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Evolution, divergence and loss of the Nodal signalling pathway: new data and a synthesis across the Bilateria

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ABSTRACT Since the discovery that the TGF-β signalling molecule Nodal and its downstream effector Pitx have a parallel role in establishing asymmetry between molluscs and deuterostomes the debate over the degree to which this signalling pathway is conserved across the Bilateria as a whole has been ongoing. Further taxon sampling is critical to understand the evolution and divergence of this signalling pathway in animals. Using genome and transcriptome mining we confirmed the presence of *nodal* and *Pitx* in a range of additional animal taxa for which their presence has not yet been described. *In situ* hybridization was used to show the embryonic expression of these genes in brachiopods and planarians. We show that both *nodal* and *Pitx* genes are broadly conserved across the Spiralia, and *nodal* likely appeared in the Bilaterian stem lineage after the divergence of the Acoelomorpha. Furthermore, both *nodal* and *Pitx* mRNA appears to be expressed in an asymmetric fashion in the brachiopod *Terebratalia transversa*. No evidence for the presence of a Lefty ortholog could be found in the non-deuterostome genomic resources examined. *Nodal* expression is asymmetric in a number of spiralian lineages, indicating a possible ancestral role of the Nodal/Pitx cascade in the establishment of asymmetries across the Bilateria.

KEY WORDS: *Nodal, Pitx, Spiralia, Bilateria, Brachiopoda*

Introduction

Nodal is a transforming growth factor-β (TGF-β) ligand that has a critical role in establishing communication among individual cells and is involved in cell differentiation and development. Nodal signals can act as morphogens and are essential at key moments and distinct tissues throughout development to determine primary body axes, ensure proper gastrulation and neural patterning, cardiogenesis, and the maintenance of embryonic stem cell pluripotency (reviewed in Shen 2007; Schier 2009; Quail *et al.,* 2013). The regulation of the Nodal pathway involves a complex network of receptors, co-receptors, antagonists and intracellular proteins, as well as positive and negative feedback loops that together define cell proliferation, differentiation, and tissue morphogenesis. The Nodal pathway is mainly restricted to embryonic and reproductive cell types and rarely activated during later development and adulthood although recent evidence suggests that this pathway is up-regulated during cancer progression (Lawrence *et al.,* 2011; Lee *et al.,* 2010; Lonardo *et al.,* 2011; Strizzi *et al.,* 2012; Topczewska *et al.,* 2006).

Nodal, like most TGF-β ligands, is synthesized as a pro-peptide that is cleaved in the secretory pathway into an amino-terminal pro-domain and a carboxy-terminal fragment that constitutes the mature form. This cleavage, together with dimerization, is essential to form an active ligand that can bind to a specific pair of receptor serine/threonine kinases, known as receptor types I and II (Shen 2007). The formation of this ligand/receptor complex brings together two type I and two type II receptors, allowing the phosphorylation and subsequent activation of Receptor I by Receptor II. While receptor type II kinases are constitutively active, type I receptors

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Abbreviations used in this paper: AP, anterior posterior; LR, left right; TGF-β, transforming growth factor-β.

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require the formation of a ligand/receptor complex for this activation. Upon activation, the ligand/receptor complex is internalized by endocytotic mechanisms. Once in the cytoplasm, the Receptor I phosphorylates Smad2/3 proteins that propagate signalling through their binding to Smad4 proteins, nuclear translocation and regulation of gene expression (see reviews in Shen 2007; Massagué 2012; Xu *et al.,* 2012).

Despite its apparent simplicity, this pathway can, however, produce highly complex patterns of target gene expression and diverse downstream effects, which can even be antagonistic in some cases. Recent studies have shown the relevance of the regulation of the signal transduction and transcription, and the epigenetic status of the cell in shaping the TGF-β -mediated transcriptional response in a cell (Massagué 2012). Hence, cell response to Nodal signalling depends on the cell context, defined by the levels of ligand/receptor complexes, the receptor activity, the activity of Smad proteins, and Smad accumulation in the nucleus, as well as by the presence of other transcription factors, histone readers, modifiers and chromatin remodellers that determine which genes will be targeted (Massagué 2012).

nodal was first identified in studies of mouse embryogenesis (Zhou *et al.,* 1993) and subsequent description and functional characterization of *nodal* homologs during early chick, frog, and zebrafish embryogenesis corroborated data from mouse (reviewed in Tian and Meng 2006). Hence, all vertebrates have in common: first, that the onset of *nodal* expression is zygotic; second, that the expression is restricted to mesodermal precursors before gastrulation and to the site of gastrulation in all three germ layers during gastrulation; and third, that *nodal* is involved in the early specification of mesoderm and endoderm as well as anterior posterior (AP) and left right (LR) axis specification (Schier and Shen 2000; Wlizla 2011). Although many similarities were identified in the core of the Nodal pathway across different vertebrate groups, the downstream targets have been found to differ between taxa. The *nodal* gene itself, another TGF-β ligand called *lefty* and the transcription factor *Pitx2* have been shown to be the only direct target genes of Nodal signalling that are shared by all vertebrates (Schier 2009). The activation of *Pitx2* in the left mesoderm by Nodal and the role of Lefty in regulating this process is a conserved cascade that determines mesoderm specification and LR determination in all vertebrates.

In addition, *nodal*, *lefty*, and *Pitx* orthologs have also been characterized in non-vertebrate deuterostomes (Morokuma *et al.,* 2002; Yu *et al.,* 2002; Duboc *et al.,* 2005; Wlizla 2011). Comparative studies on cephalochordates, urochordates, hemichordates and echinoderms have shown that in these species, as in vertebrates, Nodal signalling is necessary to establish the polarity of the embryo, (i.e. LR asymmetry and the oral-aboral and AP axes). However, unlike vertebrate *nodal*, which is expressed in the endomesoderm and is involved in its induction, non-vertebrate deuterostome *nodal* is expressed in different germ layer precursors depending on the group, and does not play a broad role in endoderm and mesoderm specification in all groups. Especially intriguing are results in echinoderms, since *nodal* and *Pitx* are expressed in the ectoderm and endoderm (Duboc *et al.,* 2010; Ohguro *et al.,* 2011; Bessodes *et al.,* 2012), and mesoderm cells are specified even when the pathway is blocked (Duboc *et al.,* 2005). In addition, as mentioned above, asymmetric expression and the actions of the Nodal pathway are conserved features of deuterostomes. However, *nodal* and *Pitx* act on the right side in echinoderms and hemichordates (grouped together in the Ambulacraria) but on the left side of the chordate embryos (cephalochordates, urochordates and vertebrates) (Hamada *et al.,* 2002; Morokuma *et al.,* 2002; Yu *et al.,* 2002; Duboc *et al.,* 2005; Wlizla, 2011). These differences have hampered the inference of the ancestral expression domains and roles of the Nodal signalling pathway in deuterostomes, as most phylogenetic evidence suggests a sister group relationship between the Chordata and Ambulacraria (see Fig 4).

Together with deuterostomes, ecdysozoans (including arthropods and nematodes, among others), chaetognaths and spiralians (including, for example, snails and brachiopods) are the main lineages of the Bilateria. Ecdysozoans, chaetognaths and spiralians are more closely related to each other than to deuterostomes, together forming the Protostomia (Aguinaldo *et al.,* 1997; Halanych 2004; Dunn *et al.,* 2008). In addition, recent morphological and molecular data support Acoelomorpha and *Xenoturbella* as sister group to all remaining bilaterians (Hejnol *et al.,* 2009). The absence of *nodal* and *lefty* orthologs in the genomes of *Drosophila* and *C. elegans* suggested that Nodal signalling was absent from ecdysozoans and initially led to the general understanding that the Nodal pathway was restricted to deuterostomes. Recently, however, a *nodal* ortholog was described in snails (Grande and Patel 2009), suggesting a new scenario where Nodal signalling evolved before the bilaterians split into the Ecdysozoa, the Chaetognatha, the Spiralia and the Deuterostomia (Grande and Patel 2010).

Outside deuterostomes, Nodal signalling has only been investigated in snails with two different types of body handedness: dextral (right-handed) and sinistral (left-handed) coiling (Grande and Patel 2009; Kuroda *et al.,* 2009). These two forms of body organization are a manifestation of LR asymmetry in snails. A maternal effect locus determines the handedness of the third division in the typical spiral cleavage in snails and consequently adult handedness (Boycott and Diver 1923; Sturtevant 1923; Boycott *et al.,* 1930; Freeman and Lundelius 1982). Hence, a dextral third cleavage will correspond to a right-handed adult and a sinistral third cleavage to a left-handed adult. Interestingly, *nodal* and *Pitx* are asymmetrically expressed and regulate LR asymmetries in snails as they do in deuterostomes (Grande and Patel 2009). However, the side of the embryo that expresses these genes is related to body handedness: *nodal* and *Pitx* are expressed on the right ectoderm of the dextral species and on the left in the sinistral species. Mechanical micromanipulation of the third cleavage chirality leads to reversal of embryonic *nodal* expression patterns and reversal of embryonic and adult handedness (Kuroda *et al.,* 2009). Although some evidence indicates that the last common ancestor of all snails had a dextral body handedness (Ponder and Lindberg 1997) the presence of both left and right-hand sided *nodal* expression means that the ancestral pattern in the Mollusca could not be inferred without other evidence from the Spiralia.

Altogether, this evidence shows that Nodal signalling arose in the lineage leading to the last common ancestor of ecdysozoans, chaetognaths, spiralians, and deuterostomes. However, the pathway has been only described in deuterostomes and snails, and the findings clearly highlight the need for further investigation in other taxa to understand the evolutionary history of the pathway. To gain new insights into the origin and evolution of Nodal signalling in the Bilateria, we have investigated the presence of *nodal*, *lefty* and *Pitx* gene orthologs in different groups of bilaterally symmetrical organisms. By surveying the genomic/transcriptomic resources

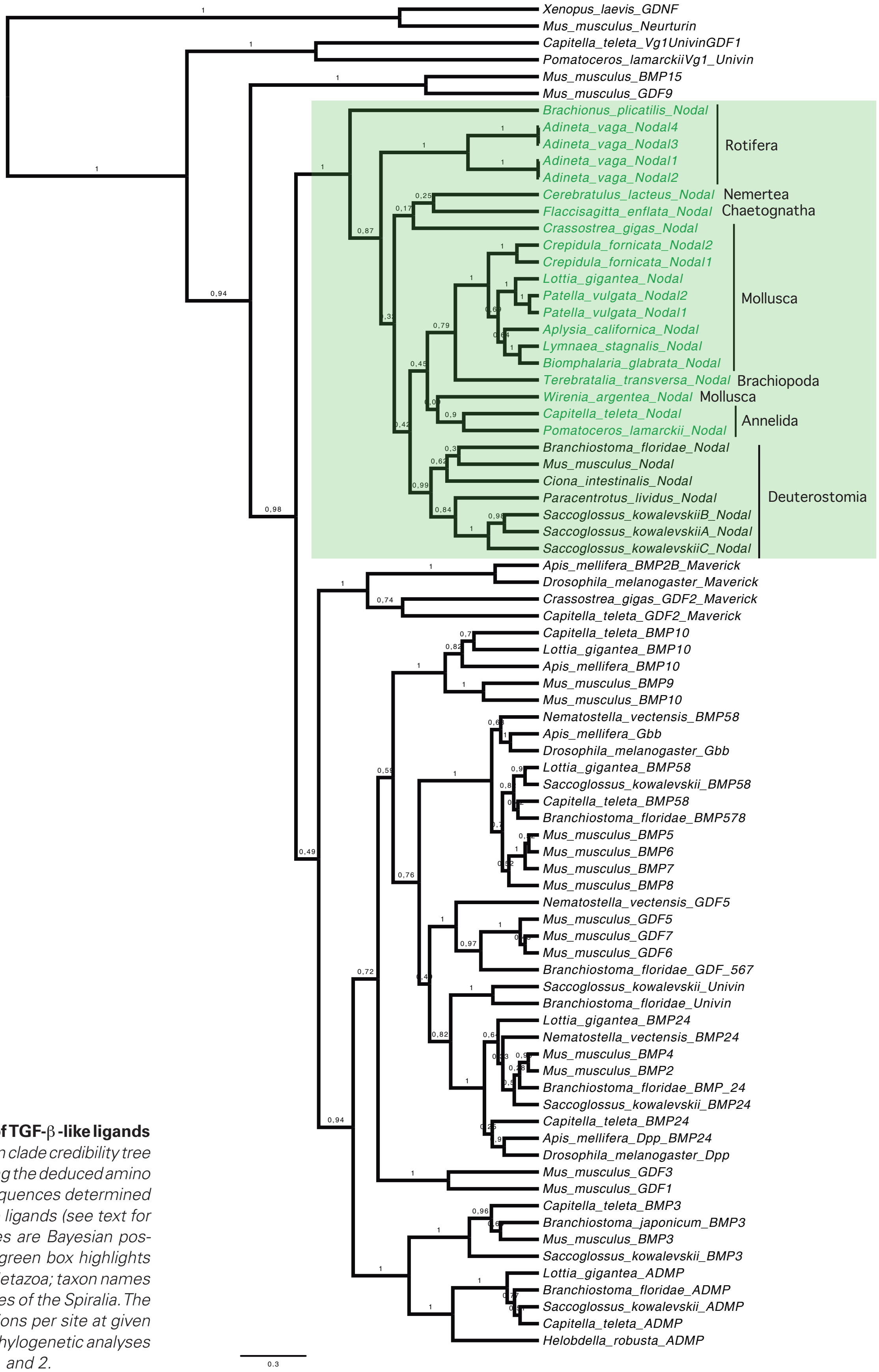


Fig. 1. Phylogenetic analysis of TGF-β -like ligands across the Metazoa. *Maximum clade credibility tree from the Beast analysis including the deduced amino acid sequences of all Nodal sequences determined so far together with TGF-β -like ligands (see text for details). Numbers above nodes are Bayesian posterior probabilities (BPP). The green box highlights Nodal sequences across the Metazoa; taxon names in green indicate representatives of the Spiralia. The scale bar represents substitutions per site at given distance. Sequences used in phylogenetic analyses can be found in Supp. Tables 1 and 2.*

available for representatives of different metazoan groups, as well as by direct amplification and sequencing, we show the existence of *nodal* and *Pitx* orthologs in several bilaterians, including rotifers, brachiopods, nemerteans, and chaetognaths. In addition, we have investigated the expression pattern of *nodal* and *Pitx* in the brachiopod *Terebratalia transversa* as well as that of *Pitx* in the embryos of the planarian *Schmidtea polychroa*, representing key taxa for further understanding of this cascade in the spiralian clade. With this new data incorporated, we have reviewed our present knowledge on Nodal signalling and interpreted it in an evolutionary context, providing strong confirmation of an ancestrally shared role for the Nodal pathway in establishing LR asymmetry, expressed on the right hand side of the body, and present before the divergence of the spiralia and deuterostome lineages from their common ancestor.

Results

Nodal in Bilateria

We searched the genomes and transcriptomes of representatives of several metazoan lineages to identify Nodal orthologs (Supp. Table 1). Furthermore, we used degenerate PCR in several species for which genomic or transcriptomic information was not available to complete our catalogue of *nodal* orthologs. We performed a phylogenetic analysis, including the newly determined amino acid sequences of Nodal, along with previously reported Nodal and

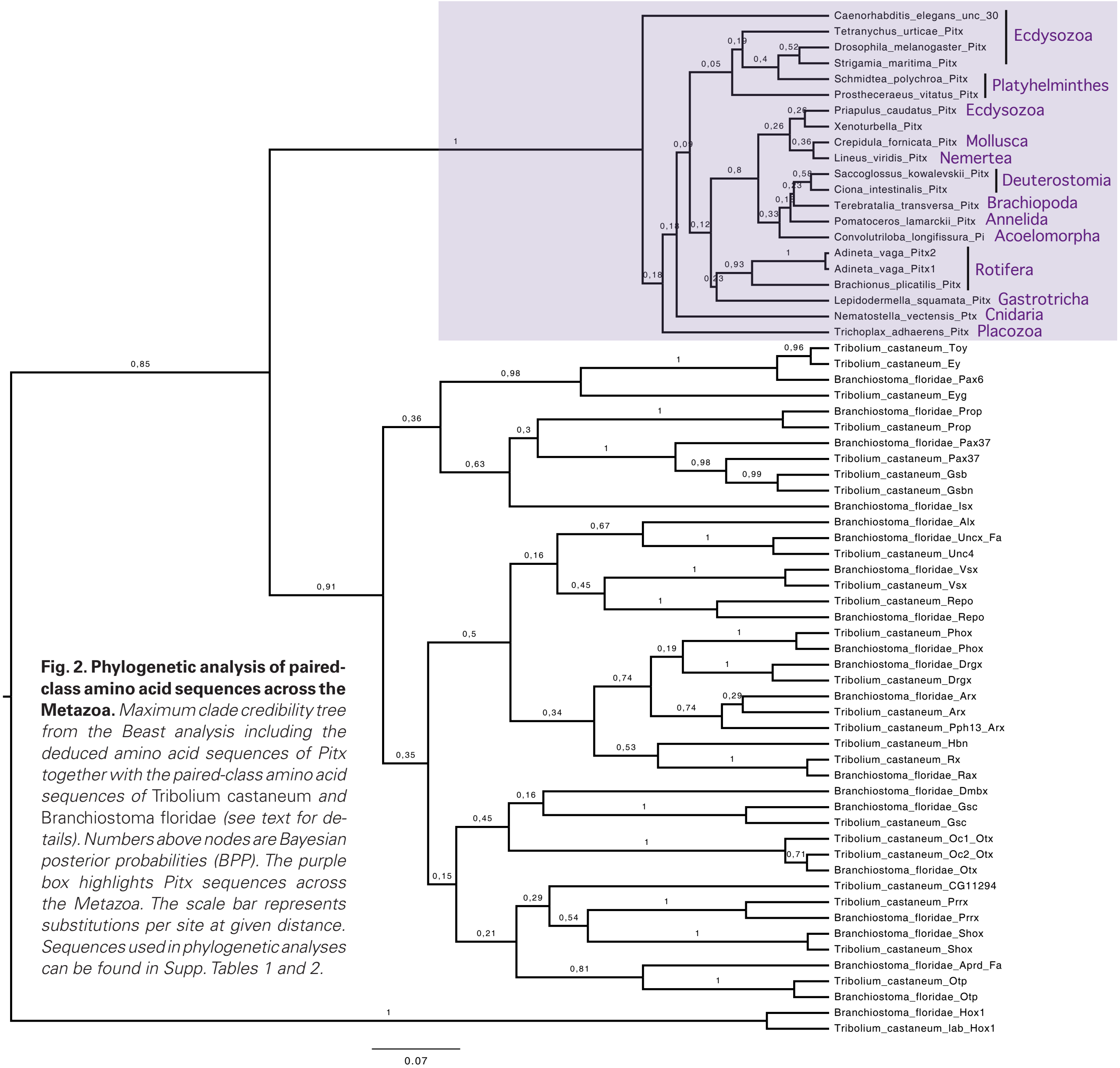


Fig. 2. Phylogenetic analysis of paired-class amino acid sequences across the Metazoa. *Maximum clade credibility tree from the Beast analysis including the deduced amino acid sequences of Pitx together with the paired-class amino acid sequences of* Tribolium castaneum *and* Branchiostoma floridae *(see text for details). Numbers above nodes are Bayesian posterior probabilities (BPP). The purple box highlights Pitx sequences across the Metazoa. The scale bar represents substitutions per site at given distance. Sequences used in phylogenetic analyses can be found in Supp. Tables 1 and 2.*

TGF-β sequences of other spiralians and deuterostomes from GenBank (Supp. Table 1, Supp. Table 2 and Supp. Fig. 1). The resulting tree confirmed with high statistical support that the newly identified Nodal sequences were indeed more closely related to the previously reported Nodal sequences than to any other TGF-β sequences. Candidate sequences were identified as orthologs when they clustered with high statistical support (BPP>0.9) with sequences of known identity. Based on this criterion, our analysis shows that a Nodal homolog protein is present in several bilaterian taxa besides deuterostomes and snails (Fig. 1).

We did not identify any potential *nodal* ortholog gene in the genomes or RNA-seq datasets of any acoelomorph or *Xenoturbella*, which have been proposed to be the sister group to all remaining Bilateria (Supp. Table 1). Blast searches were performed on the genomic datasets of several ecdysozoans without a positive identification of *nodal* (Supp. Table 1). In addition, searches in both the RNA-seq dataset for the ecdysozoan priapulid *Priapulus caudatus* and direct amplification with degenerate primers for *nodal* in this species did not provide any potential *nodal* ortholog for this group (Supp. Table 1).

We also sought *nodal* ortholog genes in several spiralians with a variety of results (Supp. Table 1). Genomic and RNA-seq datasets, as well as direct amplification with degenerate primers for *nodal,* were used to identify a potential *nodal* ortholog in the rotifers *Adineta vaga* and *Brachionus plicatilis*, the annelids *Capitella teleta* and *Pomatoceros lamarckii*, the molluscs *Patella vulgata*, *Crepidula fornicata*, and *Wirenia argentea*, the brachiopod *T. transversa*, and the nemertean *Cerebratulus lacteus*. However, genomic, RNA-seq data, and direct amplification with degenerate primers did not retrieve any potential *nodal* ortholog for the platyhelminthes *Schmidtea mediterranea, S. polychroa, Macrostomum lignano*, *Prostheceraeus vittatus*, *Prosthiostomum siphunculus, Stenostomum sthenum*, and *Schistosoma mansoni*, the gastrotrich *Lepidodermella squamata*, the cycliophore *Symbion pandora*, or the bryozoans *Bugula neritina* and *Membranipora membranacea* (Supp. Table 1). Finally, degenerate primers for *nodal* were used to identify a potential *nodal* ortholog in the chaetognath *Flaccisagitta enflata* (Supp. Table 1).

When present, a single copy of *nodal* gene was detected in most groups, except for the bdelloid rotifer *A. vaga*, where four copies were identified, and the gastropods *P. vulgata* and *C. fornicata*, where two copies were retrieved. A detailed analysis of Nodal sequences across groups showed that while the amino-terminal pro-peptide was highly variable, the carboxy-terminal was highly conserved, and contains the seven cysteine residues previously described as can be seen in positions 1, 30, 34, 62, 63, 99 and 101 of our alignment (Supp. Fig. 1).

Pitx in Bilateria

We also looked for *Pitx* orthologs in several metazoan lineages (Supp. Table 1). Genomic and RNA-seq datasets, as well as direct amplification with degenerate primers for *Pitx* were used to identify potential *Pitx* orthologs in the priapulid *P. caudatus*, *Xenoturbella bocki*, the chaetognath *F. enflata*, the gastrotrich *L. squamata*, the platyhelminthes *S. polychroa* and *P. vittatus*, the rotifers *A. vaga* and *B. plicatilis*, the annelid *P. lamarckii*, the molluscs *P. vulgata* and *C. fornicata*, the brachiopod *T. transversa* and the nemertean *Lineus viridis*. Two copies of *Pitx* were detected in the bdelloid rotifer *A. vaga*.

Phylogenetic analyses included the newly determined amino acid sequences of Pitx together with previously reported Pitx and paired-class sequences of other bilaterians and non-bilaterian metazoans available from GenBank (Supp. Table 1 and Supp. Table 2). The resulting tree confirmed with high statistical support the identity of these new sequences of *Pitx* (Fig. 2).

Expression patterns of nodal and Pitx in T. transversa

We have characterized the RNA expression patterns of *nodal* and *Pitx* in the brachiopod *T. transversa*. Following fertilization, brachiopod embryos undergo radial cleavage, gastrulation by invagination, and the formation of a planktonic larva, which finally settles to metamorphose into the adult. We did not detect any *nodal* or *Pitx* signals before the larval stages. During early and late larval stages, there is just one domain of *nodal* expression, in the mesoderm on the right side of the embryo (Fig. 3A, a, c, e, and g). In early larvae, *Pitx* expression is detected in the anterior mesoderm on both sides of the embryo, although it is clearly stronger on the right side (Fig. 3A, b and d). Two additional *Pitx* expression domains are detected in late larval stages: one symmetrical in the mesoderm of the mantle lobe and one asymmetrical in the mesoderm of the right side (Fig. 3A f and h). Embryos of *T. transversa* double-labelled for *nodal* and *Pitx* show that these expression domains are not co-localized (Fig. 3B).

Discussion

Tracing the origin of the Nodal pathway

The Nodal pathway has attracted much attention because of its critical role in deuterostome development. In particular, Nodal signalling is known to regulate gastrulation and the proper segregation of the germ layers in key model species, and helps to establish the AP and LR axes in embryos. Therefore, understanding the multifunctional nature of the pathway and its relevance in morphological variation will help in the interpretation of morphological diversification during the evolution of bilaterians.

Our work suggests that the Nodal pathway evolved early in bilaterian evolution, with the core genes *nodal* and *Pitx* present in deuterostomes, spiralians and chaetognaths (Fig. 4). Searches in the genomic and RNA-seq datasets of *Xenoturbella* and Acoelomorpha as well as direct amplification in acoelomorphs have not revealed any *nodal* ortholog. Acoelomorphs and *X. bocki* are possibly the sister groups to the Bilateria as a whole (Hejnol *et al.,* 2009, Srivastava *et al.,* 2014), although alternative placements have been proposed, notably as a sister group to echinoderms+hemichordates or sister to all remaining deuterostomes (Phillipe *et al.,* 2011) (Fig. 4). However, a newer phylogenomic analysis could not recover the result of Philippe *et al.,* 2011, instead it confirms the Acoelomorpha as earliest split from the remaining Bilateria (Srivastava *et al.,* 2014). Acoelomorphs and *X. bocki* share some ultrastructural features, including morphological characters in the digestive and nervous systems as well as a similar mode of development (Hejnol *et al.,* 2009; Nakano *et al.,* 2013). The fact that so far no LR morphological asymmetries have been described in either of these two groups and that no *nodal* ortholog has been identified in any of them may suggest that the last common ancestor of all bilaterians was morphologically symmetrical in the left-right axis. After acoelomorphs and *Xenoturbella* split from the bilaterian stem lineage, a duplicated TGF-β family member gave rise to Nodal in the bilaterian stem species, which later evolved to regulate LR specification. The alternative placement of acoelomorphs and *Xenoturbella* as a sister group to Ambulacraria and the absence of Nodal in these species would otherwise imply the loss of Nodal and its role in establishing asymmetries in these species. A broader picture of TGF-β ligand inter-relationships can be seen in other investigations, for example, Herpin *et al.,* 2004 and Pang *et al.,* 2011, where evidence from a range of basal metazoans is presented, and Kenny *et al.,* 2014 in this issue where the TGF-β signalling cassette of a range of spiralians are described in detail.

Within Spiralia, the presence of a *nodal* ortholog has been confirmed in molluscs (gastropods, bivalves, and neomeniomorphs), annelids, rotifers, brachiopods and nemerteans (Fig. 4). However, this gene could potentially be present in other groups such as bryozoans, gastrotrichs, gnathostomulids, cycliophorans and entoprocts. Unfortunately, genomic information for most of these groups is currently absent. Filling these gaps is key for a full understanding of the evolutionary history of this pathway.

Platyhelminthes, or at least those examined to date (catenulids, macrostomids, cestodes, triclads, and polyclads), seem to have lost the gene that encodes for Nodal. Morphological studies of platyhelminth embryos and adults have described perfect symmetric conformations along the bilateral axis and only rhabdocoel turbelarians show detectable asymmetries of the reproductive system (Grande 2010). Most free living platyhelminthes have male and female reproductive systems distributed symmetrically on both sides of the body. However, through the evolutionary history of rhabdocoels, the female reproductive system has been reduced on the left side and it is therefore located exclusively on the right side. In parallel, the male reproductive system has been reduced on the right side and it is therefore located on the left. Therefore, a search for the presence or absence of a *nodal* ortholog in this particular group of plathyhelminthes deserves special attention. If Nodal is absent in this group, a new, Nodal-independent mechanism for generating asymmetries in spiralians may be uncovered.

Interestingly, the *nodal* gene is present in chaetognaths. The sister-group relationship between chaetognaths and the remaining bilaterians has been under debate for decades. Chaetognaths have been related to deuterostomes (Ghirardelli 1981; Brusca and Brusca 2002), ecdysozoans (Littlewood *et al.,* 1998; Zrzavy *et al.,* 1998; Peterson and Eernisse 2001), spiralians (Matus *et al.,* 2006) and as sister taxon to Spiralia + Ecdysozoa (Giribet *et al.,* 2000; Helfenbein and Boore 2004; Marletaz *et al.,* 2006; Nesnidal *et al.,* 2013). However, new molecular and morphological evidence has supported their phylogenetic position as an independent lineage in the protostomes beside ecdysozoans and spiralians (Dunn *et al.,* 2008). Chaetognaths have retained ancestral genomic features, such as the conservation of certain genes like that encoding for the Guanidinoacetate N-methyltransferase (GAMT) enzyme, and at the same time, have acquired some derived features, such as a trans-splicing mechanism of mRNA processing (shared with many other organisms, including several spiralians). This makes them a key group for understanding early evolutionary events in the Bilateria (Marletaz *et al.,* 2008; Harzsch and Müller 2007; Harzsch *et al.,* 2009; Harzsch and Wanninger 2010; Rieger *et al.,* 2010). Further studies on the expression patterns and function of Nodal in this group will be particularly useful to understand the early role

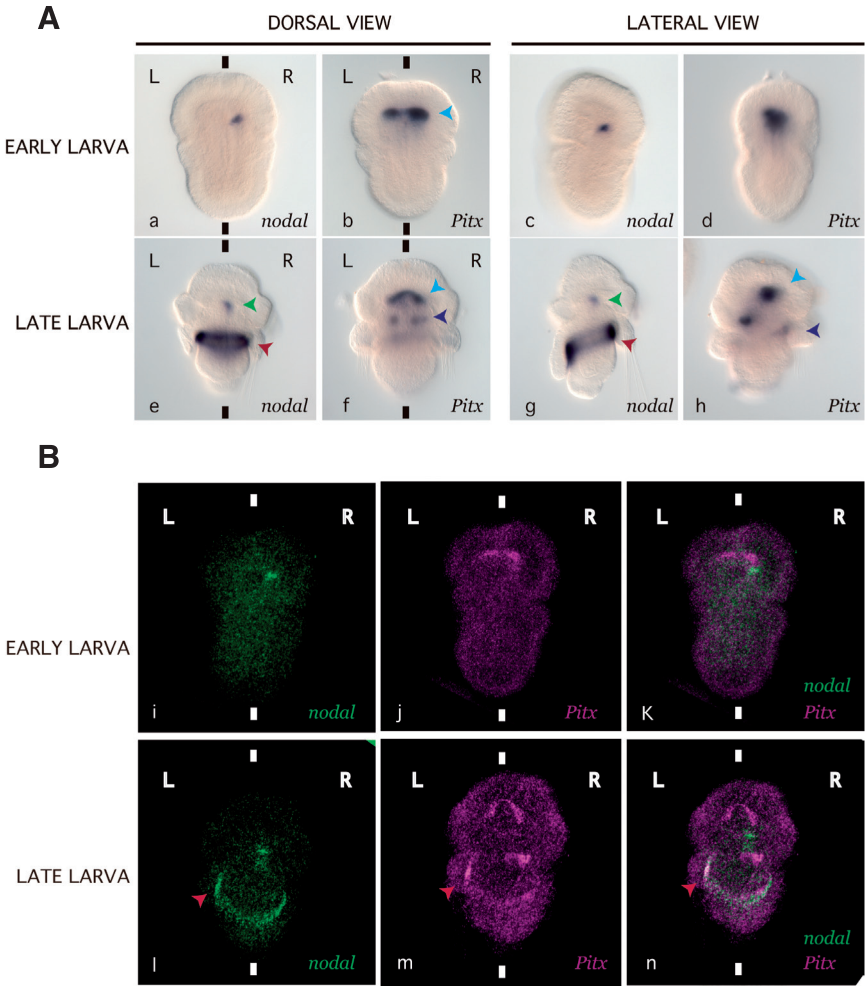


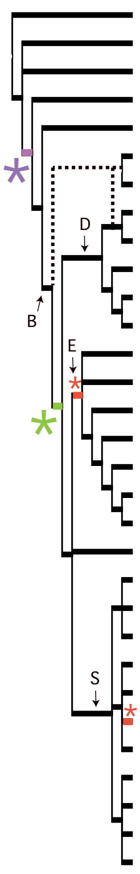
Fig. 3. Expression of *nodal* and *Pitx* in brachiopod embryos. (A) *Single colorimetric* in situ *hybridization for* nodal *and* Pitx *in the brachiopod* T. transversa. nodal *and* Pitx *are first detected in the early larva (a–d). At this stage,* nodal *is expressed in a subset of the anterior right mesoderm (a,c), while* Pitx *is detected in the entire anterior-most mesoderm (b,d), stronger in the region adjacent to the* nodal *expression domain (light blue arrowhead in b). As the larva matures,* nodal *is still detected in an anterior right mesodermal domain (green arrowhead in e,g), but* Pitx *is expressed in two ventral mesodermal domains and an asymmetric right dorsal domain (dark blue arrowhead in f,h), in addition to the anterior-most mesodermal domain already observed in the early larva (light blue arrowhead in f,h).* **(B)** *Double fluorescent* in situ *hybridization for* nodal *and* Pitx *in early and late larva in* T. transversa. *The simultaneous detection of* nodal *(green) and* pitx *(magenta) at these developmental stages demonstrates that their respective expression domains do not co-localize, but appear adjacent one to the other. In (A) panels e,g, and in (B) panels l,m,n, the red arrowhead indicates a region of probe trapping. R, right; L, left.*

of this pathway in protostomes.

Among deuterostomes, there is a single copy of the *nodal* gene in mammals, cephalochordates, urochordates, and echinoderms (Zhou *et al.,* 1993; Morokuma *et al.,* 2002; Yu *et al.,* 2002; Duboc *et al.,* 2004), whilst there are three copies in the hemichordate *Saccoglossus kowalevskii* (*nodal A*, *B*, and *C*) (Wlizla 2011), in zebrafish (*Cyclops, Squint*, and *Southpaw*) and six Nodal-related ligands in *Xenopus* (*Xnr1-6*) (Schier 2009). In spiralians, a single copy of *nodal* is present in all species examined to date, except the rotifer *A. vaga* and the gastropods *C. fornicata* and *P. vulgata*. *A. vaga* has four copies of the *nodal* gene, probably because the genomes of bdelloid rotifers show reduced tetraploidy (Flot *et al.,* 2013). Less easy to explain is the presence of two copies of *nodal* in the two distantly related gastropods *C. fornicata* (Caenogastropoda) and *P. vulgata* (Patellogastropoda). The fact that one single copy has been identified so far in other gastropods like the patellogastropod *Lottia gigantea* and the heterobranchs *Biomphalaria glabrata*, *Aplysia californica* and *Lymnaea stagnalis,* along with a lack of clear paralogy in our tree (Fig. 1) might indicate that two independent duplication events could have occurred in *P. vulgata* and *C. fornicata*. Wider genomic sampling across gastropods is necessary to test this hypothesis.

Ancestral expression domains and roles of Nodal

Previous studies in vertebrates have shown that there is a Nodal concentration and duration-dependent effect in responding cells (Gurdon and Bourillot 2001; Schier and Talbot 2005; Hagos and Dougan 2007). Therefore, the timing and expression levels of *nodal* are tightly regulated. In deuterostomes as well as in snails, the *nodal* gene is activated during cleavage stages, where it is critical in regulating gastrulation (Chea *et al.,* 2005; Grande and Patel 2009). However, the results presented here show that *nodal* is not active in brachiopods until larva stages, after gastrulation is complete and tissue layers are specified. Previously published data (e.g. Chea *et al.,* 2005; Grande and Patel 2009) suggest that the early action of this gene and its function in the regulation of the



	nodal				Pitx			
	Present/ Absent	Expression domains	Side of asym. expression	Proposed roles	Present/ Absent	Expression domains	Side of asym. expression	Regulated by nodal?
Choanoflagellata	Absent	-	-	-	Absent	-	-	No
Porifera	Absent	-	-	-	Absent	-	-	No
Ctenophora	Absent	-	-	-	Absent	-	-	No
Placozoa	Absent	-	-	-	Present	?	?	No
Cnidaria	Absent	-	-	-	Present	Oral ectoderm	Bilateral	No
Xenoturbella	Absent	-	-	-	Present	?	?	No
Acoelomorpha	Absent	-	-	-	Present	Neural precursors, neoblasts, mouth ring muscle and copulatory organs	Bilateral	No
Hemichordata	Present	Sym.: meso and ectoderm. Asym.: all three germ layers; then restricted to meso, ectoderm	Right	Posteriorization of ectoderm	Present	At the base of the proboscis on the dorsal midline. Later in a stripe along the ventral midline	?	?
Echinodermata	Present	Sym.: oral ectoderm Asym.: ectoderm	Right	Oral-aboral and LR patterning	Present	Right ectoderm near the tip of the archenteron, right celomic pouch	Right	Yes
Cephalochordata	Present	Sym.: dorsal lip, meso and ectoderm Asym.: endo, meso, ectoderm	Left	LR patterning; Mesoderm induction	Present	Gut diverticulum, Hatschek's pit, ecto and endoderm around the mouth	Left	Yes
Urochordates	Present	Sym.: endo, meso, ectoderm Asym.: ectoderm	Left	LR, neural patterning; Mesoderm induction	Present	Primordial pharynx, neural complex, sensory vesicle, mesenchyme, lateral ectoderm	Left	Yes
Vertebrata	Present	Sym. in the node. Asym.: mesoderm	Left	AP, LR, neural patterning; Endo, mesoderm induction	Present	Stomodeum, myotomes, eye, pituitary, maxillary process, lateral plate mesoderm	Left	Yes
Priapulida	Absent	-	-	-	Present	?	?	No
Nematoda	Absent	-	-	-	Present	GABAergic type D motor neurons	Bilateral	No
Tardigrada	Absent	-	-	-	?	?	?	No
Onychophora	Absent	-	-	-	?	?	?	No
Crustacea	Absent	-	-	-	Present	Posterior blastoderm, midgut, Malpighian tubules, somatic muscles, neural cells	Bilateral	No
Myriapoda	Absent	-	-	-	Present	?	?	No
Chelicerata	Absent	-	-	-	Present	?	?	No
Chaetognatha	Present	?	?	?	Present	?	?	?
Bryozoa	?	?	?	?	?	?	?	?
Cycliophora	?	?	?	?	?	?	?	?
Entoprocta	?	?	?	?	?	?	?	?
Gastrotricha	?	?	?	?	Present	?	?	?
Gnathostomulida	?	?	?	?	?	?	?	?
Platyhelminthes	Absent	-	-	-	Present	Serotonergic neurons	Bilateral	No
Rotifera	Present	?	?	?	Present	?	?	?
Annelida	Present	?	?	?	Present	?	?	?
Mollusca	Present	Asym.: Ectoderm	Dextral=Right Sinistral=Left	LR patterning	Present	Posterior dorsal and cephalic ectoderm, developing gut, visceral mass	Dextral=Right Sinistral=Left	Yes
Brachiopoda	Present	Asym.: Mesoderm	Right	?	Present	Anterior and posterior mesoderm	Right	Yes??
Nemertea	Present	?	?	?	Present	?	?	?

Fig. 4. Phylogenetic hypothesis of metazoan relationships and *nodal* and Pitx functions among Bilateria. *For each Phylum, presence and absence, expression domains and side of the body that expresses* nodal *and* Pitx *are shown. Proposed roles for* nodal *and the relationship between* nodal *and* Pitx *are also indicated. Absent means not found in genomic or RNA-seq datasets. Question marks: no information available. Boldface dashes: gene not reported in the group. E: Ecdysozoa; B: Bilateria; S: Spiralia; D: Deuterostomia. Coloured asterisks represent the inferred origins of* nodal *(green) and* Pitx *(purple) and hypothesized* nodal *losses in Ecdysozoa and Platyhelmintha (red). Dotted lines represent two alternative phylogenetic hypotheses: 1) Acoelomorpha +* Xenoturbella *are sister to remaining Bilateria (Hejnol* et al.*, 2009, Srivastava* et al.*, 2014) and 2) Acoelomorpha +* Xenoturbella *are sister to Ambulacraria (Philippe* et al.*, 2011). See text for details.*

gastrulation process may be the ancestral bilaterian condition. This implies that this has been secondarily modified in brachiopods, where *nodal* does not seem to have any role during gastrulation, and in those clades without a *nodal* gene (for example, in the Ecdysozoan lineage).

Inhibition of Nodal signalling causes disruption of germ layer induction of endoderm and mesoderm in vertebrates (Brennan *et al.,* 2001; Vincent *et al.,* 2003). However, current evidence suggests that this role is a vertebrate innovation, since normal specification has been reported in other deuterostomes (Duboc *et al.,* 2004; Morokuma *et al.,* 2002; Wlizla 2011), snails and brachiopods, as suggested by the late activation of *nodal* in the latter.

In addition, after gastrulation, *nodal* expression occurs in different germ layers depending on the group considered: the ectoderm in snails (Grande and Patel 2009), the ectoderm and endoderm in echinoderms (Duboc *et al.,* 2010; Ohguro *et al.,* 2011; Bessodes *et al.,* 2012), all three germ layers in cephalochordates, urochordates, and hemichordates, and the endomesoderm in vertebrates (Hamada *et al.,* 2002; Morokuma *et al.,* 2002; Yu *et al.,* 2002; Duboc *et al.,* 2005; Wlizla 2011). Here we have shown that in brachiopods *nodal* is restricted to the mesoderm. These data suggest that the expression domains of *nodal* have greatly diverged during the evolution of bilaterians in parallel with the diversification of its roles. With the current data, hypotheses supporting that the ancestral state is either mesodermal, ectodermal or ectoendomesodermal are equally parsimonious and only additional data in other spiralians and chaetognaths is likely to shed light on this issue.

Comparative studies have shown that there are several different early symmetry-breaking mechanisms among the Bilateria (Spéder *et al.,* 2007). However, in deuterostomes, snails and brachiopods, all these mechanisms converge in the asymmetric activation of Nodal signalling. While *nodal* expression in deuterostomes is initially symmetrical and depends on its own regulation in a positive and negative feedback loop to achieve an asymmetric pattern (Morokuma *et al.,* 2002; Yu *et al.,* 2002; Duboc *et al.,* 2005), *nodal* expression in snails and brachiopods is asymmetric from the onset, and at least in snails, it does not seem to be regulated by its own expression (Grande and Patel 2009). Further studies are needed to elucidate the ways in which asymmetric expression of *nodal* is activated in different bilaterian taxa, and its subsequent regulation.

Interestingly, there is variation in the localization of the asymmetric expression of *nodal* and the subsequent tissue-specific laterality decisions among different groups. Nodal signalling acts on the left side in chordates (vertebrates, cephalochordates and urochordates, Boorman and Shimeld 2002; Chea *et al.,* 2005; Morokuma *et al.,* 2002; Yu *et al.,* 2002) and on the right side in echinoderms and hemichordates, grouped together in the Ambulacraria, the sister group to the Chordata (Duboc and Lepage 2008). Hence, data from deuterostomes does not conclusively show the ancestral side of asymmetric expression domains, and additional data from outgroups is needed. Until now, the only *nodal* expression data on non-deuterostomes available was that from snails (Grande and Patel 2009). As previously explained, in snails the directionality of spiral cleavage at the third division defines the side of the embryo that expresses *nodal* and therefore the chirality of the adult snail. Right-handed spiral cleavage correlates with right-sided *nodal* expression and left-handed spiral cleavage correlates with left-sided *nodal* expression. It has been proposed that the ancestral snail had right-handed shell coiling (Ponder and Lindberg 1997) and therefore that the ancestral condition for snails was the expression of *nodal* on the right side of the embryo. The right-sided expression might be found in dextral snails or in spirally cleaving animals due to their mode of cleavage, rather than reflecting an ancestral bilaterian expression. Here, we show that the brachiopod *T. transversa* expresses *nodal* on the right side of its body, despite cleaving in a manner more akin to radial cleavage. This suggests that the right side expression of *nodal* is indeed the ancestral condition for spiralians and deuterostomes and that it was secondarily modified in the ancestor of all chordates.

The expression of *nodal* on the right body side in most animals outside the chordates has been used to support the hypothesis of inversion of the dorsoventral (DV) axis in the chordate lineage (Arendt and Nübler-Jung 1994; Denes *et al.,* 2007). This inversion resulted in the illusion of a reversal of the left and right axis, although the site of *nodal* expression within the body remained constant (for a more detailed explanation, see Namigai *et al.,* 2014). The side that expresses *nodal* will differentiate and acquire new features when compared to the other side of the midline. While Nodal regulates 'leftness' in mouse embryos, as inappropriate induction of *nodal* activity on the right side produces left-side morphologies (Hamada *et al.,* 2002), in echinoderms it regulates 'rightness' because Nodal signalling on the right side inhibits the formation of the rudiment in the larva (Duboc *et al.,* 2005). Therefore the opposite sides of *nodal* expression may have profound implications for the roots of morphological evolution in bilaterians.

Besides gastrulation and LR determination, Nodal signalling has functional significance in the regulation of the AP and DV axes in vertebrates, urochordates, cephalochordates and hemichordates (Schier and Shen 2000; Wlizla 2011) as well as the oral–aboral axis in echinoderms (Duboc *et al.,* 2004). By contrast, snails and brachiopods seem to define AP and DV axes independently of Nodal signalling, as these are defined in the former by the characteristics of spiralian cleavage, and in the latter, these axes are determined before the advent of *nodal* expression. Further studies in other spiralians as well as in chaetognaths will help to infer the ancestral role of the Nodal pathway in the polarization of embryos, and which roles are the result of independent co-option over evolutionary time.

Downstream of Nodal: lefty and Pitx

Although a variety of different transcriptional target genes of *nodal* have been reported in different deuterostome species, the TGF-β family member *lefty* is a common target in all of them. Nodal induces *lefty* expression, which in turn restricts the duration and the site of Nodal influence. This antagonistic interaction constitutes an example of the reaction-diffusion theoretical model (Kondo and Miura 2010). Due to its relevance as a Nodal target gene as well as its critical role in regulating Nodal signalling, we decided to check for the presence of *lefty* orthologs in other bilaterians outside the Deuterostomia. In spite of our intensive searches, no *lefty* ortholog has been found in any representative outside deuterostomes although *lefty* can be easily recognised if present, as its distinct sequence, and lack of one cysteine residue, make it identifiable both in alignments and in phylogenetic analysis. Therefore, the current data suggest that Lefty originated and was incorporated as a key regulator of the Nodal pathway in the deuterostome stem lineage.

Nodal signals activate asymmetric *Pitx* expression in deuterostomes and snails (Ryan *et al.,* 1998; Grande and Patel 2009), supporting the existence of the Nodal-Pitx cascade in the last common ancestor of deuterostomes, spiralians, ecdysozoans and chaetognaths (Fig. 4). Studies in deuterostomes and snails have shown that the asymmetric activation of *Pitx* is maintained once Nodal signals cease, and persists long enough to drive asymmetric organogenesis (Logan *et al.,* 1998; Essner *et al.,* 2000; Grande and Patel 2009). In addition to this asymmetric Nodal-dependent induction, *Pitx* is also detected in symmetric domains that persist even when Nodal function is down-regulated in both deuterostomes and snails. Therefore different, independent enhancers for *Pitx* were likely present early in bilaterian evolution, each driving a portion of its expression. Here, we have shown that *Pitx* in *T. transversa* also has both symmetrical and asymmetrical expression domains. Although functional experiments have not yet been performed, the data presented here may suggest that the asymmetric expression domain of *Pitx* is regulated by Nodal signals in brachiopods as it is in other bilaterian species.

Our phylogenetic analysis confirms the existence of *Pitx* orthologs in the Placozoa and Cnidaria, as well as in several groups of bilaterians (Fig. 2). As previously discussed, platyhelminthes and ecdysozoans seem to have lost their *nodal* ortholog but they have retained a *Pitx* ortholog. *Pitx* expression data have been recently described in the adults of planarian platyhelminthes, where it is required for the maintenance and regeneration of serotonergic neurons as well as for proper midline patterning during regeneration (Currie and Pearson 2013). Accordingly, we have also found expression of *Pitx* in isolated neurons in the ventral side of the embryo of the planaria *S. polychroa* in a bilateral, symmetric fashion (Supp. Fig. 2). In addition, *Pitx* expression has been reported for the ecdysozoans *C. elegans* and *Drosophila,* and while no asymmetrical expression pattern has been reported, *Pitx* has been related to neuronal cell differentiation (McIntire *et al.,* 1992; Vorbrüggen *et al.,* 1997). Moreover, *Pitx* expression in acoelomorphs has been detected in neural precursors (Hejnol and Martindale 2009) and in neoblasts (Chiodin *et al.,* 2013). In summary, current evidence supports the hypothesis that *Pitx* originated early outside the Bilateria, where it might have played a role in nervous cell differentiation. Once acoelomorphs and *Xenoturbella* split from the remaining bilaterians, Pitx acquired an additional role linked to the Nodal signalling pathway: regulation of LR patterning. Additionally, Pitx could have secondarily acquired a specific role in the regulation of the pituitary gland and its homologs in chordates (Boorman and Shimeld 2002).

Conclusions

Comparative developmental approaches are critical for understanding the evolution of animal form diversity. The variety of animal morphology has been shaped by a few signalling pathways, including Nodal signalling itself, that precisely regulate cell communication, division, migration, and death, among other mechanisms, throughout the evolution of metazoans. Recently, next generation sequencing techniques have allowed the production of genomic sequence data on non-model organisms at an unprecedented rate, greatly contributing to our knowledge of the diversity of signalling components across taxa and thus, to a more general understanding of their logic (Kenny *et al.,* 2014, this issue). For many years, studies of the Nodal signalling pathway have focused mainly on deuterostomes, although new genomic information presented here has shown that this pathway has indeed a broader distribution across bilaterians. While roles in gastrulation, germ layer specification and AP and DV axis specification have been described in only a subset of bilaterian clades, Nodal signalling has a critical role in LR patterning in the few bilaterians examined, suggesting that the molecular mechanisms integrating positional information of the pathway along this axis could have been established early in the evolution of bilaterians. The phylogenetic distribution of the use of the Nodal pathway for establishing asymmetries however, suggest that spiralians and deuterostomes have convergently incorporated the Nodal pathway into their development for this purpose. Additional evidence from the Spiralia, however, corroborating a role for the entirety of the pathway in a manner consistent with an ancestrally shared role would suggest that the last common ancestor of these two groups had its asymmetry patterned by this mechanism. Evidence such as that presented in this paper is therefore vital for fully understand the evolution of this undoubtedly key signalling pathway.

Here, we have described the timing and specific domain of expression of *nodal* in brachiopods. We have shown that *nodal* and *Pitx* are asymmetrically activated in the right mesoderm of the developing larva, suggesting a potential role in the development of asymmetric features. Although no asymmetries have been described for extant brachiopod species so far, consistent asymmetric folding of the commissure is a characteristic feature of a small but significant number of brachiopod fossil species (Fürsich and Palmer 2007). It remains to be seen what the specific role asymmetric activation of *nodal* and *Pitx* performs in brachiopod development and morphogenesis, but it is possible that its function is related to asymmetries in the derivatives of the mesoderm of the mantle lobe.

To date, no *nodal* ortholog has been detected in any representative of ecdysozoans or platyhelminthes. This evidence suggests that within bilaterians, the Nodal ligand has been independently lost in at least two lineages (Fig. 4). Further studies with more representatives of these groups will allow the dating of these losses. Organisms with morphological asymmetries but no Nodal ligand have to use different mechanisms to achieve their morphological asymmetries. For instance, actin and associated molecular motors such as myosins have been shown to be involved in LR determination in *Drosophila* (Spéder *et al.,* 2006, 2007). In addition, ion pumps and ion channels have been proposed to actively transport small left–right determinants in only one direction in vertebrates (Levin *et al.,* 2002) and could also be involved in early symmetry-breaking in nematodes (Huang *et al.,* 2007). However, the universality of such mechanisms is a subject of some debate, and is nowhere near as scientifically established as the role of the Nodal/Pitx cascade in establishing asymmetry.

Finally, although the Nodal-Pitx cascade seems well conserved across the Bilateria with the obvious exception of the Ecdysozoa and Platyhelminthes, this does not seem to be the case for its regulators. While current data have shown that highly elaborate mechanisms control the regulation and the target genes of this pathway in examined model organisms in the Deuterostomia, our results show that the key regulator Lefty, which controls many aspects of this process, is a deuterostome novelty. This raises many questions as to the identity of regulators of this key pathway in other bilaterians, and, ultimately, the ancestral form of the ancestral regulatory cassette controlling this fundamental signalling cascade, which are sure to be the focus of future research.

Materials and Methods

Gene identification

Potential *nodal* and *Pitx* orthologs were found in RNA-seq data, and complete genomic sources (Supp. Table1) using tBlastn (Altschul *et al.,* 1990) with conserved regions of known gene homology. In addition, the *nodal* gene for the mollusc species *C. fornicata* and *W. argentea*, the annelid *P. lamarckii*, the chaetognath *F. enflata* and the nermertean *C. lacteus* were first amplified with degenerate primers (with sequences available from the authors on request). The SMARTer™ RACE kit (Clontech) was used to amplify longer 3' and 5' sequence. In addition, the *Pitx* gene for the chaetognath *F. enflata* was first amplified with degenerate primers, with sequences available from the authors on request. Translation into protein sequence was carried out using MacVector v12.7 (Olson 1994), assuming standard codon usage.

Phylogenetic analyses

Phylogenetic analyses included the newly determined amino acid sequences of Nodal, as well as the known Nodal sequences of other spiralians and deuterostomes and other TGF-β superfamily members available from GenBank (Accession No. given in Supp. Table 2). In addition, the newly determined amino acid sequences of Pitx together with other Paired-class homeodomain containing genes of metazoans were subject to phylogenetic analysis (Supp. Table 2). Sequence alignments were visualized and aligned with Mesquite version 2.75 OSX (Maddison and Maddison 2011) and Clustal X version 2.1 (Larkin *et al.,* 2007). Conserved domains were identified and alignments trimmed to include only these areas for further analysis. Gene orthology was determined by phylogenetic analyses in BEAST v1.8.0 (Drummond *et al.,* 2012). The optimal substitution model for each gene was selected with Prot-test v.2.4 (Abascal *et al.,* 2005). In all cases, the best model selected was LG+I+G (Le and Gascuel 2008). For tree search, we used a birth-death prior under a strict clock model. The analyses were run for 20,000,000 generations and trees were sampled every 2,000 generations. Convergence of parameter estimates was visually checked in Tracer v1.5 (Rambaut and Drummond 2007). After a burn-in of 10%, all values had effective sample sizes (ESSs) above 200, indicating good mixing. The post-burn-in samples of trees were summarized with TreeAnnotator v1.8.0, distributed as part of the BEAST package.

Preparation and culture of embryonic material and whole mount in situ hybridization

Gravid *T. transversa* adults were collected from the coast near Friday Harbor Laboratories (USA) during their reproductive season and spawned as described previously (Santagata *et al.,* 2012). Embryos were collected at different stages of development up to the late larva stage. Fixation and *in situ* hybridization of embryos and larvae were performed following an established protocol (Passamaneck *et al.,* 2011; Santagata *et al.,* 2012). Planarian embryos were collected and in situ hybridization on these samples were performed as previously described (Martín-Durán *et al.*, 2010). Stained embryos and larvae were imaged with a Zeiss Axiocam HRc connected to a Zeiss Axioscope Ax10 using bright field Nomarsky optics. All images were analyzed with Photoshop CS5 (Adobe).

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