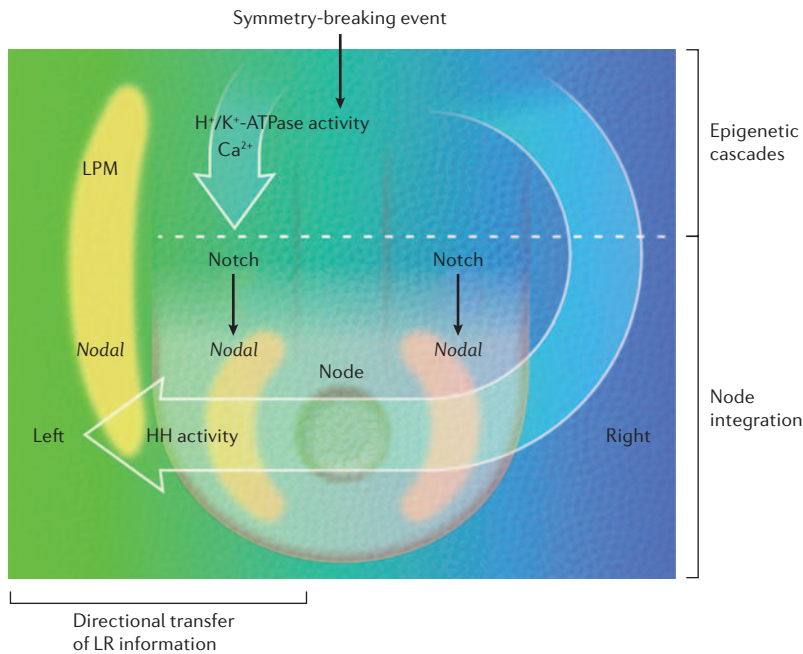


Figure 3 | Cascades of early laterality information. A putative hierarchy of steps in the early phases of left–right (LR) asymmetry determination in vertebrate embryos is overlaid on a background that represents a dorsal view of the posterior part of a generalized embryo, anterior to the top. An early, unknown symmetry-breaking event generates laterality information in *Xenopus laevis*, chick, zebrafish, and perhaps also in the mouse, which is transferred to the node by means of epigenetic cascades. Known epigenetic mechanisms that form part of such cascades include the LR differences in membrane voltage potential that are generated by differences in H^+/K^+ -ATPase activity¹⁹ and the downstream asymmetry in extracellular Ca^{2+} levels²⁵. Other epigenetic mechanisms are likely to exist, but their identity and the nature of their relationships with known epigenetic and genetic steps remain to be uncovered. Epigenetic information converges at the embryo node through parallel pathways. Laterality cues are translated by the Notch signalling pathway, generating a perinodal domain of *Nodal* expression^{30,31}, which is required for the establishment of *Nodal* expression in the left lateral plate mesoderm (LPM)^{28,29}. Lateralization of hedgehog (HH) signalling is achieved in the chick by restricting *SHH* expression to the right side of the node¹⁶, a process that depends on H^+/K^+ -ATPase activity¹⁹. In the mouse, lateralization of HH signalling is probably achieved by the leftward transfer of nodal vesicular parcels (and their associated SHH cargo) by the nodal flow⁵⁵. A probable outcome of the lateralization of HH signalling in both species is to provide a left-sided bias to the transfer of the perinodal expression domain of *Nodal* to the LPM.

Midline barrier

A physical and/or molecular barrier that separates the right and left halves of the vertebrate embryo so that the action of long-range side-specific signals does not affect the other side. Physical elements of the barrier are exemplified by the midline derivatives of the node: the notochord and the floorplate. The best-understood molecular component of the midline barrier is the divergent TGF β signal LEFTY1.

loaded with cargo above a threshold level and seems to be regulated by FGF signalling. Among the cargo that is carried by NVPs are SHH and retinoic acid (RA)⁵⁵, which is consistent with earlier evidence that signalling pathways activated by these molecules synergize during the establishment of LR asymmetries⁵⁶. Moreover, inhibition of FGF signalling, which blocks NVP production, results in a loss of the left-sided accumulation of intracellular Ca^{2+} , and both effects can be rescued by either SHH or RA⁵⁵. Intriguingly, although Indian hedgehog (IHH) can rescue Ca^{2+} accumulation after inhibition of FGF signalling, this rescue is bilateral and cannot rescue NVP production⁵⁵. These results could account, at least in part, for the more severe LR phenotype of *Shh/Ihh* compound mutants when compared with *Shh* mutants⁵⁷. However, it is likely that further roles of IHH in LR patterning remain to be discovered.

The existence of the NVP transport mechanism does not rule out other more traditional forms of morphogen gradient formation in the node. Proteins in the range of 20–40 kDa are distributed LR asymmetrically by the nodal flow of both mouse and rabbit embryos⁴⁸, which suggests a scheme that is much more complex than the long-standing ‘morphogen X’ view: instead, various molecules with diverse physicochemical properties could be distributed LR asymmetrically by different mechanisms with specific dynamics (FIG. 4). The final outcome of the nodal flow could be a pleiotropy of effects, resulting from the combined actions of several different morphogens.

A conserved role for HH signalling? The evidence described above also suggests a novel early role of hedgehog (HH) signalling in the specification of mouse LR asymmetries, in addition to its well-established later function in the formation of the midline barrier^{53,56,58}. The nature of this early role of SHH will require further investigation, but the similarity between this mechanism and the restriction of *SHH* expression to the left side of Hensen’s node in the chick¹⁶ is intriguing. If a leftward accumulation of SHH that occurs as a result of nodal flow biases the transfer of *NODAL* expression to the left LPM, this would be consistent with the absence of *Nodal* expression that is seen in the LPM of mouse embryos in which HH signalling is profoundly downregulated (smoothed homologue (*Smo*) mutants or *Shh Ihh* double mutants; REF. 57). Findings in *X. laevis*, zebrafish, chicks and mice have suggested an evolutionary divergence in the function of SHH during LR patterning^{53,59,60}, but these can be reinterpreted in light of this early role of SHH. Overexpression of *Shh* or banded hedgehog (*Bhh*) in the right side of chick and *X. laevis* embryos, respectively, induces bilateral expression of *Nodal* in the LPM^{16,61}, and misexpression of *shh* in the right side of zebrafish embryos results in defects in heart and viscera asymmetries⁶². These findings cannot be easily accounted for by a role of HH proteins in the formation of the midline barrier, but are consistent with an earlier role of HH activity in the transfer of *Nodal* expression to the LPM.

The findings described above indicate that HH signalling becomes lateralized around the embryo node — by transcriptional regulation in the chick embryo, or by means of the nodal flow in the mouse — therefore determining the transfer of LR information, in the form of *Nodal* expression, towards the left LPM. However, despite the intellectually satisfying evolutionary conservation that is implicit in this proposal, aspects at least need further clarification. First, it is unclear why a lateralized distribution of SHH in the mouse node⁵⁵ is not reflected in some LR asymmetry in the expression of Patched homologue 1 (*Ptch1*) (REF. 57), which is the SHH receptor and downstream target of HH signalling, the expression of which is widely used as a proxy for HH signalling⁶³. Second, the analysis of HH signalling in the context of ciliary function and LR patterning might be further complicated by the fact that cilia seem to be intrinsic components of the HH signalling machinery (BOX 4).

Somitogenesis

The process of metameric segmentation of chordate embryos. In this process, paired blocks of paraxial mesoderm (somites) are specified and segmented following a stereotypical species-specific sequence. In vertebrates, somites form in a bilaterally symmetrical fashion and give rise to bilaterally symmetrical structures, such as the skeletal muscles, the axial skeleton and parts of the dermis.

Rudiment

A structure that is present in the larvae of sea urchins that gives rise to most of the adult tissues. The process of rudiment specification is left–right asymmetrical, originating from the left coelomic pouch and its adjacent lateral ectoderm.

Downstream of the node

The transient LR asymmetries that are established around the node during gastrulation are transferred to and stabilized in the LPM by the early stages of somitogenesis. The hallmark of this phase of LR patterning is the expression of NODAL transcripts along the left LPM, which is conserved in all vertebrate embryos that have been analysed so far, including chick¹⁶, mouse^{64,65}, *X. laevis*⁶⁶, zebrafish^{67,68}, quail⁶⁹ and rabbit⁷⁰ embryos.

The establishment and maintenance of broad domains of *Nodal* expression in the left LPM depends on complex interactions among positive and negative regulators of TGF β signalling, which have been studied in detail in the past 5 years^{47,72}. In summary, *Nodal* expression in the left LPM is positively regulated by other TGF β signals, such as GDF1 and NODAL itself, and depends on the presence of members of the EGF–CFC (epidermal growth factor–cripto/FRL-1/cryptic) family of NODAL co-receptors. By virtue of these autocatalytic loops, *Nodal* expression rapidly extends towards both the anterior and posterior to occupy most of the left LPM. Conversely, divergent ligands of the TGF β family such as left–right determination factors 1 and 2 (**LEFTY1** and **LEFTY2**), the expression of which is positively regulated by NODAL, antagonize NODAL function and expression, therefore creating a negative-feedback loop that restricts the time and extent of *Nodal* expression in the LPM. The combination of both positive and negative regulatory loops of *Nodal* expression results in a marked amplification of the LR asymmetries that are established around the node

— so that vast fields of cells are informed as to their left identity — at the expense of generating a signal that is intrinsically short-lived.

The expression of transcripts that encode the paired-like homeodomain transcription factor **PITX2** has been proposed to mediate the stabilization of the left identity of LPM cells and their derivatives^{1–5}. Similar to *Nodal*, the expression of *Pitx2* is limited to the left LPM, but it is maintained until much later in development and is still evident in the primordia of organs that will eventually be asymmetrical, such as the heart and gut^{73–77}. Based on expression patterns in the LPM, it is generally accepted that the left-sided expression of *Pitx2* is initiated by NODAL. Further support for this idea comes from the correlation of *Nodal* and *Pitx2* expression in a range of experimental conditions^{2,4}, as well as from the identification of *Pitx2* as a direct target of NODAL⁷⁸. However, it should be noted that *Pitx2* expression has been shown to occur in the absence of NODAL function in at least three instances — mutation of the gene that encodes the ion channel PKD2 in the mouse⁷⁹, mouse embryos that are deficient in the serine protease **FURIN**⁸⁰ and downregulation of Notch activity^{25,30,31} — indicating that requirements for *Pitx2* expression other than NODAL remain to be uncovered.

The left-sided expression of the *Nodal* and *Pitx2* signalling module is highly conserved among vertebrate embryos. Importantly, the origin of these asymmetries predates the vertebrate lineage, as they are also found in invertebrate chordates (reviewed in REFS. 1,81,82). LR asymmetrical *Nodal* and *Pitx2* expression is found in all chordate taxa, including vertebrates, cephalochordates (lancelets) and urochordates (or tunicates, including ascidians). Moreover, recent evidence indicates that the involvement of NODAL and PITX2 in the regulation of LR patterning is also conserved in non-chordate deuterostomes such as echinoderms (sea urchins)²¹. A hallmark of LR asymmetries in the sea urchin embryo is the formation of the adult rudiment on the left side of the body (REF. 83 and references therein). Duboc *et al.*²¹ found that the choice between formation of this structure on the left versus the right side depends on the activity of a conserved NODAL–PITX2 signalling module. Intriguingly, both *Nodal* and *Pitx2* are expressed on the right side of the sea urchin embryo, as opposed to the left side which is typical of chordates. It is currently unclear whether this divergence could be accounted for by the postulated inversion of the dorsal–ventral axis during bilaterian evolution, or simply reflects different conventions for how the body axes are labelled among taxa.

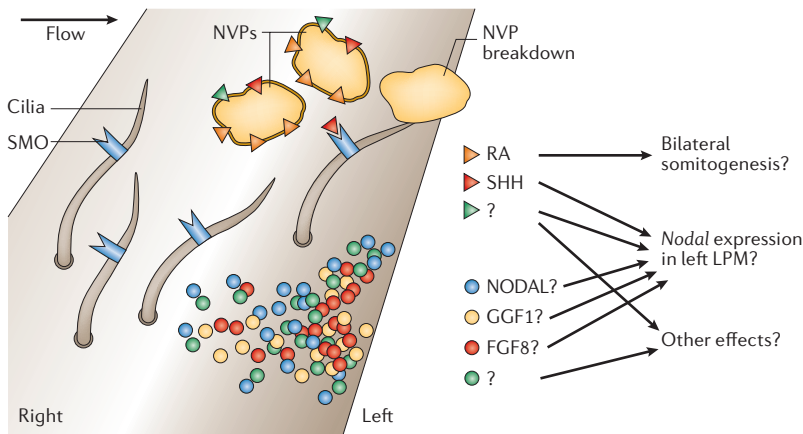


Figure 4 | Possible pleiotropic effects of the nodal flow. Different mechanisms might coexist for transporting signalling molecules in response to the nodal flow, allowing the left-sided accumulation of various molecular species. Nodal vesicular parcels (NVPs) transfer sonic hedgehog (SHH), retinoic acid (RA), and possibly other lipophilic molecules (green triangle) towards the left side of the node, where they are released by cilia-mediated NVP breakdown⁵⁵. Cilia might also participate in receiving hedgehog (HH) signalling^{40,41,43}, which is indicated by the localization of smoothened (SMO) to primary cilia⁹⁸. In parallel, left-sided accumulation of proteins of 20–40 kDa (dots of various colours in the figure) might be achieved by the nodal flow⁴⁸. Most secreted factors proposed to serve as nodal flow morphogens in the light of genetic evidence — such as NODAL, growth differentiation factor 1 (GDF1) or fibroblast growth factor 8 (FGF8) — lie within this molecular weight range. The left-sided accumulation of a range of ‘morphogens’ might result in a pleiotropy of downstream effects. Some possibilities are listed on the right part of the figure.

Higher levels of integration

The transfer of laterality information from the node to the LPM requires the establishment of transient LR asymmetrical domains of activity of signalling pathways that have key roles in processes other than that of LR patterning, such as the Notch, FGF and Wnt pathways. In particular, these signalling pathways also control the generation of somites, a process that takes place in the vicinity of the node⁸⁴. Paradoxically,

Box 4 | The hedgehog–cilia connection

Additional elements of complexity have been recently added to the roles of hedgehog (HH) signalling and cilia function in the context of mouse left–right (LR) visceral patterning. HH signalling is not only essential for patterning the floorplate^{94,95} (therefore contributing to establishing a midline barrier that prevents spreading of left-sided signals to the right side of the embryo^{53,56,96,97}), but is also likely to be lateralized by the cilia-mediated directional transfer of sonic hedgehog (SHH)-containing ‘nodal vesicular parcels’ (NVPs⁵⁵). The nodal cilia, in turn, not only create the nodal flow^{32,33} and contribute to sensing it (by a mechanosensing mechanism^{8,45} and/or by breaking NVPs and releasing their contents⁵⁵), but also seem to have an active role in the detection of HH signalling.

Support for this latter role of cilia comes from the analysis of mice that are mutant for genes that encode intraflagellar transport proteins, in which the absence of cilia is associated with phenotypic defects that are characteristic of HH deficiency^{40,41,43}. Recent analyses by Corbit *et al.*⁹⁸ have discovered that the localization of smoothed homologue (the cellular transducer of HH signals^{99,100}) to primary cilia is required for HH signalling in mammalian cells in culture and zebrafish embryos, lending further support to the idea that cilia function as HH signalling sensors. These findings necessitate the re-evaluation of the LR phenotypes that have been described in mouse mutants that lack nodal monocilia. For example, the previously noted differences between mouse mutants with immotile versus absent nodal monocilia, which prompted the proposal of the ‘two-cilia’ model⁸, could be alternatively explained in the light of a further role of cilia in sensing HH signalling.

somites are formed bilaterally in a highly symmetrical fashion. Recent evidence from our laboratory and others uncovered the existence of a mechanism that is triggered by RA signalling that shields somites from LR asymmetrical influences^{20,85,86}. Downregulation of RA signalling in mouse^{85,86}, chick⁸⁶ or zebrafish²⁰ embryos results in the appearance of uneven numbers of somites on each side of the embryo. Importantly, in all cases, the LR asymmetry in somitogenesis was found to be strongly biased towards one side. By contrast, manipulation of the LR information cascade at the level of H⁺/K⁺-ATPase activity, Notch signalling or cilia function in the zebrafish²⁰, or at the level of cilia function in the mouse⁸⁶, resulted in randomized LR asymmetries in somite formation. These findings indicate that LR asymmetrical patterning and bilaterally symmetrical elongation of the anterior–posterior axis are actively coordinated at the level of the node. Importantly, somitogenesis in the invertebrate chordate amphioxus (lancelet) is markedly LR asymmetrical, with the left somites forming faster than the right ones (see REF. 87 and references therein), which raises the possibility that the active coordination of LR and anterior–posterior axes could be an innovation of vertebrates.

The exact mechanism by which RA signalling buffers LR asymmetrical cues remains unclear. Moreover, it is also unknown how RA signalling becomes lateralized in response to the LR information cascade, although analyses of the expression patterns of RA-metabolizing enzymes and receptors²⁰ indicate that this lateralization is likely to take place at a post-transcriptional level. In this respect, the mechanism of NVP transfer recently uncovered by Tanaka *et al.*⁵⁵ emerges as an attractive possibility. One of the cargo components of NVPs is RA, the distribution of which is efficiently lateralized within the node through directional transport and fragmentation NVPs⁵⁵. It will be interesting to determine whether the bilaterally symmetrical formation of somites can be experimentally separated from normal LR asymmetrical patterning by manipulating the levels of RA locally in NVPs. The presence of both SHH and RA in NVPs

makes it tempting to speculate that the same mechanism that generates or amplifies LR asymmetrical information in the node is responsible for buffering the unwanted influence of this LR asymmetrical information during somitogenesis.

Conclusions

More than 10 years have elapsed since the seminal work that identified a genetic cascade displaying LR asymmetrical expression in the chick embryo¹⁶. Our understanding of how vertebrate LR patterning is determined and governed has since increased in an extraordinary fashion. Furthermore, the investigation of LR patterning has highlighted new biological models, providing unique opportunities to explore the mechanisms that translate epigenetic information into stable domains of gene expression. Working hypotheses such as that of nodal flow^{32,33} have been approached both theoretically^{46,49} and experimentally^{47,48} to a high level of detail, and provide a testable framework for the conversion of molecular chirality into LR asymmetry. Mechanisms for the generation of electrochemical gradients¹⁹, sensing of fluid flow by cilia⁴⁵, molecular sensing of extracellular ion levels²⁵, or morphogen transport through NVPs⁵⁵ might well have roles in other biological processes.

One important unresolved issue in the field of LR patterning is the extent to which disparities in the mechanisms of asymmetry determination in different species reflect divergent ways to specify the LR axis, or rather represent distinctive details within a design that shows overall evolutionary conservation. The fact that the final outcome of the process (stereotypical LR asymmetries in organ morphology and placement) is consistently shared between different species, as are some early and intermediate steps (the requirement for H⁺/K⁺-ATPase-activity and the left-sided expression of *Nodal–Pitx2*), would seem to argue in favour of a general conservation. But, if this is the case, what are the ‘distinctive details’ that are regulated by the species-specific mechanisms? Do they depend on specific embryo morphologies or on general evolutionary trends? Does the nodal flow represent a

Floorplate

Ventral region of the early neural tube of vertebrate embryos. The medial part of the floorplate is formed by cells that originate in the node or organizer, which induces floorplate-like characteristics in the neural ectoderm-derived cells of the lateral floorplate. The floorplate has important roles during ventral nervous system patterning, including the specification of motor neurons and interneurons, and the differentiation of oligodendrocytes.

symmetry-breaking mechanism, as has been proposed for the mouse^{32,33}, or is it a mechanism that amplifies pre-existing laterality information, as seems to be the case for the zebrafish²⁰? The answer to these questions will probably require the systematic analysis of the role of each

mechanism in species that represent different developmental plans among the vertebrates. The use of alternative species to the mainstream vertebrate model organisms — such as the rabbit^{48,70}, quail⁶⁹ and medaka fish⁴⁸ — promises to provide further insights into these questions.

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Competing interests statement

The authors declare no competing financial interests.

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