Embryonic chirality and the evolution of spiralian left–right asymmetries

José M. Martín-Durán, Bruno C. Vellutini and Andreas Hejnol

Sars International Centre for Marine Molecular Biology, University of Bergen, Thormøhlensgate 55, Bergen, 5006 Norway

Cite this article: Martín-Durán JM, Vellutini BC, Hejnol A. 2016 Embryonic chirality and the evolution of spiralian left–right asymmetries. Phil. Trans. R. Soc. B 371: 20150411. http://dx.doi.org/10.1098/rstb.2015.0411

Accepted: 1 June 2016

One contribution of 17 to a theme issue ‘Provocative questions in left–right asymmetry’.

Subject Areas: developmental biology, evolution, molecular biology

Keywords: Spiralia, Nodal, Pitx, left–right axis, evolution

Author for correspondence: Andreas Hejnol e-mail: andreas.hejnol@uib.no

Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.3515265.

The group Spiralia includes species with one of the most significant cases of left–right asymmetries in animals: the coiling of the shell of gastropod molluscs (snails). In this animal group, an early event of embryonic chirality controlled by cytoskeleton dynamics and the subsequent differential activation of the genes Nodal and Pitx determine the left–right axis of snails, and thus the direction of coiling of the shell. Despite progressive advances in our understanding of left–right axis specification in molluscs, little is known about left–right development in other spiralian taxa. Here, we identify and characterize the expression of Nodal and Pitx orthologues in three different spiralian animals—the brachiopod Novocrania anomala, the annelid Owenia fusiformis and the nemertean Lineus ruber—and demonstrate embryonic chirality in the biradial-cleaving spiralian embryo of the bryozoan Membranipora membranacea. We show asymmetric expression of Nodal and Pitx in the brachiopod and annelid, respectively, and symmetric expression of Pitx in the nemertean. Our findings indicate that early embryonic chirality is widespread and independent of the cleavage programme in the Spiralia. Additionally, our study illuminates the evolution of Nodal and Pitx signalling by demonstrating embryonic asymmetric expression in lineages without obvious adult left–right asymmetries.

This article is part of the themed issue ‘Provocative questions in left–right asymmetry’.

1. Introduction

Bilaterally symmetrical animals exhibit two orthogonal main body axes, namely the anteroposterior and the dorsoventral axes, which establish a plane of symmetry that runs longitudinally along the midline of the animal, and defines the left–right axis of the organism [1]. In many species, the left and right body regions are mirror images of each other, and thus there is an exact correlation between the organs and structures on each side. In other organisms, however, body parts develop asymmetrically along the left–right axis [2,3]. We humans exhibit a common example of this situation, with our heart located on the left side of the body.

One of the most beautiful examples of left–right asymmetries occurs in the direction of coiling of the shell of snails (figure 1a). Snails are molluscs and members of the Spiralia, which is one of the two major clades of the Protostomia [4–7]. The Spiralia comprises a broad diversity of animal forms [8,9], including meiofaunal taxa (e.g. rotifers and gastrotrichs) and large macrobenthic organisms (e.g. segmented annelids and ribbon worms; figure 1b). There are not only colonial forms, such as bryozoans (figure 1c), but also sessile animals, like brachiopods (figure 1d), and behaviourally complex animals like octopuses. Moreover, there is also variation in the life cycles, with taxa showing direct development, groups with intermediate larval forms and parasites. This vast developmental, morphological and ecological diversity contrasts with a seeming simplicity of the left–right axis in most spiralian taxa, which is most
four animal micromeres that can be shifted either dextrally or sinistrally with respect to the vegetal macromeres. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs. Anomala quadrata is a spiral-cleaving embryo displaying embryonic chirality at the eight-cell stage. The asymmetric division of the four blastomeres at the four-cell stage forms four smaller cells (micromeres) and four larger cells (macromeres) in the animal and vegetal pole, respectively. However, the micromeres do not align completely parallel to the animal–vegetal axis, but shift either dextrally (i.e. to the right) or sinistrally (i.e. to the left) with respect to the macromeres (figure 1e). If this first asymmetric division were dextral, the next division would be sinistral and vice versa. The alternation of the left–right orientation of the mitotic spindles during cleavage is what eventually causes a spiral arrangement of the micromeres when observed from the animal pole, hence the name of this mode of cleavage. The dextral chirality is more common and genetically dominant, often symmetrical (table 1). The most extreme asymmetry is that of the shell and internal organs of gastropod molluscs, and to a less extent the digestive system of other molluscs, annelids, brachiopods and rotifers (table 1).

Despite the absence of major left–right morphological asymmetries in most adult and larval forms, an inferred ancestral feature present in many lineages of the Spiralia is the spiral-cleaving embryo, a programme of highly stereotypical cell divisions that displays embryonic chirality (figure 1e) [21–23]. With the third round of zygotic divisions, a typical spiral-cleaving embryo becomes eight cells. These divisions are asymmetric and occur in the direction of the animal–vegetal axis, so that four smaller cells (micromeres) and four larger cells (macromeres) form in the animal and vegetal pole, respectively. However, the micromeres do not align completely parallel to the animal–vegetal axis, but shift either dextrally (i.e. to the right) or sinistrally (i.e. to the left) with respect to the macromeres (figure 1e). If this first asymmetric division were dextral, the next division would be sinistral and vice versa. The alternation of the left–right orientation of the mitotic spindles during cleavage is what eventually causes a spiral arrangement of the micromeres when observed from the animal pole, hence the name of this mode of cleavage. The dextral chirality is more common and genetically dominant, often symmetrical (table 1). The most extreme asymmetry is that of the shell and internal organs of gastropod molluscs, and to a less extent the digestive system of other molluscs, annelids, brachiopods and rotifers (table 1).

Despite the absence of major left–right morphological asymmetries in most adult and larval forms, an inferred ancestral feature present in many lineages of the Spiralia is the spiral-cleaving embryo, a programme of highly stereotypical cell divisions that displays embryonic chirality (figure 1e) [21–23]. With the third round of zygotic divisions, a typical spiral-cleaving embryo becomes eight cells. These divisions are asymmetric and occur in the direction of the animal–vegetal axis, so that four smaller cells (micromeres) and four larger cells (macromeres) form in the animal and vegetal pole, respectively. However, the micromeres do not align completely parallel to the animal–vegetal axis, but shift either dextrally (i.e. to the right) or sinistrally (i.e. to the left) with respect to the macromeres (figure 1e). If this first asymmetric division were dextral, the next division would be sinistral and vice versa. The alternation of the left–right orientation of the mitotic spindles during cleavage is what eventually causes a spiral arrangement of the micromeres when observed from the animal pole, hence the name of this mode of cleavage. The dextral chirality is more common and genetically dominant, often symmetrical (table 1). The most extreme asymmetry is that of the shell and internal organs of gastropod molluscs, and to a less extent the digestive system of other molluscs, annelids, brachiopods and rotifers (table 1).

Figure 1. The Spiralia, embryonic chirality and the distribution of cleavage modes. (a) The marine snail Annulobalbis auristemma (credit Alvaro E. Migotto). (b) Juvenile nemertean of Lineus ruber. (c) Adult zooids in a bryozoan colony of Membranipora membranacea. (d) Adult specimen of the brachiopod Novocrania anomala. (e) Spiral-cleaving embryos display embryonic chirality at the eight-cell stage. The asymmetric division of the four blastomeres at the four-cell stage forms four animal micromeres that can be shifted either dextrally or sinistrally with respect to the vegetal macromeres. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs. In molluscs, there is a direct correspondence between this chirality and the direction of coiling of the shell and internal organs.
Table 1. Left–right asymmetries in adult and larval forms of Spiralia.

| group                  | left–right axis       |
|------------------------|-----------------------|
| Gnathostomulida        | Symmetrical           |
| Micrognathozoa         | Symmetrical           |
| Rotifera               | Symmetrical. Asymmetries in the jaws (trophi) in some species, and unpaired gonad often displaced to one side in Monogononta [10] |
| Gastrotricha           | Symmetrical           |
| Platyhelminthes        | Symmetrical. Asymmetries in the ciliary band of some polyclad larvae [11], gonads of rhabdocoels [12], and neural morphology/physiology in polyclad larvae and triclads [13,14] |
| Mollusca               | Asymmetry in shell coiling and internal body organization in Gastropoda [15]. Minor asymmetries, mostly affecting the digestive system, in Polyplacophora, Bivalvia and Scaphopoda [12,16]. |
| Annelida               | Symmetrical. Asymmetries in buccal apparatus of some polychaetes [2,17] and digestive system of Capitella teleta [18] |
| Nemertea               | Symmetrical. Asymmetric eye in paleonemertean larva [19] |
| Phoronida              | Symmetrical           |
| Brachiopoda            | Symmetrical. Anus in the right side in the Lingulacea & Discinacea [20] |
| Bryozoa                | Symmetrical. Asymmetry in the colony coiling [2] |
| Entoprocta             | Symmetrical           |
| Cyclophora             | Symmetrical           |
| Orthonectida           | Symmetrical           |
| Dicyemida              | Symmetrical           |

connection with the early embryonic chirality and final left–right morphology is still unknown in most spiralian taxa. Even more importantly, virtually nothing is known about the early embryonic chirality and development of the left–right axis in those spiralian lineages that have lost spiral cleavage (figure 1f).

In this study, we characterize the expression of members of the Nodal signalling pathway in three spiralian taxa with different embryogenesis, life histories and adult morphologies, and analyse the embryonic chirality of a bira-dial-cleaving spiralian. We show asymmetric expression of nodal in the brachiopod Neoschnia anomala (O. F. Müller, 1776), and of Pitx in the annelid Owenia fusiformis Delle Chiaje, 1844, as well as symmetrical expression of Pitx in the nemertean Lineus ruber (Müller, 1774). We further describe symmetric expression of Pitx in Priapulus caudatus Lamarck, 1816, a member of the Priapulida, which seems to be the most evolutionarily conservative taxon in the Ecdysozoa [45,46], the sister group of the Spiralia. Additionally, we provide evidence for embryonic chirality in the bryozoan Membranipora membranacea (Linnaeus, 1767), a spiralian that lost the stereotypical spiral cleavage, and thus does not show the early, dextral or sinistral asymmetric cell divisions. Altogether, our findings improve our understanding of the evolution of the Nodal signalling pathway in metazoans and provide a more comprehensive view of the establishment of left–right chirality during spiralian development.

2. Material and methods

(a) Animal collections and embryo fixation

Adult specimens of N. anomala were collected from the coasts near Espeland Marine Biological Station (Norway) during the months of September and October. They were spawned as described elsewhere [47]. Gravid specimens of O. fusiformis were collected near Station Biologique de Roscoff, and spawned as previously reported [48]. Adult worms of L. ruber were collected, maintained and spawned as previously described [49]. Gravid adults of P. caudatus were collected from Gullmarsfjorden (Fiskebäckskil, Sweden) during November, and spawned as described elsewhere [46]. Finally, kelp blades with ripe colonies of the bryozoan M. membranacea were collected from floating docks in Hjellestadosen (Bergen, Norway), kept in water tanks with constant running seawater and spawned as previously described [50].

For all the different species, embryos at the desired developmental stage were fixed in 4% paraformaldehyde diluted in seawater for 1 h at room temperature. For P. caudatus, the eggshell was permeabilized with 0.05% thioglycolate and 0.01% pronase for 30 min at 9°C before fixation. Larval and juvenile stages of N. anomala and L. ruber were relaxed in 7.4% magnesium chloride before adding the paraformaldehyde. After fixation, samples were washed several times in phosphate buffer saline supplemented with 0.1% Tween 20. Samples were dehydrated through a graded methanol series and stored in pure methanol at −20°C.

(b) Gene expression analyses

Full-length sequences of nodal in N. anomala, and Pitx in O. fusiformis, L. ruber and P. caudatus were identified from RNAseq data of mixed embryonic stages. Protein alignments were constructed with MAFFT v. 7 [51] and poorly aligned regions were removed with Gblocks v. 0.91b [52]. RAxML v. 8 [53] was used to infer gene orthologies (electronic supplementary material, figure S1). Resulting trees were formatted with FigTree and Illustrator CS6 (Adobe). Fixed embryos of N. anomala, O. fusiformis, L. ruber and P. caudatus were used to perform colorimetric whole mount in situ hybridization following previously described protocols [46,49]. After developing the signal, samples were stored in 70% glycerol and imaged with an AxioCam HRc connected to an Axioscope Ax10 (Zeiss), using bright field Nomarski optics. Images were analysed with Photoshop CS6 (Adobe), and figure plates made with Illustrator CS6 (Adobe). Contrast and brightness were adjusted always to the whole image and not to specific parts of it.

(c) Live microscopy of bryozoan development

We transferred cleaving M. membranacea embryos to a glass slide coated with poly-L-lysine, where they were mounted under a coverslip sealed with Vaseline. We imaged the slide under a four-dimensional microscope [54] and acquired 60 optical planes of the embryo every 40 s using differential interference contrast.
3. Results

(a) Expression of nodal in the brachiopod Novocrania anomala

The brachiopod *N. anomala* shows radial cleavage, gastrulation by invagination and the formation of a radially symmetrical gastrula (figure 2a) [20,47]. During anteroposterior elongation in the mid and late gastrula, the vegetal blastopore moves posteriorly along the ventral midline and closes (figure 2a). After this, the embryo differentiates into a bilobed larva, with an anterior apical lobe, and a posterior mantle lobe with three pairs of chaetae (figure 2a). During elongation, the mesoderm forms four pairs of pouches distributed along the anteroposterior axis [20,47]. The first anterior pouch will form the mesoderm of the apical lobe, and the other three consecutive pouches will originate each pair of chaetae bundles.

We identified a single orthologue of *nodal* in *N. anomala* (electronic supplementary material, figure S1a). We did not find a clear orthologue of *Pitx* in our transcriptomic data, although *Pitx* is present in the related brachiopod species *T. transversa* [42]. Gene expression analysis during the embryonic development showed that *nodal* was only detected at the end of anteroposterior axial elongation, on the anterior right mesodermal pouch of the late gastrula (figure 1b). This expression was maintained in the differentiated larva (figure 1b).

(b) Expression of Pitx in the annelid Owenia fusiformis

The annelid *O. fusiformis* shows stereotypical asymmetric spiral cleavage, with the D quadrant being only slightly larger than the other quadrants [48]. After cleavage, the embryo forms a hollow blastula, and gastrulates by invagination, forming a radial early gastrula (figure 3a). At this stage, the internal endoderm bends and forms a U-shape, and the mesoderm grows into two lateral bands [48]. A subequatorial ciliary band forms, together with a bundle of chaetae in the posterior dorsal area, eventually resulting in the formation of the distinctive mitraria larva of oweniids (figure 3a) [48,55].

We did not identify an orthologue of *nodal* in our RNAseq data of *O. fusiformis*, but we detected an orthologue of *Pitx* (electronic supplementary material, figure S1b). The analysis of the expression of *Pitx* during the embryonic development of *O. fusiformis* showed weak asymmetrical expression in one cell on the right side of the embryo at the late gastrula–early mitraria stage (figure 3b). The internal location of the staining suggests that the *Pitx*-positive cell is part of the growing lateral mesodermal bands, as described for the sister species *Owenia collaris* [48]. This expression was restricted to this stage, and not observed in mature mitaria larvae.

(c) Expression of Pitx in the nemertean Lineus ruber

The nemertean *L. ruber* shows a characteristic indirect development that involves the formation of an adelphaphagic intracapsular larva [49,56]. Early cleavage is of the spiral type, and results in the formation of a blastula with a small blastocoele. After invagination of the endomesoderm, the radial gastrula develops into the Schmidt’s larva (figure 3c) [49,56]. This intracapsular larva consists of a temporary epidermis, and a set of epidermal imaginal discs from which the juvenile will form: a pair of cephalic discs, a pair of trunk discs, one proboscis disc, one pharyngeal disc and a blind gut rudiment. The Schmidt’s larva can feed on other siblings contained within the same egg capsule, growing in size. After around 18–20 days of development, the larva metamorphoses into the juvenile, which involves the shedding of the larval epidermis, and the differentiation of the juvenile tissues and organs (figure 3c).

As with *O. fusiformis*, we identified an orthologue of *Pitx* in the available transcriptomic data (electronic supplementary material, figure S1b), but not of *nodal*. The analysis of its expression revealed that Pitx was first expressed symmetrically in a few internal anterior mesenchymal cells of the Schmidt’s larva (figure 3d). This position corresponds to the place of formation of the proboscis rudiment [49]. In late larval stages, two additional symmetrical domains of expression appeared, which seem to locate where the ventral pair of nerve cords forms (figure 3d). After metamorphosis,
Pitx was detected in the proboscis and ventral nerve cords (figure 3d).

(d) Expression of Pitx in the outgroup taxon Priapulus caudatus

The priapulid P. caudatus exhibits holoblastic radial cleavage [57]. Gastrulation occurs by invagination, and is followed by the division of the embryo in an anterior introvert region and a posterior trunk region (introvertula stage; figure 3e) [46]. After differentiation of the larval tissues, the introvert retracts inside the trunk region, and the embryo eventually hatches by protruding the introvert against the hatching cap of the eggshell (figure 3e). The first hatching larva is non-feeding, and subsequent rounds of moulting lead to the formation of the definitive adult tissues [58–60].
As in other studied members of Ecdysozoa, *P. caudatus* lacks a nodal orthologue [42]. We could identify, however, a Pitx gene (electronic supplementary material, figure S1). We detected the first expression of Pitx in the gastrula, on a group of endomesodermal cells of the animal pole (figure 3f). With the formation of the introvertula, we observed two distinct expression domains: a pair of bilaterally symmetrical ectodermal cells on the ventral side of the introvert, which probably correspond to neural tissue; and a broader expression on the anterior dorsal mesoderm of the introvert (figure 3f).

4. Discussion

(a) nodal, Pitx and the genetic control of left–right development in Spiralia

The TGF-β ligand nodal is asymmetrically expressed along the left–right axis in echinoderms and hemichordates (on the right side), molluscs (on the right or left side, depending on body handedness) and chordates (on the left side), and is functionally required to properly develop this axis in most of these organisms [31–37,39]. Recently, a study showed expression of nodal on the right side of the anterior mesoderm in the late gastrula embryo of the rynchonelliform brachiopod *T. transversa* [42], but its function and influence on the left–right patterning is unknown. In this study, we identified larval body (figure 4). However, we noticed that in 9 out of 11 embryos, the right blastomere at the four-cell stage is sister to the blastomere giving rise to posterior structures, while in two embryos the pattern is mirrored, the left blastomere is the one sister to the posterior blastomere.
a new nodal orthologue in the craniid brachiopod *N. anomala*, but failed to recover a nodal member in the annelid *O. fusiformis* and *L. ruber*. However, the presence of nodal in other members of the Annelida and Nemertea [42] indicate that these absences are probably not real gene losses, but subsampling transcriptomic issues. The expression of nodal in the brachiopod *N. anomala* demonstrated a similar timing and location to that in *T. transversa* (figure 2b), albeit these two species differ significantly in the mode of gastrulation and mesoderm development [47,63]. Since the last common ancestor of *T. transversa* and *N. anomala* corresponds to the last common ancestor to all brachiopods [64], our findings indicate that the most probable ancestral expression of nodal in brachiopods was in the anterior right, mature mesoderm. This contrasts with the expression in gastropod molluscs, where nodal is already expressed at relatively early stages (32-cells) and in ectodermal derivatives of the shell and head region [31]. However, there are no data available on the expression of nodal in other groups of molluscs, and in particular, in those without strong left–right asymmetries like the early branching polycladophorans. Thus, the ancestral expression of nodal for this group, and Spiralia generally, is still unclear (figure 5).

The homeobox transcription factor Pitx is a downstream regulator of the Nodal signalling pathway, and thus appears asymmetrically expressed on the side of nodal expression in members of the Deuterostomia and gastropod molluscs [31,32,36,38]. In the studied molluscs, Pitx is additionally expressed in endodermal and cephalic ectodermal domains [31]. In brachiopods, however, Pitx is expressed symmetrically, although stronger on the right, nodal-positive side of the anterior mesoderm [42]. In platyhelminth species that lack a nodal orthologue, Pitx is expressed in different neuronal populations, and controls the regeneration of the serotoninergic nervous system and the body midline [42–44]. Our results provide first evidence of expression of Pitx in annelids and nemerteans (figure 3b,d). Interestingly, Pitx is expressed symmetrically in the nemertean *L. ruber*, in the nervous system and proboscis, while it is expressed asymmetrically in one anterior right mesodermal cell in the annelid *O. fusiformis*. No expression during early cleavage and development was observed in either of these two spiralian. Altogether, these findings give a complex picture of the evolution of Pitx expression in Spiralia (figure 5). When out-group lineages, such as priapulids (figure 3f) are considered, it appears that expression of Pitx associated with the nervous system at mid–late stages of development is probably ancestral. However, further analysis of Pitx in relation to nodal expression in those lineages with both genes will be essential to better understand the evolution of this genetic cassette in spiralian.

Altogether, the expression and functional data on nodal and Pitx suggest that they are likely involved in the morphological differentiation of the left–right axis in the Spiralia, with asymmetric expression of one or two genes in at least molluscs, annelids and brachiopods (figures 2 and 3) [31,42]. However, the absence of expression of nodal and Pitx in the earliest cleavage stages in all studied species, when embryonic chirality is established, indicate that a separate upstream genetic mechanism defines the left–right axis in spiralian embryos [29]. In this regard, a recent report showed that a tandemly duplicated, diaphanosom-related formin gene (*Ldia2*) is asymmetrically expressed as early as in two-cell stage embryos and maps to the genomic region associated with the inheritance of body handedness in the pond snail *Lymnaea stagnalis* [30]. Formins are involved in actin, and thus cytoskeletal, dynamics...
[65]. Interestingly, the chemical disruption of this gene during the earliest zygotic divisions leads to the loss of chiral twist in dextral-cleaving embryos [30]. In wild-type sinistral cleaving embryos of *L. stagnalis*, *Ldia2* shows a truncated version. Therefore, these observations suggest that *Ldia2* controls embryonic chirality and that chiral dimorphism evolved with the appearance of a non-functional *Ldia2* recessive allele in *L. stagnalis* [30]. Nonetheless, other mollusc species with sinistral forms do not show the truncated version in their formin genes, which indicates that the genetic basis of embryonic chirality is probably multifactorial. These recent advances are a first step towards understanding the molecular grounds that connect cytoskeleton dynamics and embryonic chirality in spiralian embryos. Further investigations will uncover how these early symmetry breaking events influence the later left–right axis differentiation programme controlled by *nodal* and *Pitx*.

(b) Embryonic chirality and left–right asymmetries in Spiralia

The dextral or sinistral shift of the animal micromeres, and thus the presence of embryonic chirality, is a defining feature of spiralian cleavage and Spiralia as a whole. However, there are multiple cases of loss of this developmental programme (figure 5), either in major groups (e.g. gastrotrichs, rotifers, brachiopods and bryozoans) or in particular lineages within otherwise spiral-cleaving groups (e.g. in cephalopod molluscs and neoporan Platyhelminthes) [21,66,67]. Often, the loss of spiral cleavage is associated with the evolution of a radially symmetrical programme of zygotic divisions, with no obvious cellular and/or morphological asymmetries. The bryozoan *M. membranacea* and the brachiopod *N. anomala* display, for instance, this type of development [47,62]. Remarkably, our four-dimensional microscopy approach to study the earliest embryogenesis of *M. membranacea* demonstrates that there is in fact chiral dimorphism in these biradially cleaving embryos, with the right-handed form being more common than the left-handed, as is also observed in molluscs (figure 4). Whether the same molecular programme involved in controlling embryonic chirality in spiral-cleaving embryos is also playing a role in the early specification of the left–right axis in biradial-cleaving spiralian is unknown.

Altogether, the asymmetric expression of *nodal*/ *Pitx* in different lineages, the presence of embryonic chirality in radial cleaving embryos, and the spiral cleavage itself demonstrate that the presence of left–right asymmetries during development is widespread in the Spiralia. It remains paradoxical, however, that these evident embryonic differences in the cellular fate and molecular profile of the left and right sides are later on not translated into morphological asymmetries in most of the adult and larval forms of the Spiralia.

5. Conclusion

Early cytoskeleton dynamics and the subsequent asymmetric activation of the Nodal signalling pathway control the direction of coiling of the shell of gastropod molluscs [29–31], which is one of the most striking cases of left–right asymmetries in animals. Importantly, the presence of embryonic chirality during the first zygotic divisions, which is a defining feature of spiralian development [21,22], is also observed in lineages that have lost the ancestral spiral cleavage, such as the bryozoan *M. membranacea*. Similarly, other spiralian without obvious morphological asymmetries in their adult and larval forms, such as the brachiopods *T. transversa* [31] and *N. anomala*, and the annelid *O. fusiformis*, show asymmetric expression of *nodal* and/or *Pitx* at some point of their embryonic development. Altogether, these evidences indicate that embryonic left–right asymmetries are widespread in the Spiralia, albeit their exact impact on the development of the definitive adult morphology is still unclear.

Ethics. The animal research reported in this study adheres to local ethical guidelines.

Data accessibility. All DNA sequences have been deposited in GenBank (accession numbers KU885445–KU885448).

Authors’ contributions. J.M.M.-D. and A.H. designed the study. J.M.M.-D. carried out the gene expression analyses. B.C.V. conducted the live microscopy analysis of bryozoan development. All authors analysed the data. J.M.M.-D. drafted the manuscript, and B.C.V. and A.H. edited the text.

Competing interests. We have no competing interests.

Funding. This work has been funded by the Sars core budget to A.H.

Acknowledgments. We thank all past and present members of the Hejpil laboratory for support and discussions, and in particular, Aina Barve, Anlau Badddington, Carmen Andrikou and Daniel Thiel for help with animal collections. We thank Harald Hausen and Oliver Vöcking for their help with *O. fusiformis* spawnings, as well as Justine Diaz for a critical read of this manuscript.

References

1. Blekemishev WN. 1969 Principles of comparative anatomy of invertebrates. Chicago, IL: University of Chicago Press.
2. Palmer AR. 2009 Animal asymmetry. *Curr. Biol*., 19, R473–R477. (doi:10.1016/j.cub.2009.04.006)
3. Palmer AR. 1996 From symmetry to asymmetry: phylogenetic patterns of asymmetry variation in animals and their evolutionary significance. *Proc. Natl Acad. Sci. USA* 93, 14 279–14 286. (doi:10.1073/pnas.93.25.14279)
4. Kokot KM. 2016 On 20 years of Lophotrochozoa. *Org. Divers. Evol.* 16, 329–343. (doi:10.1007/ s13127-015-0261-3)
5. Struck TH et al. 2014 Platyzoan paraphyly based on phylogenetic data supports a noncoelomate ancestry of Spiralia. *Mol. Biol. Evol.* 31, 1833–1849. (doi:10.1093/molbev/msu143)
6. Laumier CE et al. 2015 Spiralian phylogeny informs the evolution of microscopic lineages. *Curr. Biol.* 25, 2000–2006. (doi:10.1016/j.cub.2015.06.068)
7. Dunn CW et al. 2008 Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature* 452, 745–749. (doi:10.1038/ nature06614)
8. Wanninger A (ed). 2015 Evolutionary developmental biology of invertebrates. Vienna, Austria: Springer.
9. Nielsen C. 2012 Animal evolution: interrelationships of the living phyla. Oxford, UK: Oxford University Press.
10. Fontaneto D, De Smet WH. 2015 Rotifera. In *Handbook of zoology*. 3. Gastrotricha and gnathifera (ed. A Schmidt-Rhaesa). Berlin, Germany: De Gruyter.
11. Lacalli TC. 1992 The nervous-system and ciliary band of Müller larva. *Proc. R. Soc. Lond.* B 217, 37–58. (doi:10.1098/rspb.1992.0093)
12. Ludwig W. 1932 Das Rechts-links problem im Tierreich und beim Menschen. Berlin, Germany: Springer.
13. Nogu T, Yuan YF, Sorocco D, Perez-Tomas R, Levin M. 2005 Eye regeneration assay reveals an invariant functional left-right asymmetry in the early bilaterian, Dugesia japonica. *Laterality* **10**, 193 – 205. (doi:10.1080/135547603420001440)

14. Lantfranchi A, Bedini C. 1986 Electron-microscopic study of larval eye development in Turbellaria Polycladida. *Hydrobiologia* **132**, 121 – 126. (doi:10.1007/BF00462388)

15. Morton JE. 1967 Molluscs. London, UK: Hutchinson & Co.

16. Matsukuma A. 1996 Transposed hinges: a polymorphism of bivalve shells. *Molluscan Stud.* **62**, 415 – 431. (doi:10.1093/mollus/62.4.415)

17. Paxton H. 2009 Phylogeny of Eunicida (Annelida) based on morphology of jaws. *Zoosymposia* **2**, 241 – 264.

18. Boyle MJ, Seaver EC. 2008 Developmental expression of foxa and gata genes during gut formation in the polychaete annelid, *Capitella sp.*. *Evol. Dev.* **10**, 69 – 105. (doi:10.1111/j.1525-142X.2008.00123.x)

19. Nielsen C. 1991 The development of the brachiopod *Crania* (Neoconcha) anomala (OF Müller) and its phylogenetic significance. *Acta Zool.* **72**, 7 – 28. (doi:10.1111/j.1463-6395.1991.tb00312.x)

20. Hejnol A. 2010 A twist in time—the evolution of *Priapulus* (Priapulida). *Integr. Comput. Biol.* **402**, 254 – 265. (doi:10.1023/A:1003756912738)

21. Lambert JD. 2010 Developmental patterns in spiralan embryos. *Carr. Biol.* **20**, R72 – R77. (doi:10.1016/j.cub.2009.11.041)

22. Henry JJ, Martindale MQ. 1999 Conservation and innovation in spiralan development. *Hydrobiologia* **402**, 255 – 265. (doi:10.1023/A:1003756912738)

23. Palmer WF, Lindberg DR. 1997 Towards a phylogeny of gastropod mollusks: an analysis using morphological characters. *Zool. J. Linn. Soc.* **119**, 83 – 265. (doi:10.1111/j.1096-3642.1997.tb00137.x)

24. Liu MM, Davey JW, Banerjee R, Han J, Yang F, Abboaker A, Blaxter ML, Davison A. 2013 Fine mapping of the pond snail left-right asymmetry (chirality) locus using RAD-Seq and fibre-FISH. *Plas. ONE* **8**, e70676. (doi:10.1371/journal.pone.0070676)

25. Schillithuizen M, Davison A. 2005 The convoluted evolution of snail chirality. *Naturwissenschaften* **92**, 504 – 515. (doi:10.1007/s00114-005-0045-2)

26. Shibazaki Y, Shimizu M, Kuroda R. 2004 Body handedness is directed by genetically determined cytoskeletal dynamics in the early embryo. *Carr. Biol.* **14**, 1462 – 1467. (doi:10.1016/j.cub.2004.08.018)

27. Crampton HE. 1894 Reversal of cleavage in a sinistral gastropod. *Ann. NY Acad. Sci.* **8**, 167 – 170. (doi:10.1111/j.1749-6632.1894.tb55419.x)

28. Kuroda R, Endo B, Abe M, Shimizu M. 2009 Chirality bistomere arrangement dictates zygotropic left-right asymmetry pathway in snails. *Nature* **462**, 790 – 794. (doi:10.1038/nature08597)

29. Davison A et al. 2016 Forebrain is associated with left-right asymmetry in the pond snail and the frog. *Curr. Biol.* **26**, 654 – 660. (doi:10.1016/j.cub.2015.12.071)

30. Grande C, Patel NH. 2009 Nodal signalling is involved in left-right asymmetry in *Nassaria*. *Nature* **457**, 1007 – 1011. (doi:10.1038/nature07603)

31. Levin M, Johnson RL, Stern CD, Kuehn M, Tabin C. 1995 A molecular pathway determining left-right asymmetry in chick embryogenesis. *Cell* **82**, 803 – 814. (doi:10.1016/0092-8674(95)90777-8)

32. Duboc V, Lapage T. 2008 A conserved role for the nodal signaling pathway in the establishment of dorso-ventral and left-right axes in deuterostomes. *J. Exp. Zool. B Mol. Dev. Evol.* **310**, 41 – 53. (doi:10.1002/jzce.21212)

33. Morokuma J, Ueno M, Kawanishi H, Saiga H, Nichida H. 2002 *HrNodal*, the ascidian nodal-related gene, is expressed in the left side of the epidermis, and lies upstream of *HrNodal*. *Dev. Genes Evol.* **212**, 439 – 446. (doi:10.1002/dge.v212:2002-0242-3)

34. Yuan YF, Sorocco D, Perez-Tomas R, Levin M. 2005 Eye regeneration assay reveals an invariant functional left-right asymmetry in the early bilaterian, *Dugesia japonica*. *Integr. Comput. Biol.* **30**, 721 – 238. (doi:10.1006/icbe.2000.9857)

35. Martin-Duran JM, Janssen R, Wennberg S, Budd GE, Hejnol A. 2012 Deuterostomic development in the protostome *Prasipoda caudata*. *Curr. Biol.* **22**, 2161 – 2166. (doi:10.1016/j.cub.2012.09.037)

36. Freeman G. 2000 Regional specification during embryogenesis in the craniform brachiopod *Crania anomala*. *Dev. Biol.* **227**, 219 – 238. (doi:10.1006/dbio.2000.9857)

37. Smart TI, Von Dassow G. 2009 Unusual development of the mitraria larva in the polychaete *Owennia collaris*. *Biol. Bull.* **217**, 253 – 268.

38. Martin-Duran JM, Vellutini BC, Hejnol A. 2015 Evolution and development of the adephagophic, intracapsular Schmidt’s larva of the nemertean *Lineus ruber*. *Evol. Dev.* **6**, 28. (doi:10.1186/s13227-015-0023-5)

39. Reed CG. 1987 Phyllum Bryozoa. In *Reproduction and development of marine invertebrates of the Northern Pacific Coast*: data and methods for the study of eggs, embryos, and larvae (ed. MF Strathmann), pp. 494 – 510. Seattle, WA: University of Washington Press.

40. Katoh K, Standley DM. 2013 MAFFT multiple sequence alignment software version 7, improvements in performance and usability. *Mol. Biol. Evol.* **30**, 772 – 780. (doi:10.1093/molbev/msu101)

41. Talavera G, Castresana J. 2007 Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst. Biol.* **56**, 564 – 577. (doi:10.1080/1063515070142164)

42. Stamatakis A. 2014 RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**, 1312 – 1313. (doi:10.1093/bioinformatics/btu103)

43. Hejnol A, Schnabel R. 2006 What a couple of dimensions can do for you: comparative developmental studies using 40 microscopy—examples from tardigrade development. *Integr. Comp. Biol.* **46**, 151 – 161. (doi:10.1093/icb/ii102)

44. Wilson DP. 1932 On the mitralia larva of *Owennia fusiformis* Delle Chiaje. *Philos. Trans. R. Soc.* **221**, 231 – 334. (doi:10.1098/rstb.1932.0004)

45. Schmidt GA. 1937 Vergleichende embryologische Studien über die Typen der Embryonalanpassungen bei Hirudineen und Nemertinen. *Z. Morph.*
57. Wennberg SA, Janssen R, Budd GE. 2008 Early embryonic development of the priapulid worm *Priapulus caudatus*. *Evol. Dev.* 10, 326 – 338. (doi:10.1111/j.1525-142X.2008.00241.x)

58. Martin-Duran JM, Hejnol A. 2015 The study of *Priapulus caudatus* reveals conserved molecular patterning underlying different gut morphogenesis in the Ecdysozoa. *BMC Biol.* 13, 29. (doi:10.1186/s12915-015-0139-z)

59. Wennberg S, Janssen R, Budd GE. 2009 Hatching and earliest larval stages of the priapulid worm *Priapulus caudatus*. *Invert. Biol.* 128, 157 – 171. (doi:10.1111/j.1744-7410.2008.00162.x)

60. Martin-Durán JM, Wolff GH, Strausfeld NJ, Hejnol A. 2016 The larval nervous system of the penis worm *Priapulus caudatus* (Ecdysozoa). *Phil. Trans. R. Soc. B* 371, 20150050. (doi:10.1098/rstb.2015.0050)

61. Gruhl A. 2010 Ultrastructure of mesoderm formation and development in *Membranipora membranacea* (Bryozoa: Gymnolaemata). *Zoomorphology* 129, 45 – 60. (doi:10.1007/s00435-009-0099-3)

62. Reed CG. 1991 Bryozoa. In *Reproduction of marine invertebrates. VI. Echinoderms and lophophorates* (eds AC Giese, JS Pearse, VB Pearse). Pacific Groove, CA: Boxwood Press.

63. Freeman G. 1993 Regional specification during embryogenesis in the articulate brachiopod *Terebratalia*. *Dev. Biol.* 160, 196 – 213. (doi:10.1006/dbio.1993.1298)

64. Cohen BL, Bitner MA. 2013 Molecular phylogeny of rhynchonellide articulate brachiopods (Brachiopoda, Rhynchonellida). *J. Paleol.* 87, 211 – 216. (doi:10.1666/12-100R.1)

65. Evangelista M, Zigmond S, Boone C. 2003 Formins: signaling effectors for assembly and polarization of actin filaments. *J. Cell Sci.* 116, 2603 – 2611. (doi:10.1242/jcs.00611)

66. Martin-Duran JM, Egger B. 2012 Developmental diversity in free-living flatworms. *Evodevo* 3, 7. (doi:10.1186/2041-9139-3-7)

67. Watase S. 1891 Studies on cephalopods. I. Cleavage of the ovum. *J. Morph.* 4, 247 – 302. (doi:10.1002/jmor.1050040302)