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HYPOTHESIS

The evolution and conservation of left-right patterning mechanisms

Martin Blum‡, Kerstin Feistel, Thomas Thumberger* and Axel Schweickert

ABSTRACT

Morphological asymmetry is a common feature of animal body plans, from shell coiling in snails to organ placement in humans. The signaling protein Nodal is key for determining this laterality. Many vertebrates, including humans, use cilia for breaking symmetry during embryonic development: rotating cilia produce a leftward flow of extracellular fluids that induces the asymmetric expression of Nodal. By contrast, Nodal asymmetry can be induced flow-independently in invertebrates. Here, we ask when and why flow evolved. We propose that flow was present at the base of the deuterostomes and that it is required to maintain organ asymmetry in otherwise perfectly bilaterally symmetrical vertebrates.

KEY WORDS: Cilia, Evolution, Left-right asymmetry, Left-right organizer, Leftward flow

Introduction

Symmetry is a guiding principle for the construction of animal body plans. Apart from sponges, which are considered the most basal branch of the animal phylogenetic tree (see Box 1), all other phyla are characterized by one or several planes of symmetry along their longitudinal axis. In radially symmetrical cnidarians, such as the freshwater polyp *Hydra*, multiple planes of symmetry can be drawn. All other major animal phyla belong to the bilateria, which are marked by one plane of symmetry along the head to tail axis, perpendicular to the dorsal-ventral axis. It has been suggested that symmetry is used as a measurement of genetic fitness of a potential mate in sexual selection (Brown et al., 2005). Asymmetry, in that respect, is widely considered a defect. However, asymmetry is also ubiquitously encountered in nature. This ranges from the chirality of biomolecules, to functional asymmetries in symmetrical structures, to the overt morphological asymmetries of organs.

In vertebrates, visceral and abdominal organs are asymmetrically positioned with respect to the two main body axes (Fig. 1). This arrangement, termed situs solitus (see Glossary, Box 2), is rarely altered. Only ∼1/10,000 humans shows a mirror image of the normal organ display (situs inversus; see Glossary, Box 2). Other vertebrate asymmetries, such as left and right handedness, vary with much higher frequencies in human populations and are not covered here. Asymmetric organ morphogenesis and placement is initiated during embryogenesis. In the early vertebrate neurula embryo, three genes – those encoding Nodal, its feedback inhibitor Lefty and the homeobox transcription factor Pitx2 – become asymmetrically expressed in the left lateral plate mesoderm (LPM). This so-called Nodal cascade (see Box 3) is a conserved feature of vertebrate left-right (LR) axis formation. The functional importance of this asymmetric expression has been demonstrated in all classes of vertebrates (Yoshiba and Hamada, 2014). However, the mechanism of symmetry breakage, i.e. how *Nodal*, the first asymmetric gene, is initially induced in the left LPM, remains a matter of much debate.

In recent years, various models have been put forward to explain how symmetry is first broken in early embryos. The flow model (Fig. 2) claims that cilia on the LR organizer (LRO; see Glossary, Box 2) of early developing embryos produce a leftward fluid flow in the extracellular space (Hamada, 2008; Hamada et al., 2002; Hirokawa et al., 2012). This flow occurs a few hours before induction of the Nodal cascade; LROs, which feature a surprising diversity of morphologies, are transient structures that disappear once the Nodal cascade is induced (Blum et al., 2007, 2009b). By contrast, the early determinants/ion-flux model acknowledges that cilia may play a role in symmetry breakage in some species, such as mouse, but proposes that symmetry breakage is initiated much earlier – in the zygote and during early cleavage divisions (reviewed by Levin, 2005; Vandenberg and Levin, 2009, 2010, 2013). In this model, early determinants, in particular ion channels, set up voltage gradients that lead to the asymmetric distribution of small molecules. In particular, the candidate molecule serotonin has been suggested to govern asymmetric Nodal cascade activation in the LPM at a much later stage (Fukumoto et al., 2005; Vandenberg et al., 2013).

Genetic and experimental data clearly support the flow model in fish, amphibians and some mammals (mouse, rabbit, human) (Hirokawa et al., 2012). However, despite considerable efforts in many laboratories over a long period of time, LROs have not been found in the chick embryo and seem to be absent in the pig (Gros et al., 2009), a finding on which the early model capitalizes. In addition, the Nodal cascade is already present in mollusks; through an unknown mechanism, the spiral cleavage pattern observed in these animals places *Nodal* and *Pitx2* asymmetrically in the early larva. This asymmetry governs shell coiling in much the same way as organ placement in vertebrates is under the control of the Nodal cascade (Grande and Patel, 2009; Kuroda et al., 2009; Patel, 2009).

Here, we examine the evolution of asymmetry and of leftward flow. We infer that the Nodal cascade was already present in the last common ancestor of bilateria, the urbilateria (see Glossary, Box 2). We argue that the gastrointestinal (GI) tract was the first functionally asymmetrical organ system, and that the gut tube was asymmetrical in urbilateria. We hypothesize that a flow-based mechanism of symmetry breakage exists in the entire deuterostome lineage (Box 1). We propose that vertebrate evolution depended on maintaining LR organ asymmetries on a background of a now perfectly bilaterally symmetrical axial skeleton. Our hypotheses, which are based on evolutionary reasoning, imply that leftward flow is a synapomorphy (see Glossary, Box 2) of the deuterostomes. Our proposals provide a

Institute of Zoology, University of Hohenheim, 70593 Stuttgart, Germany.
*Present address: Centre for Organismal Studies, Im Neuenheimer Feld 230, University of Heidelberg, 69120 Heidelberg, Germany.

‡Author for correspondence (martin.blum@uni-hohenheim.de)

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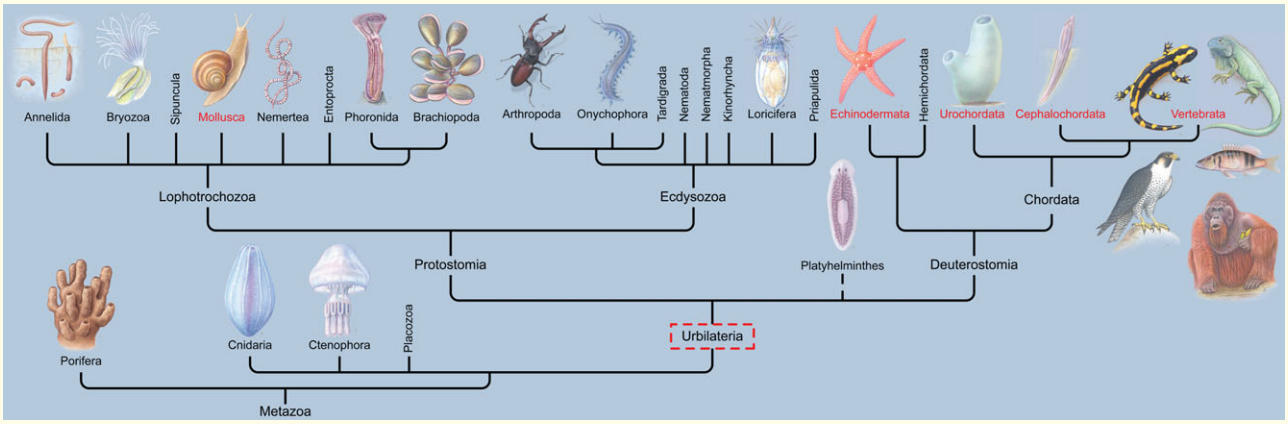
Box 1. Phylogenetic tree of the major animal clades



The lineage relationships between phyla, based on the Tree of Life project (tolweb.org), is outlined. The last common ancestor of protostomes and deuterostomes, a hypothetical bilaterally symmetrical animal called urbilateria, is also included (outlined in red). Phyla in which Nodal cascade genes have been identified are marked in red text. The presence of Nodal cascade genes in both protostomes and deuterostomes suggests that urbilateria also possessed the Nodal cascade (Carroll et al., 2004).

coherent hypothesis on the evolution and conservation of LR patterning mechanisms that is testable and, we hope, will provoke investigations in different model organisms throughout the animal kingdom.

Why did organ asymmetry evolve?

If one considers the functional relevance of organ asymmetry in humans, the heart is certainly the most striking case. It is not only placed asymmetrically in the chest, but also is built asymmetrically such that the left and right atria and ventricles differ with respect to

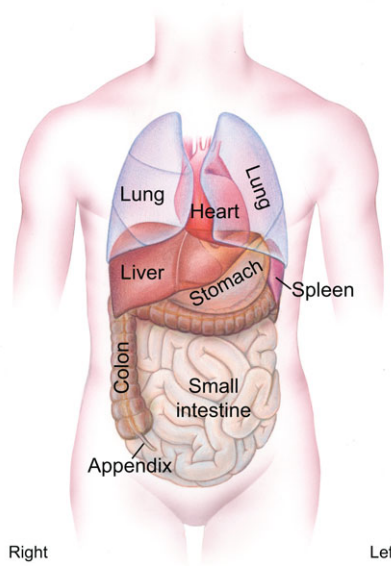


Fig. 1. Asymmetric organs. In humans, asymmetric organs are found in the chest (heart, lung) and abdomen (stomach, spleen, liver, small and large intestine). The apex of the heart, which is placed at the midline, points to the left side. Lungs differ with respect to lobation: two lobes are found on the left and three lobes on the right side. The stomach and spleen are positioned on the left, whereas the liver and appendix are found on the right. In addition, the small intestine and colon coil asymmetrically.

pumping performance and wiring to arteries and veins (Ramsdell, 2005). In evolutionary terms, however, a primitive heart was nothing more than a linear contractile muscle that pumped hemolymph, facilitating the distribution of nutrients throughout the body (Carroll et al., 2004). Such a pump did not require asymmetric morphogenesis or asymmetric placement in the body. There are many examples of such primitive hearts in extant animals, including the cardiac tube in *Drosophila*. The fly heart, which constitutes the entire cardiovascular system, is a simple muscular pump, working in an open circulation. Flies are even viable without their heart, supporting a role for the heart as a facilitator of nutrient circulation (Medioni et al., 2009). Lung asymmetries, too, seem not to be functionally relevant but might reflect space constraints in the thorax resulting from asymmetric heart placement. We therefore hypothesize that the digestive tube, or GI tract, was the first organ system to undergo asymmetric organ morphogenesis during evolution. Invertebrates, such as snails, feature GI tracts that, from mouth to anus, exceed the length of the main body axis (Fig. 3A), a situation encountered in all extant vertebrates. In carnivores, such as cats, this ratio is ~3:1. It increases from 6:1 in omnivores (humans) to ~10:1 in herbivores such as horses, and is particularly high in ruminants [>20:1 (Nickel et al., 1995)].

It is not just length, but also compartmentalization of the GI tract that is seen universally in animals. The mouth, esophagus, stomach and gut each represent modules with distinct functions and physiological specializations. Both length and modularization are functionally relevant for the efficient recovery of nutrients from ingested food. Interestingly, compartmentalization of the digestive system is already present in protozoans such as the ciliate *Paramecium*. Here, the length of the passage of food vacuoles exceeds the longitudinal dimension of the cell, the phagosome-lysosome system is extremely plastic, and acidification occurs only in certain parts of the system (Allen and Fok, 2000; Fok and Allen, 1990). A compartmentalized GI tract that exceeds body length will inevitably need to be packaged asymmetrically. Furthermore, achieving this in a reliable manner, as opposed to stochastic placement within the body cavity, would no doubt be advantageous both with respect to nutrient recovery and for the prevention of

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Box 2. Glossary

Archenteron. The primitive gut tube that forms during gastrulation. Sometimes also termed the gastrocoel (cavity of the gastrula embryo), it transiently harbors the left-right organizer in amphibian and mammalian embryos. The mouse left-right organizer, before its renaming as the node/posterior notochord, was also referred to as the archenteron (Blum et al., 2009a,b; Theiler, 1972). Relationships are less clear in bony fish and birds, in which the archenteron is ill-defined.

Ciliopathies. A collective term that describes a diverse group of human syndromes caused by ciliary dysfunction. These include Bardet-Biedl syndrome, polycystic kidney disease, nephronophthisis, Meckel-Gruber syndrome, Joubert syndrome and Senior-Løken syndrome, all of which are associated with laterality defects (Fliegauf et al., 2007).

Left-right organizer (LRO). A ciliated epithelium that either represents the posterior part of, or is located at the posterior end of, the notochord. LROs come in different shapes and sizes, ranging from flat to indented, dome-shaped to spherical. Cilia on the LRO are polarized and rotate in a clockwise fashion to produce a leftward fluid flow in the extracellular space.

Situs solitus. The stable asymmetric arrangement of organs in the chest (heart, lung) and abdomen (stomach, spleen, liver, colon, intestine).

Situs inversus. An inversion in the asymmetric arrangement of organs. Such inversion is very rare and occurs in ~1/10,000 humans.

Synapomorphy. A synapomorphic trait is a character shared by two or more taxa and in their most recent common ancestor.

Urbilateria. The term urbilateria has been coined by Eddy De Robertis to describe the common ancestor of all bilateral symmetrical animals (De Robertis, 2008; De Robertis and Sasai, 1996). Although, in all likelihood, a fossil urbilaterian will never be found given the complex circumstances of successful fossilization, cladistic (deductive) logics were applied to characterize urbilateria as a light-sensing, motile, worm-like creature with a heart-like pump, a regionalized gut and nervous system (Carroll et al., 2004).

malfunctions. The many problems associated with intestinal malrotation in humans, which has been estimated to occur in as many as 1/500 births, support this reasoning (Burn and Hill, 2009; Stewart et al., 1976; Sutherland and Ware, 2009).

When did organ asymmetry evolve?

The Nodal cascade, which is responsible for asymmetric organ morphogenesis and placement in the vertebrates, is found in deuterostomes and protostomes alike (Chea et al., 2005). This suggests that the last common ancestor was also characterized by the presence of a Nodal cascade. This hypothetical animal at the base of all bilaterally symmetrical species has been dubbed urbilateria (De Robertis and Sasai, 1996) (see Glossary, Box 2). Although no fossil record of such an animal is known to date, nor likely to exist, it has been described as a creature that possessed a heart-like pump, body appendages, a light-sensing primitive eye, and compartmentalization of the nervous system and digestive tract (Carroll et al., 2004). Based on our consideration of the Nodal cascade, we hypothesize that the urbilaterian GI tract was asymmetrically arranged (Fig. 3B).

Among the protostomes, Nodal has so far only been described in mollusks, which belong to the lophotrochozoa (Box 1), which includes snails and slugs. Gain- and loss-of-function experiments in snails have unequivocally shown that Nodal cascade asymmetry is responsible for shell coiling, i.e. asymmetric organ placement (Grande and Patel, 2009). Many species from phyla of the other major protostome group, the ecdysozoa (Box 1), also display marked morphological and functional asymmetries. The nematode *C. elegans*, for example, undergoes LR asymmetric rotation within the eggshell, cleaves asymmetrically, loses sensory rays in an asymmetric manner and shows lateralization of the nervous system (summarized in Burdine and Caspary, 2013). In *Drosophila*, the genital disc and the gut rotate asymmetrically, a process driven by an actin-based mechanism (Petzoldt et al., 2012). However, the genomes of *C. elegans* and *Drosophila melanogaster* have been sequenced without discovery of a *Nodal* homolog, indicating that *Nodal* might have been lost in ecdysozoa. Given the low degree of nucleotide and protein homology between snail and deuterostome *Nodal* (Grande and Patel, 2009), however, one should not render a premature judgment. In addition, both *C. elegans* and *Drosophila melanogaster* represent highly derived species in their respective phyla. Finally, it remains to be seen whether cnidarians have a *Nodal* gene. Certainly, there are no apparent organ asymmetries in *Hydra* (Technau and Steele, 2011). However, asexual reproduction through budding *in Hydra* occurs asymmetrically along the body column and thus creates an asymmetry (Bode, 2011). The molecular mechanisms of asymmetric bud morphogenesis have not been elucidated (Böttger and Hassel, 2012; Meinhardt, 2012), but if *Hydra* has a *Nodal* gene, it would be likely to act in this process.

Flow is error-prone and expensive: why should it evolve?

In snails, asymmetric induction of the Nodal cascade occurs via chiral blastomere arrangement at the 8-cell stage, providing a strong argument in favor of symmetry breakage through early determinants (Kuroda et al., 2009). We envisage a scenario reminiscent of asymmetric cell division in *C. elegans*, whereby cell polarity governs asymmetric cell division (Li, 2013; Munro and Bowerman, 2009; Sawa, 2012). Remnants of spiral cleavage are still present in the

Box 3. The Nodal cascade

The Nodal signaling cascade is active in the left LPM (Hamada et al., 2002). Nodal, a member of the TGFβ growth factor family, induces transcription of three target genes: *Nodal* itself, providing a positive-feedback loop; *Lefty*, which encodes a Nodal inhibitor and acts in a negative-feedback loop; and *Pitx2*, which encodes a homeobox transcription factor. The Nodal cascade spreads rapidly throughout the LPM using a self-enhancement and lateral-inhibition mechanism (Nakamura et al., 2006). Upstream, the cascade is regulated by members of the DAN family of proteins, such as Coco and Cerberus, which act as Nodal antagonists. Coco is a secreted protein that is co-expressed with Nodal in somitic LRO cells and is downregulated as an immediate target of cilia-driven leftward flow. Coco repression liberates Nodal to signal and/or transfer to the left LPM. Although Nodal and Pitx2 are conserved, variations on the theme have been described in vertebrates, mostly in chick, in which a highly divergent set of asymmetrically transcribed genes exists (reviewed by Raya and Izpisúa-Belmonte, 2006): the snail-related transcription factor cSnR is active in the right LPM, whereas Lefty is missing in the left LPM. Instead, caronte, a cerberus/Dan-related growth factor, is active in the left paraxial mesoderm. Curiously, the homeobox transcription factor Nkx3.2 is asymmetrically expressed in the mouse and chick LPM, but on opposite sides [right in mouse versus left in chick (Schneider et al., 1999)]. It remains to be seen whether and how these chick-specific asymmetries relate to the node rotation observed in chick. Targets of the Nodal cascade can also differ. For example, the neuropeptide galanin was shown to be asymmetrically expressed, in a flow-dependent manner, on the left side of the mouse heart anlage. As galanin asymmetry was not detected in *Xenopus* embryonic heart, this species-specific difference might relate to the much higher complexity of the mammalian four-chambered heart (Schweickert et al., 2008). Galanin might have been co-opted to the Nodal cascade through evolution of Pitx2 binding sites in its promoter, an option that has yet to be tackled experimentally.

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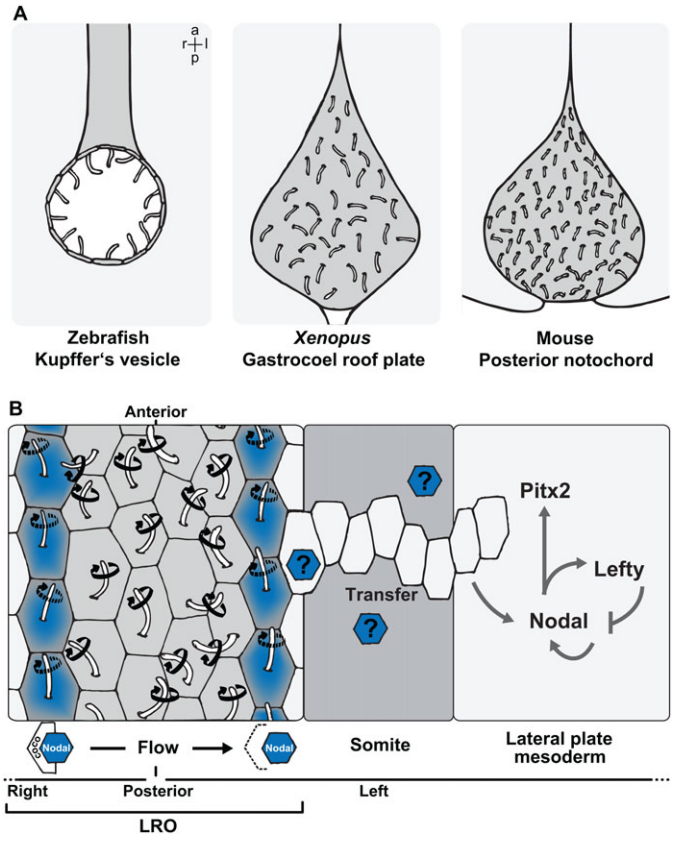

Fig. 2. Left-right organizers and the flow model of symmetry breakage. (A) Left-right organizers (LROs) come in different forms (Blum et al., 2007). In zebrafish, the LRO is known as Kupffer's vesicle and is a closed sphere. In *Xenopus*, the gastrocoel roof plate (GRP) acts as the LRO and is a flat triangular to diamond-shaped epithelium. In mouse, the LRO (the posterior notochord/'node') is an indentation at the distal tip of the egg cylinder. In all cases the LRO is positioned at the posterior pole of the notochord (gray). Axes are indicated: a, anterior; p, posterior; l, left; r, right. (B) Depiction of leftward flow at the ciliated epithelium of an LRO. Motile and polarized cilia (positioned at the posterior pole of cells) rotate in a clockwise fashion to produce a leftward fluid flow in the extracellular space. Flow is sensed by unpolarized cilia on cells bordering the LRO. In mouse and *Xenopus* these cilia have been described as being immotile (Boskovski et al., 2013; McGrath et al., 2003). These cells express both Nodal and the Nodal inhibitor Coco. As a result of flow, Coco becomes downregulated on the left side (Hojo et al., 2007; Nakamura et al., 2012; Schweickert et al., 2010), thereby derepressing and liberating Nodal protein. Also shown is the transfer of an unidentified asymmetric signal (likely to be Nodal protein; blue octagon labeled with question mark) to the left lateral plate mesoderm (LPM), where the Nodal cascade is induced. Nodal transfers across the somites and intermediate mesoderm (not shown) to the LPM, where it induces its own transcription and that of its feedback inhibitor Lefty as well as expression of Pitx2.

vertebrates. In the frog *Xenopus*, for example, it has been shown that the first cleavage division is inherently chiral (Danilchik et al., 2006). If mollusks use spiral cleavage to induce *Nodal* asymmetrically, why, then, should leftward flow evolve? What could be the advantage of having such a complex machinery for the sole reason of inducing *Nodal* mRNA transcription on the left side of the neurula embryo?

It is possible that, if it was more reliable or 'cheaper' than a cell polarity-based mechanism, flow might yield increased fitness. A detailed consideration of leftward flow, however, provides proof of the contrary, and the spontaneous rate of situs inversus in snails is fortunately known. When snails such as the Burgundy snail *Helix pomatia* are raised in France for consumption, corkscrew-like pincers are used to extract the meat from the shell. These tools do not easily fit into shells with inverted torsion, allowing ready identification of these so-called snail-kings. A snail-king is discovered in ∼1/20,000 specimens, closely matching the rate of situs inversus in humans (Brunner, 1999). Therefore, if one assumes that human embryos employ leftward flow for symmetry breakage, which is well justified on the basis of cilia mutants resulting in laterality syndromes (Goetz and Anderson, 2010; Norris and Grimes, 2012; Shiraishi and Ichikawa, 2012), both mechanisms appear equally reliable at first glance.

Vertebrate LR axis specification using flow is, however, exceedingly error-prone. It has been estimated that congenital heart defects occur in up to 1% of live human births (Liu et al., 2013), of which some 3% are considered to arise from defects in the LR pathway (Sutherland and Ware, 2009). Individual ciliopathies (see Glossary, Box 2), a sizable number of which are associated with LR defects (Fliegauf et al., 2007; Gerdes et al., 2009; Norris and Grimes, 2012; Oh and Katsanis, 2012), tend to be relatively rare ($\leq 10^{-4}$). If these syndromes are considered in combination, it has been estimated that ∼1/300 humans is affected by some form of ciliopathy. Other mutations, for example those occurring in Nodal cascade genes, also result in human LR defects (Bisgrove et al., 2003; Sutherland and Ware, 2009). In zebrafish and *Xenopus*, depending on the clutch of eggs, LR defects can be observed in up to 10% of wild-type embryos (Danos and Yost, 1995; Lohr et al., 1997; Long et al., 2003), further supporting our conclusion that LR axis determination via leftward flow is, in fact, error-prone.

To make things worse, flow is expensive. The embryo invests a lot of energy to specify and pattern the gastrula embryo for the transient emergence of an LRO, which has no other function than to break symmetry. Ciliogenesis and cilia polarization within the LRO require an elaborate cooperation of growth and transcription factors and their respective target genes and processes. In addition, once flow has been sensed at the left margin of the LRO, a complicated transfer system has to be set in motion to transport (an) asymmetric cue(s) from the LRO to the left LPM (Fig. 2). Ablation experiments

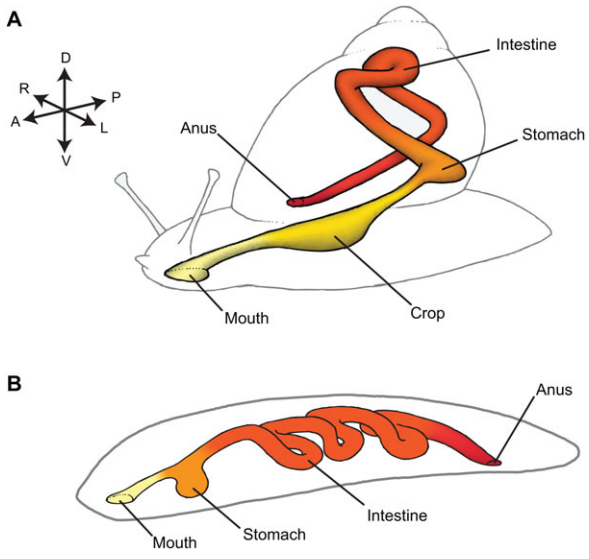

Fig. 3. Organ asymmetry evolved to store a regionalized and long gut tube. (A) The regionalized (as represented by the color gradient) gastrointestinal (GI) tract of a snail. Note that its length exceeds that of the main body axis. A compartmentalized GI tract that exceeds body length will inevitably be packaged asymmetrically. (B) We hypothesize that the urbilaterian GI tract was also regionalized and asymmetrically arranged. D, dorsal; V, ventral; other axes as Fig. 2.

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in the freshwater fish medaka and in *Xenopus* have shown that, despite LR defects, development proceeded normally when Kupffer's vesicle, which is the fish LRO, was manually destroyed, or upon removal of the superficial mesoderm, which is the LRO precursor in the frog (Bajoghli et al., 2007; Blum et al., 2009a). Following Nodal cascade induction, LROs rapidly integrate into the notochord and somites (Brennan et al., 2002; Shook et al., 2004; Yamanaka et al., 2007). Snails certainly use an easier and cheaper way of rendering *Nodal* asymmetric.

In search of a solution: evolutionary considerations

Faced with two apparently very distinct modes of symmetry breakage – one that is flow-based and one that requires early determinants – we now consider more basal chordates, which turns out to be a rewarding exercise.

Insights from echinoderms

To begin this evolutionary reflection, we look at echinoderms, the other major deuterostome phylum aside from the chordata (Box 1). In particular, we focus on LR asymmetry in sea urchins, which has been studied in great depth. Although apparently radially symmetrical, echinoderms belong to the bilateria and develop via a larval stage that is bilaterally symmetrical; radial symmetry of the adult only develops during metamorphosis (McClay, 2011). Sea urchin larvae show asymmetrical expression of Nodal cascade genes, interestingly in the primitive gut (the archenteron; see Glossary, Box 2), although in only a single patch of staining (for a recent review see Molina et al., 2013). From the archenteron, coelomic pouches or sacs bud off in a symmetrical fashion. The coelomic tissue may be considered as a structure homologous to the LPM, as it splits the LPM horizontally in vertebrates. It is important for the future development of the sea urchin; the rudiment of the adult animal, an imaginal disc-like structure (Molina et al., 2013), only develops from the coelom on one side. In this case, it is the side on which *Nodal* is not expressed. Asymmetric *Nodal* expression in the archenteron, however, is responsible for the development of the adult rudiment; when Nodal signaling was inhibited after gastrulation, an ectopic rudiment formed, whereas ectopic Nodal expression prevented rudiment formation (Duboc et al., 2005). These experiments also confirmed that the LR axis becomes fixed only after gastrulation, a conclusion that was derived previously from the culture of LR-bisected embryos (Aihara and Amemiya, 2001; McCain and McClay, 1994).

Nodal cascade asymmetry in sea urchins has been described as right-sided, in contrast to the left-sided cascade in the LPM of vertebrates (Molina et al., 2013). In the absence of a notochord or neural tube, the definition of left and right relies exclusively on the position of the mouth, which is considered to open on the ventral side. In fact, the oral ectoderm of the sea urchin embryo, from which the mouth develops, expresses all of the genes that are typically expressed on the dorsal side of vertebrates, such as *chordin*, *nodal* and *goosecoid* (Li et al., 2013). In addition, gene regulatory networks between sea urchins and vertebrates are apparently inverted with respect to the dorsal-ventral axis (Molina et al., 2013). If one considers the possibility that the mouth of the sea urchin larva might open on the dorsal side (i.e. due to the apparent inversion of the dorsal-ventral axis) then the left and right sides would also flip, and the sea urchin larva would display a left asymmetric Nodal cascade like all other deuterostomes (Blum et al., 2009b). How, then, is this asymmetry set up in the larva?

We speculate that echinoderms possess archenteron cilia that produce a directed fluid flow in much the same way as chordate

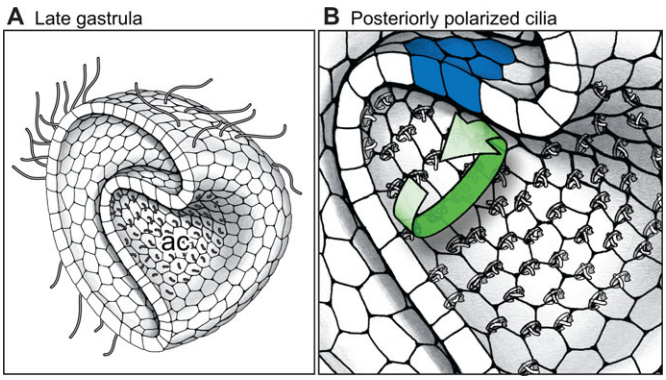

Fig. 4. Cilia in the sea urchin gastrula embryo. Schematic of the dorsal half of a late gastrula sea urchin embryo. We speculate that archenteron (ac) cells are ciliated (A), and that cilia are polarized and produce a leftward fluid flow (B, green arrow). Nodal-expressing cells at the archenteron tip are in blue.

archenteron cilia, and that it is this fluid flow that induces the asymmetric Nodal cascade (Fig. 4). Two published observations support this notion. First, archenteron cilia have been described in gastrula embryos of the feather star *Comanthus japonica* (Holland, 1976), which belongs to another, more basal, class of echinoderms. The second lead is more indirect and relates to the coelomic pouches before the development of the adult rudiment. In larvae of the sea urchin *Temnopleurus hardwickii*, it has been reported that the coelomic sacs undergo a tube-like extension when they separate from the archenteron tip (the future esophagus) and organize into a ciliated epithelium with motile 9+2 cilia (Hara et al., 2003). Remarkably, these cilia produce a directed fluid flow (Ruppert and Balser, 1986). In addition, the tubulin staining pattern observed in these larvae strongly suggests that the archenteron tip cells are also ciliated, at least at the (late) stage when the coelom buds off (Hara et al., 2003). It would be worthwhile investigating whether polarized and motile LR cilia are indeed present earlier, before asymmetric *Nodal* expression, and whether flow, as we predict, induces the asymmetric Nodal cascade.

Interestingly, monocilia are also found covering the epidermis of neurula embryos of ascidians, which are considered a sister group to the vertebrates (Box 1). These cilia show similarities to LR cilia, as they are polarized and ~5 μm in length, and it is the motility of these cilia that is responsible for the chiral, anticlockwise rotation of the embryo (neurula rotation). Interfering with this process alters LR asymmetry, for example asymmetric *Nodal* expression, in the ascidian *Halocynthia roretzi* (Nishide et al., 2012; Thompson et al., 2012). It has been proposed that such epidermal motile cilia, which are commonly used for locomotion and swimming by most non-chordate embryos (such as the sea urchin pluteus larva), might have been re-adapted for symmetry breakage (Nishide et al., 2012). It is therefore tempting to speculate that the gastrocoel/archenteron cilia of vertebrates evolved because superficial cells, from which the archenteron derives and which bear motile cilia, became internalized during gastrulation. As the longitudinal extension of the archenteron corresponds to the anterior-posterior axis, it is not difficult to imagine that cilia became polarized to the posterior pole of cells, using global anterior-posterior cues, which await identification even in the vertebrates. If this were the case, this polarization would inevitably result in a leftward fluid flow. Molecular data support our reasoning: manipulation of Notch signaling or of the ion pump ATP4 affects asymmetric *Nodal* expression in the sea urchin archenteron (Bessodes et al., 2012). A role of Notch in vertebrate LR axis determination has long been

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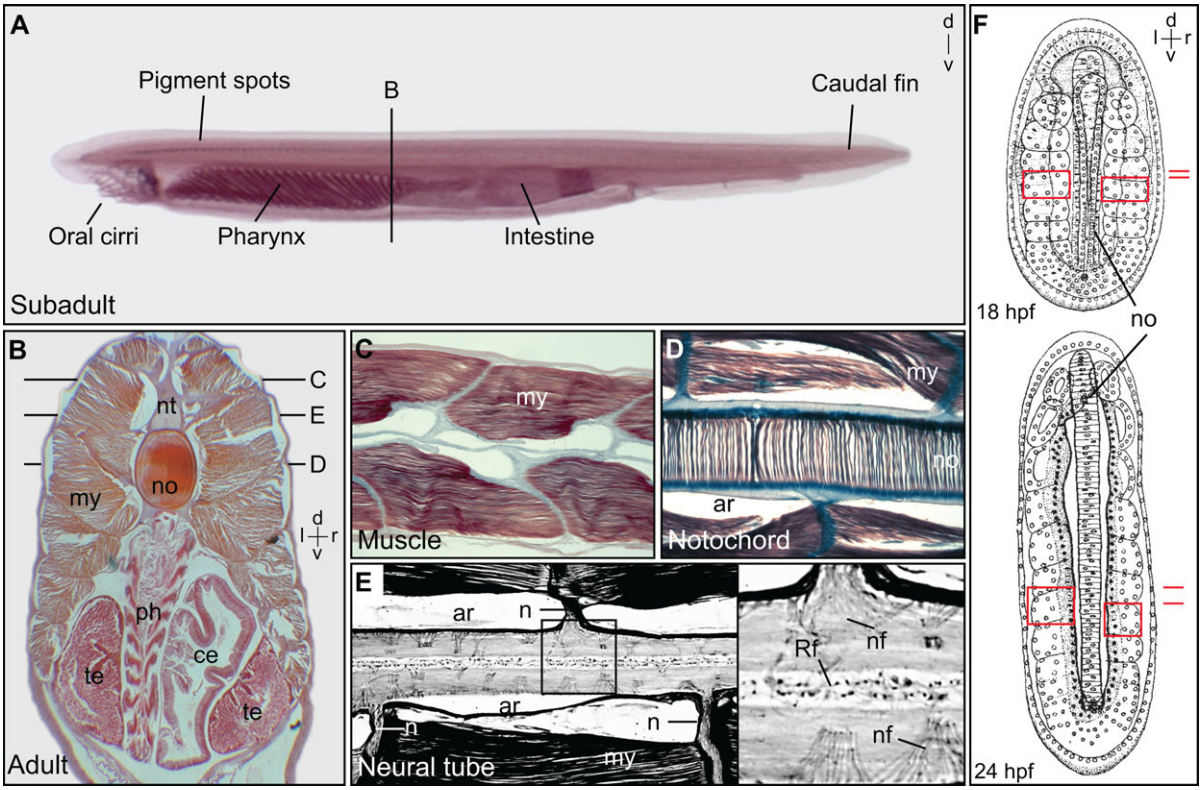

Fig. 5. Asymmetry in amphioxus. (A) Subadult animal (before differentiation of the gonads). Histological transverse (B) and longitudinal (C-E) sections of adult animals. The longitudinal sections were taken at the level of the dorsal muscle, dorsal to the neural tube (nt; C), at the level of the notochord (no; D) and at the level of the neural tube (E). Note the asymmetric body plan as reflected in the placement of the pharynx (ph), cecum (ce) and testes (te), and the alignment of muscles [myomeres (my)], nerve (n) and nerve fibers (nf). ar, fixation artifact; Rf, Reissner's fiber. (F) Reproduction of original drawings from Conklin's 1932 description of embryogenesis in amphioxus [reproduced with permission (Conklin, 1932)]. At 18 h post-fertilization (18 hpf, top), the somites are symmetrically aligned. However, by 24 hpf (bottom), somitogenesis has become out of register, as is obvious from the seventh somite onwards. The fourth (top) and seventh (bottom) somites are boxed in red. d, dorsal; l, left; r, right; v, ventral.

known (Krebs et al., 2003; Przemeck et al., 2003). In addition, it has recently been shown that Notch signaling governs the ratio and distribution of motile and non-motile sensory cilia on the frog LRO (Boskovski et al., 2013). ATP4 plays a dual role in the frog: it is required for the induction of the cilia transcription factor *Foxj1* and for cilia polarization, under the control of non-canonical and canonical Wnt signaling, respectively (Walentek et al., 2012). In conclusion, we speculate that polarized archenteron cilia in sea urchins produce a leftward fluid flow that is responsible for the asymmetric induction of *Nodal*.

Lessons from amphioxus

We now consider amphioxus (also known as lancelets), which belong to the chordate subphylum of the cephalochordates (Box 1). This subphylum is evolutionarily ancient: fossils were reported from the Burgess shale, i.e. they date back ~500-550 million years to the Cambrian (Putnam et al., 2008). Remarkably, the body plan of all of the ~45 extant species, as well as that of fossil amphioxus, is asymmetrical (Fig. 5): the mouth and anus open on the left side, the midgut cecum, where digestion and absorption take place, extends along the right side, and the cone-shaped muscle segments (myomeres) are asymmetrically arranged on the left and right side of the body. Although mostly buried in the sand, where they live as filter-feeders, animals occasionally swim in an undulating manner owing to the asymmetric muscle alignment (Liem et al., 2001).

Because of its position at the base of the chordates, rooting the vertebrates in the phylogenetic tree (Box 1), the embryology of amphioxus has been studied in great detail (Bertrand and Escriva, 2011; Holland et al., 2004). The first asymmetry that was described concerns the alignment of the somites on the left and right sides of the notochord (Schubert et al., 2001). Somites and the notochord represent evolutionary novelties of the chordates, which makes this observation all the more interesting. Remarkably, somites form asymmetrically on both sides of the amphioxus notochord, with the left side being slightly advanced compared with its counterpart on the right. It has been a long-standing debate as to when this asymmetry first appeared during development. Cerfontaine claimed asymmetries from the first pair of somites onward (Cerfontaine, 1906), whereas Hatschek and Conklin agreed that the first 7-8 somites develop in a symmetrical manner (Hatschek, 1893; Conklin, 1932) (Fig. 5F). However, Conklin noted that the viewing perspective plays a role in judging these asymmetries, which is why he was "not inclined to place much weight upon this observation" (Conklin, 1932). More recently, molecular studies using the homeobox gene *Mox* to mark the forming somites clearly showed asymmetry from the fifth somite onwards, without resolving the dispute surrounding the first pairs (Minguillón and Garcia-Fernandez, 2002).

How do somites become asymmetrical during early somitogenesis? Perhaps not too surprisingly, the Nodal cascade in its entirety is conserved in amphioxus. *Nodal*, *Lefty* and *Pitx2* genes have been cloned and their expression patterns described during early development (Boorman and Shimeld, 2002; Yu et al., 2002, 2007). In the late gastrula/early neurula amphioxus embryo, *Nodal* is found in two patches in the roof of the archenteron (Fig. 6) (Yu et al., 2002).

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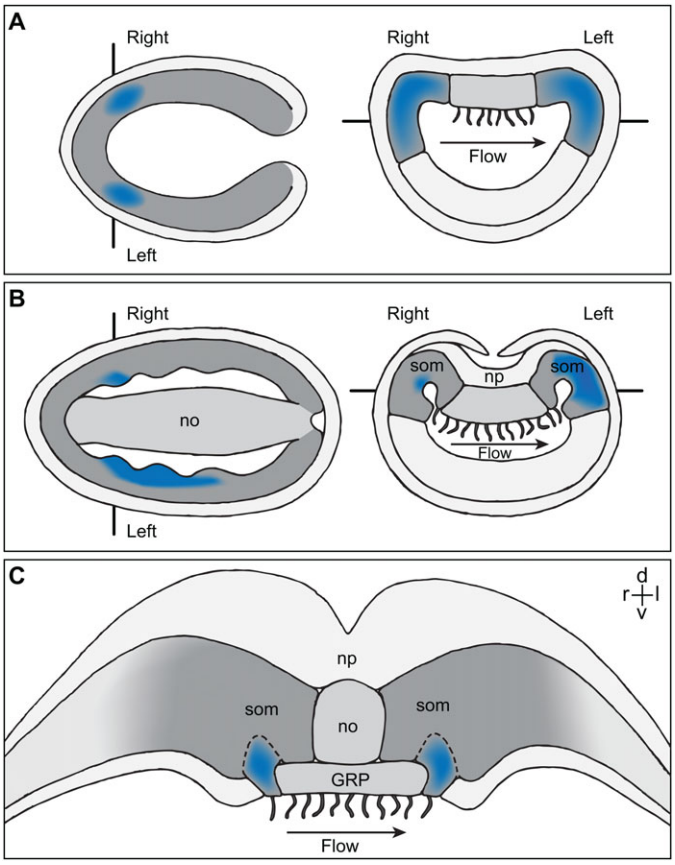

Fig. 6. Homology between amphioxus and *Xenopus* gastrocoel roof plates. Schematics of the gastrocoel roof plates (GRPs) of amphioxus (A,B) and *Xenopus* (C). Dorsal views of late gastrula (A) and 11.5 h neurula (B) embryos are shown (left) together with transverse sections (right) at the levels indicated. *Nodal* mRNA expression (blue) in amphioxus becomes asymmetric during early neurulation. Cells that are Nodal positive at late gastrula bud off from the archenteron roof to form the somites. At later stages, the epithelium in between the Nodal-positive cells likewise buds off to become the notochord (not indicated). Drawn according to Yu et al. (Yu et al., 2002). Cilia and flow at the notochordal part of the archenteron are a hypothetical prediction of the authors. (C) Schematic transverse section through a stage 17 *Xenopus* neurula embryo. Drawn according to Schweickert et al. (Schweickert et al., 2007). Note the striking homology between amphioxus and *Xenopus* GRPs: in both cases, lateral cells expressing Nodal are fated to become somites while central cells fold off to form (amphioxus) or integrate into (frog) the notochord. no, notochord; np, neural plate; som, somite.

Remarkably, this expression of *Nodal* marks the pre-somitic mesoderm, as these cells in the archenteron roof bud off to give rise to the somites shortly thereafter (Bertrand and Escriva, 2011; Holland et al., 2004; Yu et al., 2002). Concomitant with the budding off of these cells, *Nodal* expression becomes asymmetrical, with much stronger signals on the left than right side (Yu et al., 2002). Interestingly, the cells in between these pre-somitic *Nodal*-positive cells also bud off to give rise to the notochord (Fig. 6) (Yu et al., 2002). If one compares this arrangement in the archenteron roof of amphioxus with that in the gastrocoel roof plate (GRP; i.e. the LRO) of *Xenopus*, striking similarities become apparent (Fig. 6). The fate of the frog GRP cells is identical to that of the equivalent cells of amphioxus: *Nodal*-positive lateral cells integrate into the somites, while central cells fold off to become part of the notochord (Fig. 6C) (Shook et al., 2004). In addition, it is these very cells of notochordal fate that in the frog are ciliated and produce a leftward fluid flow, which is sensed by the somitic lateral *Nodal*-positive cells (Boskovski et al., 2013; Schweickert et al., 2007). In one of the extant cephalochordates, *Branchiostoma belcheri tsingtauense*, ciliated archenteron cells have indeed been described (Hirakow and Kajita, 1991), substantiating the similarities between *Xenopus* and amphioxus.

We therefore propose that the axial cells in the archenteron roof of amphioxus possess polarized LR cilia that produce a fluid flow from right to left (Fig. 6A,B). This reasoning is further supported by the recent description of the expression of a *cerberus* gene in amphioxus (Le Petillon et al., 2013). Amphioxus *cerberus* is homologous to frog *Coco*, mouse cerberus-like 2 (*Dand5*) and zebrafish *charon* (*dand5*), which all encode Nodal inhibitors that become downregulated in a flow-dependent manner on the left side of the LRO (Box 3) (Hojo et al., 2007; Nakamura et al., 2012; Schweickert et al., 2010). As in vertebrates, amphioxus *cerberus* is initially expressed in a bilaterally symmetrical fashion, co-expressed with *Nodal*. During early somitogenesis, it becomes downregulated on the left, i.e. appears asymmetrically expressed on the right (Le Petillon et al., 2013). In vertebrates this asymmetry is the result of cilia-driven leftward flow (Schweickert et al., 2010), lending strong support to the hypothesis that leftward flow was already present in the cephalochordates at the base of the vertebrate tree. It will be rewarding to investigate cilia in amphioxus. The prediction based on vertebrate LR cilia would be to find motile cilia that are ~5 µm long, polarized to the posterior pole and perhaps exhibit a mix of different axoneme types, such as the 9+0, 9+2 and 9+4 configurations described in both rabbit and mouse (Caspary et al., 2007; Feistel and Blum, 2006).

One striking difference between asymmetric *Nodal* expression in sea urchin and amphioxus embryos is the split *Nodal* domain in the latter (Fig. 6A,B). As these domains contain descendants of the organizer, which expresses *Nodal* in a single domain in the early gastrula (Yu et al., 2002), this split merely reflects the fate of the organizer: axial *Nodal*-negative notochordal cells and lateral (paraxial) *Nodal*-positive somitic cells. In sea urchins, the hypothesized leftward flow would displace the entire *Nodal* domain to the left. In amphioxus, flow across the epithelium of the notochordal plate would render the *Nodal* domain asymmetrical, using all of the players of vertebrate LROs, but without the need for further transfer into the LPM, as the entire mesoderm derives from the archenteron roof (notochordal plate and somites; Fig. 6B) (Yu et al., 2002).

LR patterning during vertebrate evolution

Our line of argument predicts leftward flow as a synapomorphy of the deuterostomes. Its presence throughout the vertebrates is thus no surprise, although its apparent loss in some species awaits explanation. Remarkably, cilia and flow are not found in chick embryos; here, asymmetric cell migration renders Hensen's node (the organizer) and, in due course *Nodal* expression, asymmetrical (Gros et al., 2009). The emu, as a representative of a more primitive bird, also has an asymmetric Hensen's node (Nagai et al., 2011). It is therefore conceivable that all birds may lack LR cilia and leftward flow. The analysis of more basal reptiles will thus be a worthwhile enterprise. Node asymmetry and a lack of notochordal cilia are not restricted to birds: the pig embryo resembles the chick blastodisc in many aspects, including node and *Nodal* asymmetry (Gros et al., 2009).

Before we suggest a solution for the riddle that chick and pig apparently lack cilia and flow, we wish first to discuss a more fundamental problem for the evolution of vertebrates. It is difficult to imagine that the body plan of the cephalochordates, in particular somite asymmetry, is compatible with the development of a perfectly bilateral axial skeleton. The morphogenesis of vertebrae requires somites to develop in register, and the same holds true for

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muscles that connect to the vertebral column and allow for synchronous locomotion. As mentioned above, cephalochordates swim in an oscillating manner (Liem et al., 2001) due to asymmetric myomeres that are connected to the notochord, i.e. the functional equivalent of the vertebral column in amphioxus. Vertebrates thus have two options: (1) to abandon flow and invent a new way to induce *Nodal* asymmetrically in the left LPM; (2) to shield somites from the influence of flow and transfer the asymmetric signal [Nodal protein, in all likelihood (Yoshiba and Hamada, 2014)] across the somitic tissue without leaving an imprint.

We favor the second option, particularly because a shielding mechanism that acts in both mouse (a flow species) and chick (a no-flow species) has been described. When embryos were depleted of retinoic acid, either genetically (mouse) or through drug treatment (chick), somite formation became out of register, with a delay of somitogenesis on the right side, precisely as is observed in amphioxus (Vermot and Pourquié, 2005; Vermot et al., 2005). Another observation fits with this reasoning: in mouse, it has been shown that retinoic acid-containing vesicles arise at the apical surface of the LRO and that vesicles transfer to the left side of the LRO with flow (Tanaka et al., 2005). The evolution and the mechanism of such shielding, which we predict was not present in cephalochordates, await elucidation.

Finally, it should be noted that vertebrates have minimized the problem of shielding the somites from leftward flow, as most of the mesoderm does not bud off from the archenteron as it does in amphioxus. The LRO cells, however, still stick out. They produce and sense the flow, create the asymmetric signal and send the signal to the LPM. In addition, where these cells have been studied it has been shown that they are of mesodermal fate and integrate into the notochord [frog and mouse (Brennan et al., 2002; Shook et al., 2004; Yamanaka et al., 2007)] as well as into somites [frog and fish (Long et al., 2003; Shook et al., 2004)]. Histology and scanning electron microscopy of frog neurula embryos clearly showed that the sensory lateral GRP cells of somitic fate are already a part of the somite and that just their apical surface sticks out, and only until flow has been received (Schweickert et al., 2007, 2010). In the zebrafish LRO, the *Nodal* gene *southpaw* is co-expressed with the presomitic marker *spadetail* (Long et al., 2003), suggesting that a somitic fate of flow-sensing cells is conserved from amphioxus to at least fish and amphibians. We believe that it is these cells that require shielding from flow.

A role for early determinants in vertebrate symmetry breaking?

The scenario outlined above is of course hypothetical, as cilia-driven leftward flow has not yet been described in echinoderms or amphioxus. If flow only evolved in the vertebrates, a shielding mechanism would have had to evolve at the same time. Furthermore, flow should have been present at the base of the vertebrates. In line with this, primitive fish (sturgeon), which gastrulate like amphibians, have a ciliated GRP as in the frog (Blum et al., 2009b; Bolker, 1993). The LRO/Kupffer's vesicle in bony fish is also ciliated. We thus suggest that leftward flow represents the ancestral mode of symmetry breakage in vertebrates.

Species that display flow at an LRO are characterized by the expression of the cilia transcription factor *Foxj1* in the precursor tissue of the LRO, i.e. the primary embryonic organizer or node. Expression of Foxj1 correlates with motile cilia in all cases investigated so far. In zebrafish and *Xenopus*, loss of Foxj1 function deletes motile cilia, whereas ectopic Foxj1 expression gives rise to ectopic motile cilia (Stubbs et al., 2008; Yu et al., 2008). We have previously shown that *Foxj1* mRNA is expressed in the Hensen's node of pig (Gros et al., 2009). The pig, like chick, lacks cilia at the posterior notochord, which otherwise resembles the ciliated LRO of the rabbit (Feistel and Blum, 2006). *Foxj1* expression in the chick embryo has not yet been published; however, the chick Hensen's node does express the dynein heavy chain gene *Dnah11* (previously known as left-right dynein, *lrd*) (Essner et al., 2002), which is induced by *Foxj1* (Stubbs et al., 2008) and is required for the motility of LRO cilia (Supp et al., 1997). Based on these data, we suggest that chick and pig both inherited the molecular *Foxj1/Dnah11* module, which in other vertebrates sets up leftward flow at the LRO. It has been suggested that, in mouse, as few as two cilia are sufficient to produce and sense LRO flow (Shinohara et al., 2012), so it is possible that chick and pig might have a tiny LRO with just a few motile and sensory cilia, which thus far have gone unnoticed. We consider this possibility unlikely, however, because many laboratories (including our own) have looked in vain for chick cilia in the past. Instead, we suggest that chick and pig have lost the functionality of the *Foxj1/Dnah11* module, perhaps through loss of a promoter element in a *Foxj1* target gene or through an epigenetic mechanism.

A precedent for such a loss is set by the limbless snakes, an adaptation of the tetrapod body plan to a new form of locomotion. The python lineage represents a transitional stage that retains a pelvic girdle and rudimentary hindlimbs. During development, a hindlimb bud still forms but it lacks a functional apical ectodermal ridge (AER) and therefore does not elongate. Recombination with a chick AER or simply providing the AER signaling molecule FGF induces leg bud outgrowth, demonstrating that the molecular machinery for limb outgrowth, with the sole exception of the inducing AER signal, is present in the python (Cohn and Tickle, 1999; Graham and McGonnell, 1999). Thus, loss of a structure or process is not necessarily accompanied by the loss of the genetic modules required to set it into motion and, vice versa, the presence of a genetic module without the respective structure or process represents a tell-tale sign of a function now lost during evolution.

If chick and pig lack a functional Foxj1 module and hence do not use cilia, how do they break symmetry and induce the Nodal cascade on the left side? The fact that the ion pump ATP4 acts hierarchically upstream of asymmetric cell migration in the chick has been taken as support for the early determinants/ion-flux model of symmetry breakage, acting before the onset of gastrulation in a cilia/LRO-independent manner (Gros et al., 2009; Vandenberg and Levin, 2013). ATP4 is an important player in the ion-flux model, and asymmetric expression of ATP4 in the 4-cell *Xenopus* embryo has been described. This asymmetry has been proposed to set up a voltage gradient that drives serotonin through gap junctions to the right side of the embryo, where it represses the Nodal cascade to break symmetry early on (for a recent review see Vandenberg and Levin, 2013). However, recent work, mostly in *Xenopus* but also in mouse, has examined the role of the central components of the ion-flux model in LRO-based symmetry breakage. For example, ATP4 was shown to be symmetrically expressed in frog embryos and to control ciliogenesis and cilia polarization (Walentek et al., 2012). Serotonin was also found in a symmetrical fashion and was shown to be required for specification of the LRO precursor in the frog, the so-called superficial mesoderm (Beyer et al., 2012a). Finally, gap junction communication has been implicated in the transfer of asymmetric cue(s) from the LRO to the left LPM in frog and mouse (Beyer et al., 2012b; Saund et al., 2012; Viotti et al., 2012). Together, these recent findings render the ion-flux model of symmetry breakage unlikely.

Symmetry breaking in chick and pig

How then do chick and pig, which have no functional LRO and do not use early determinants, break symmetry? Our last hypothesis, which

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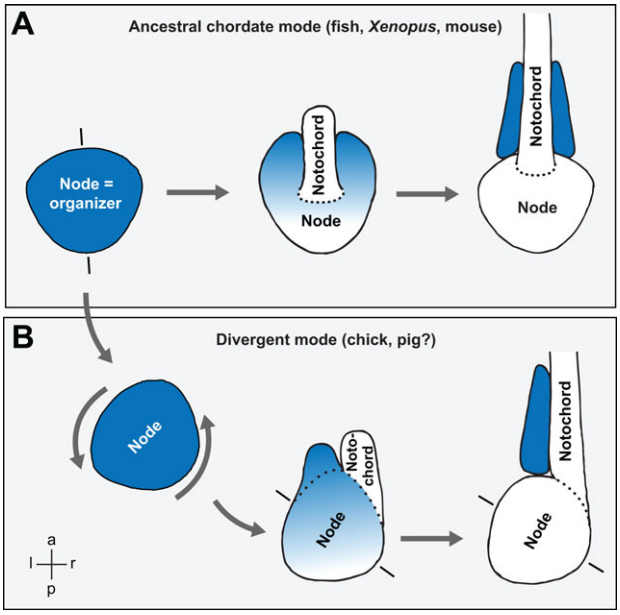

Fig. 7. Divergent modes of symmetry breakage in vertebrates. (A) Ancestral flow-based mode of chordates. The early gastrula organizer (node), which expresses Nodal (blue), differentiates into the notochord and somites during early neurulation. The notochord does not express Nodal and thus splits the Nodal-positive domain down the middle. Nodal-positive cells later integrate into the somites. (B) Divergent mechanism in chick and pig. The organizer (node) rotates during early gastrulation, which renders the node left-asymmetric. The notochord emerges over the right shoulder of the node and does not split the Nodal domain. Nodal thus becomes displaced to the left without the need for flow.

suggests a solution to this riddle, rests on descriptive embryology and on a continuation of the evolutionary considerations outlined above. Let us recall the specifics of *Nodal* asymmetry in echinoderms and cephalochordates: there is a single asymmetric domain in the sea urchin archenteron but a split domain in the amphioxus gastrocoel, which only becomes asymmetric during late gastrulation (Figs 4 and 6). We hypothesize that, at least in the chordates, these domains are direct descendants of the previous *Nodal* domain in the organizer tissue at the onset of gastrulation. In many cases, *Nodal* expression is transiently off during involution of the organizer tissue. A continuity can occasionally be seen in mouse, i.e. expression in the node, which splits in its anteriormost aspect (Blum et al., 2007). The main difference in the chordates compared with echinoderms is the fate of *Nodal*-positive organizer cells, which develop into notochord and somites in chordates (Spemann and Mangold, 1924). Following involution (and specification of the notochord as the axial mesodermal component) the *Nodal* domain splits down the middle, with central cells being *Nodal* negative and lateral cells retaining *Nodal* expression (Fig. 4). In the case of LRO flow, the central, *Nodal*-negative cells bear motile, polarized cilia, whereas the lateral, *Nodal*-positive cells sense flow.

Remarkably, chick and pig embryos do not exhibit a split *Nodal* domain. Rather, the organizer/node *Nodal* domain is displaced to the left side in its entirety, in chick through leftward migration of cells at Hensen's node (Cui et al., 2009; Gros et al., 2009). Recently, Viebahn and colleagues performed histological analyses of chicken nodes at different stages of development, showing that the right shoulder of the node differs from the left shoulder following node rotation (Tsikolia et al., 2012). In particular, they describe that notochordal cells emerge via the thickened right shoulder of the node (Tsikolia et al., 2012). A continuity between the right part of the node and the notochord was already described by Wetzel (Wetzel, 1929), the discoverer of node asymmetry in chick. Based on these observations, we hypothesize that leftward node rotation leaves the organizer *Nodal* domain undivided, because the notochord emerges on the right side of Hensen's node (Fig. 7). Such a mechanism of leftward *Nodal* displacement should be very robust and perhaps less error-prone than LRO flow. In agreement with this notion, no spontaneously occurring alterations of Nodal cascade gene expression in chick and pig embryos have been reported in the literature, in contrast to frog and fish. How could leftward node rotation have evolved? We can suggest no solution for this fascinating question at this time. Unraveling the precise role of ATP4 in the context of node rotation might be particularly rewarding as, to date, this ion pump represents the only shared component between LRO flow and node rotation.

Conclusions

Our examination of LR asymmetry in an evolutionary context has led us to three conclusions covering the base of bilateria, the deuterostome tree and species-specific differences between vertebrates. (1) Based on cladistic logics, we hypothesize that urbilateria used the Nodal cascade for asymmetric morphogenesis of the gut tube. The Nodal cascade itself might have been lost in ecdysozoa, and the elucidation of molecular mechanisms underlying LR asymmetries in these species might lead to the identification of novel homologies between protostomes and deuterostomes, with the potential to sharpen the evolutionary view on the origin of animal LR asymmetry. (2) We speculate that a cilia-driven mechanism of symmetry breakage exists throughout the deuterostomes. We predict in which tissue and at what embryonic stage LR cilia and leftward flow should be present in sea urchin and amphioxus embryos, but descriptive and functional experiments will be required to prove or refute this hypothesis. Not working on these species ourselves, we hope to have provided some leads for future investigations. (3) Finally, we highlight that traits, characters and processes can get lost during evolution if other mechanisms are able to compensate. The absence of leftward flow in chick and pig, and perhaps in all birds and also in other mammals, might represent such a case. Under the control of at least one common determinant, i.e. the ion pump ATP4, a new process has evolved to guide an as yet poorly understood mechanism for breaking symmetry based on asymmetric cell movements. In addition, the leftward displacement of an undivided organizer *Nodal* domain can activate the asymmetric LPM signaling cascade in much the same way as if induced by flow, but this mechanism should be cheaper, more robust and less error-prone. In the future, descriptive and functional work in both established and novel model organisms, combined with evolutionary thinking and reasoning, should hopefully provide a promising path through which we can expand our understanding of the origin and diversification of animal LR asymmetry.

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Competing interests

The authors declare no competing financial interests.

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