Cells & Development xxx (xxxx) xxx

Contents lists available at ScienceDirect

Cells & Development

journal homepage: www.journals.elsevier.com/cells-and-development

'Three signals - three body axes' as patterning principle in bilaterians

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ARTICLE INFO

Keywords:
FGF
Rspo2
axis formation
Spemann-Mangold organizer
Urbilateria

ABSTRACT

In vertebrates, the three orthogonal body axes, anteroposterior (AP), dorsoventral (DV) and left-right (LR) are determined at gastrula and neurula stages by the Spemann-Mangold organizer and its equivalents. A common feature of AP and DV axis formation is that an evolutionary conserved interplay between growth factors (Wnt, BMP) and their extracellular antagonists (e.g. Dkk1, Chordin) creates signaling gradients for axial patterning. Recent work showed that LR patterning in *Xenopus* follows the same principle, with R-spondin 2 (Rspo2) as an extracellular FGF antagonist, which creates a signaling gradient that determines the LR vector. That a triad of anti-FGF, anti-BMP, and anti-Wnt governs LR, DV, and AP axis formation reveals a unifying principle in animal development. We discuss how cross-talk between these three signals confers integrated AP-DV-LR body axis patterning underlying developmental robustness, size scaling, and harmonious regulation. We propose that *Urbilateria* featured three orthogonal body axes that were governed by a Cartesian coordinate system of orthogonal Wnt/AP, BMP/DV, and FGF/LR signaling gradients.

1. Introduction

The work of Hans Spemann and Hilde Mangold to whom this *Festschrift*-volume is dedicated, is inextricably linked to the inquiry into embryonic body axes formation. Body axes are imaginary reference lines used to describe the orientation and alignment of anatomical structures within an organism. Bilaterians have two main body axes. The anteroposterior (AP, oral-aboral, or long axis) axis signifies the direction from the head to the tail in animals and defining the front-to-back orientation of organs and body parts. The dorsoventral axis distinguishes the top-to-bottom orientation of anatomical structures. While Bilateria appear outside bilaterally symmetric, inside, many animals display variable levels of asymmetry. Thus, the LR body axis runs perpendicular to AP and DV axis and refers to the asymmetrical orientation and positioning of visceral organs and structures, such as heart, lungs, liver, and spleen, along the left and right sides of the main body axis.

Hans Spemann is best known for his work on the Spemann-Mangold organizer, which determines AP and DV axes. However, he also studied left-right (LR) body axis development exploring *situs inversus*, the inversion of LR organ asymmetry (reviewed in (Blum et al., 2009)). Spemann became interested in this topic while conducting constriction experiments in early amphibian embryos. Constriction experiments were popular in early experimental biology around 1900 following a famous experiment by German embryologist Hans Driesch in 1891. Working in sea urchin embryos, Driesch separated the two 2-cell stage blastomeres and obtained two complete but undersized sea urchins' larva. This result revealed embryonic regulation and showed, contrary to previous results of Wilhelm Roux, that the fate of each embryonic cell is not yet determined at the 2-cell stage. Following in these footsteps, Spemann separated the first two blastomeres of salamander eggs by constriction along the first cleavage furrow and obtained two complete twin embryos in the majority of cases (1901) (Spemann, 1901, 1902, 1903). However, he noted that the right twin tended to show *situs inversus* of the heart and the intestine. Spemann realized that *situs inversus* is an intriguing outcome whose analysis may provide insights into the underlying causes of these asymmetries, and concluded:

In cases of double and twin formations, which can be induced in Triton embryos by median incision or constriction, the left anterior end or the left twin exhibits the normal situs. In contrast, the right anterior end or the right twin often shows situs inversus. This asymmetry of the situs cannot arise only at the moment when it becomes externally visible; rather, it must originate from a typical asymmetry of the structure in earlier stages. It must already be present in the fertilized egg. [author's translation]. Spemann suggested that the cytoplasm of the unfertilized egg contains a bilateral-asymmetric 'microstructure' that

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https://doi.org/10.1016/j.cdev.2024.203944
Received 8 May 2024; Received in revised form 5 August 2024; Accepted 5 August 2024
Available online 8 August 2024
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determines LR asymmetry. Meanwhile, in snails and *C. elegans*, formins were identified to regulate LR patterning via the actin cytoskeleton (Abe and Kuroda, 2019; Middelkoop et al., 2021).

In 1906, Spemann induced *situs inversus* by manipulating neurula stage amphibian embryos. He removed the medial part of the medullary plate with underlying mesendoderm and replaced it after rotating it by 180 degrees. He obtained *situs inversus* not only of the intestine derived from the rotated mesendoderm but also of the heart, which was surprising, because the heart and its precursors were not touched during the operation. Thus, these experiments indicated that the positioning of the dorsal mesendoderm can influence that of the heart (Spemann and Falkenberg, 1919). Today we know that the lateral plate mesoderm that was likely associated with the rotated neural plate contains signaling molecules (e.g. Nodal) that regulate LR patterning. Finally, Spemann's student Hedwig Wilhelmi conducted extirpation experiments, aiming to identify LR determinants in salamander gastrulae. She observed that extirpation of a piece on the left side of the dorsal blastopore lip induced *situs inversus* (Wilhelmi, 1921). This region of the amphibian embryo coincides with the precursor of what today is known as the left-right organizer (LRO; see below) (Schweickert et al., 2010; Shook et al., 2004).

Mechanisms underlying LR axis determination are extensively reviewed (Forrest et al., 2022; Grimes, 2019; Hamada, 2020; Hamada and Tam, 2020; Little and Norris, 2021). Here, we focus on our recent findings that in the frog *Xenopus* an Rspo2-FGF system determines the LR vector (Lee et al., 2024). We address the evolutionary consequences of the emerging principle in animal development that the formation of the LR, DV, and AP axes is governed by a triad of anti-FGF, anti-BMP, and anti-Wnt signaling.

2. The LRO and ciliary fluid flow

The year 2023 marked the 25th anniversary of a landmark paper in LR axis formation (Nonaka et al., 1998). The Hirokawa group examined the process by which LR symmetry breaking occurs (Nonaka et al., 1998). They discovered in early mouse embryos, that the LR axis depends on a leftward fluid flow produced by motile cilia located in the node, a region now called the left-tight organizer (LRO).

The LRO is the embryonic structure where ciliary fluid flow breaks LR symmetry (Hamada and Tam, 2020). It is located in the embryonic midline: the ventral node in the mouse, the gastrocoel roof plate in *Xenopus*, and Kupffer's vesicle in zebrafish, all of which carry motile cilia that produce a leftward flow for LR patterning. However, not all vertebrates employ motile cilia and leftward flow for symmetry breaking as the LRO as e.g. pig and chick lack motile cilia (Gros et al., 2009). Human embryos on the other hand do employ ciliary flow for LR patterning, as deduced from the fact that cilia deficiencies lead to laterality defects and heterotaxy (HTX). These conditions are associated with a spectrum of abnormalities ranging from malformations to misarrangements of organs along the LR axis (Grimes and Burdine, 2017; Sutherland and Ware, 2009; Zhu et al., 2006). Notably, HTX is linked to congenital heart defects and organ dysfunction in live births (Zhu et al., 2006), emphasizing the medical importance of understanding LR specification mechanisms.

Leftward flow activates in the left lateral plate mesoderm (LPM) the Nodal-Pitx2 signaling cassette, a positive feedback loop that orchestrates organ situs development (Hamada, 2020). Yet, the first gene whose asymmetric expression is triggered by leftward flow is *Dand5/Cerl2/Charon,* which acts upstream of the Nodal-Pitx2 signaling cassette. Leftward flow induces asymmetric mRNA degradation of *Dand5* transcripts on the left LRO margin (Hojo et al., 2007; Maerker et al., 2021; Nakamura et al., 2012; Schweickert et al., 2010). *Dand5* encodes an antagonist of Nodal signaling and hence unilateral *Dand5* inhibition on the left activates the Nodal-Pitx2 signaling cascade LPM (Blum and Ott, 2018; Shiratori and Hamada, 2014).

While there is consensus that the ciliary flow-derived LR asymmetry pathway is common to fish, amphibians, and mammals, a key question is the mechanism whereby leftward flow acts mechanistically. A prediction of Nonaka et al. was that leftward flow produces a gradient of putative morphogen across the midline, which patterns the LR axis. This has been termed the chemosensation hypothesis (Hirokawa et al., 2006; Nonaka et al., 1998; Tanaka et al., 2005).

Simulations show that asymmetric transport of signaling molecules is a viable mode for breaking LR asymmetry (Ferreira et al., 2017). The distribution of fluorescently labeled proteins in mouse and rabbit LRO revealed that ciliary movement causes concentration gradients of extracellular proteins ranging from 15 to 50 kDa, with LR concentration variations of up to ~10-fold (Okada et al., 2005). Thus, leftward flow may create a signal concentration gradient to promote left-specific (sinistral) cell fates. To avoid ambiguities, we use the terms sinistral (left) and dextral (right).

The other main hypothesis explaining how leftward flow acts is mechanosensation, whereby immotile crown cell sensory cilia surrounding the node detect fluid flow (McGrath et al., 2003; Tabin and Vogan, 2003; Yoshiba et al., 2012; Yuan et al., 2015). Intracellular calcium flux downstream of flow, stronger on the left side of the node, is thought to downregulate *Dand5*. Studies on polycystic kidney disease indicate that PKD1 and PKD2 localized in cilia mediate flow sensation, and loss of PKD2 function results in defective situs determination. Additionally, the PKD paralogue PKD1L1 is essential for LR determination and can facilitate flow-dependent calcium signals in vitro (Field et al., 2011; Kamura et al., 2011; Tanaka et al., 2023). Recent work in mouse and fish has convincingly demonstrated that ciliary force sensing is necessary and sufficient for embryonic laterality (Djenoune et al., 2023; Katoh et al., 2023).

3. *Xenopus* Rspo2 is an FGFR antagonist that creates a dextrosinistral FGF signaling gradient

The chemosensation hypothesis for LR patterning has fallen in some disrespect because of the strong evidence supporting mechanosensation on the one hand and lack of a Hirokawa morphogen candidate on the other. Notably, any plausible LR morphogen should epistatically act downstream of leftward flow but upstream of asymmetric *Dand5* expression, which has not been shown for any secreted molecule.

We have recently introduced R-spondin 2 (Rspo2) as a candidate for a Hirokawa morphogen in *Xenopus* embryos. Rspo2 is member of a small protein family of secreted proteins that is best known as Wnt signaling agonists and potent stem cell growth factors (Box 1). *Rspo2* is expressed in the LRO and by gain- and loss-of-function experiments, it is necessary and sufficient for organ laterality and LR specification. Specifically, Rspo2 acts as sinistralizing signal and operates upstream of *dand5*, whose expression downregulates the induction of Nodal-Pitx2 cassette. Addition of Rspo2 protein can rescue *pitx2* expression in embryos when leftward flow is inhibited either mechanically by methylcellulose injection or by inhibiting ciliogenesis. Thus, Rspo2 fulfills key criteria for a Hirokawa morphogen. Given the prominent function of R-spondins in Wnt signaling, it came as a surprise that in LR patterning Rspo2 acts as an FGF receptor antagonist: Rspo2 via its TSP1 domain binds Fgfr4 and promotes its membrane clearance by ZNRF3-dependent endocytosis (Box 1). Thus, Rspo2 is not an instructive signal but instead it restricts the dextralizing function of FGF signaling. Concordantly, at flow-stage, FGF signaling acts dextralizing and, as per phospho-Erk staining, forms a MAPK signaling gradient across the LRO. The FGF signaling gradient is high on the dextral- and low on the sinistral side, with at least 6-fold phospho-Erk difference across the LRO. Similarly, steep morphogen gradients were reported in Wnt-AP body axis patterning (6-fold nuclear β-catenin) (Kiecker and Niehrs, 2001) and BMP-DV patterning (5-fold phospho-Smad5) (Zinski et al., 2017). The FGF signaling gradient is equalized when leftward flow is inhibited or Rspo2 is deficient at the LRO.

Is there a physiological relevance of FGF signaling difference across the LR axis manifesting as a gradient? At first glance, the LR axis is

distinguished from the other two body axes by its binary, quantal nature of handed laterality, questioning if it represents a legitimate body axis (King and Brown, 1999) and the relevance of a graded LR signal. However, evidence for a non-binary, quantitative nature of LR axis formation comes from patients with laterality defects and heterotaxy syndrome, also known as *situs ambiguous*. Mild laterality defects involve subtle abnormalities in the arrangement or orientation of thoraco-abdominal organs, such as minor variations in the positioning of organs. Severe laterality defects involve significant misplacement or malformation of organs, including *situs ambiguous* with multiple organ involvement, severe congenital heart defects, and anomalies like asplenia. Thus, heterotaxy syndrome exhibits a spectrum of LR defects, suggesting gradations rather than strict binary outcomes in visceral organ positioning (Forrest et al., 2022; Shapiro et al., 2014).

In summary, the three key insights from the recent work in *Xenopus* are: i) Rspo2 is a candidate Hirokawa-morphogen that promotes sinistral

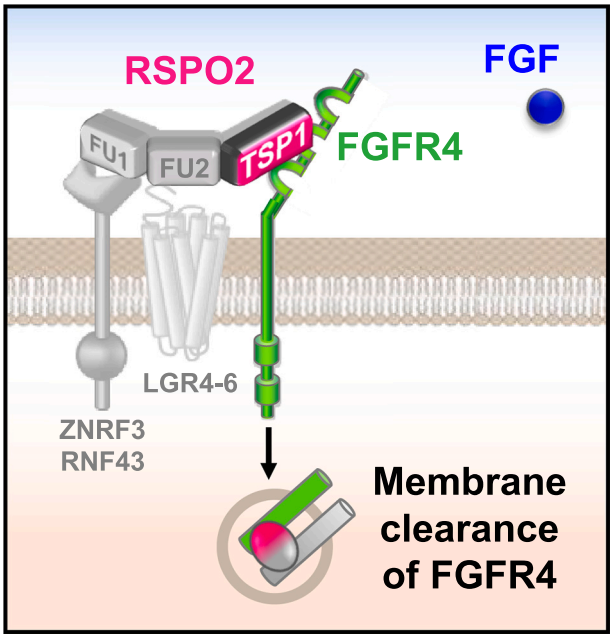
Box 1

R-spondins as multifunctional modulators of growth factor signaling

The R-spondin family consists of four secreted ~30 kDa proteins (RSPO1–4). They have a modular structure with two furin-like repeats (FU1, FU2) and a thrombospondin 1 (TSP1) domain (de Lau et al., 2014; Lee et al., 2020). RSPOs are known for enhancing Wnt signaling and affecting cell differentiation, proliferation, regeneration, tissue homeostasis, and cancer (Chartier et al., 2016; de Lau et al., 2014; Hao et al., 2016; Kazanskaya et al., 2004; Seshagiri et al., 2012). RSPOs also facilitate long-term Wnt-driven adult stem cell development, allowing organoids to be maintained (Huch et al., 2013; Sato et al., 2009). Mechanistically, the FU2 domain of RSPOs interacts with adult stem cell markers leucine-rich repeat-containing G-protein coupled receptors (LGR4-6) and recruits the transmembrane E3 ubiquitin ligase ZNRF3/RNF43 via the FU1 domain (de Lau et al., 2014; Hao et al., 2016; Hao et al., 2012; Koo et al., 2012). The ternary complex of RSPO-LGRs-ZNRF3/RNF43 sequesters ZNRF3/RNF43 for ubiquitination and lysosomal degradation. This releases Wnt receptors Frizzled and LRP5/6 from degradation, promoting their cell surface accumulation and enhance Wnt signaling (de Lau et al., 2014; Hao et al., 2016; Hao et al., 2012). In certain cases, LGR4–6 is dispensable for RSPO2 and RSPO3 to inhibit ZNRF3/RNF43 function and potentiate Wnt signaling (Lebensohn and Rohatgi, 2018; Szenker-Ravi et al., 2018).

In vivo roles of R-spondin 2

Rspo2 was first identified as a secreted Wnt agonist in Xenopus, and knockdown of *rspo2* causes muscle abnormalities (Kazanskaya et al., 2004). Zebrafish, *rspo2* null mutants show malformation of fin and rib skeleton (Tatsumi et al., 2014). *Rspo2* mutant mice display perturbed Wnt signaling and a broad spectrum of developmental defects, including distal limb truncation, craniofacial and laryngeal-tracheal malformation, hypoplasia and branching defects of the lung, kidney malformation, and defects in the ovarian follicle maturation (Bell et al., 2003; Bell et al., 2008; De Cian et al., 2020; Jin et al., 2011; Nam et al., 2007). A recent study identified *RSPO2* mutations in human fetuses with severe limb defects linking to LGR-independent role of RSPO2 in potentiating Wnt signaling (Szenker-Ravi et al., 2018). Rspo2 enhances not only canonical- but also non-canonical Wnt/planar cell polarity signaling, which is implicated in *Xenopus* morphogenesis and mouse osteoblast differentiation (Friedman et al., 2009; Ohkawara et al., 2011). Beyond acting as Wnt agonist, Rspo2 is also a BMP receptor type 1 A antagonist in *Xenopus* dorsoventral patterning (Lee et al., 2020). *Rspo2* is expressed outside the Spemann organizer and serves as a negative feedback inhibitor of *bmp4* to achieve robust ventralizing Bmp signaling during DV axial patterning. As discussed in this review, Rspo2 is also a FGFR4 antagonist in LR axis determination, by interacting with Fgfr4 via its TSP domain to form quaternary complex of Rspo2-Lgr-Znrf3-Fgfr4, leading to Fgfr4 endocytosis and degradation ((Lee et al., 2024) and Figure Box1). Moreover, in *Xenopus* gastrula, Rspo2 is reported to inhibit FGF2 signaling and required for mesoderm formation (Reis and Sokol, 2020).



Box 1.

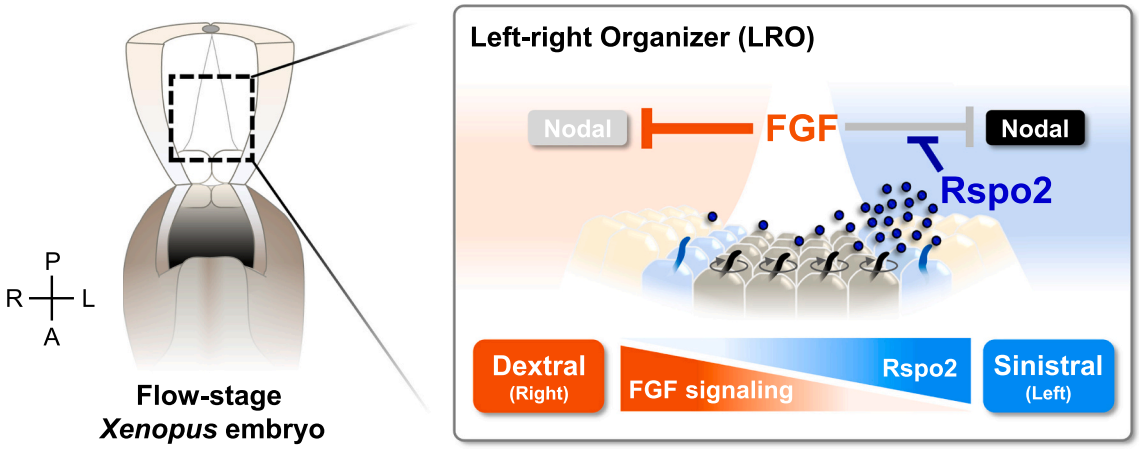


Fig. 1. Rspo2 creates an FGF signaling gradient during LR specification.
In the ciliary flow-stage *Xenopus* LRO, FGF signaling inhibits the Nodal-Pitx2 cascade. Rspo2 is transported by ciliary leftward flow to the left side of the embryo where it inhibits FGF signaling. This asymmetric anti-FGF function of Rspo2 establishes a dextrosinistral FGF signaling gradient across the LRO and activates the Nodal-Pitx2 cascade on the left. A, anterior; P, posterior; D, dorsal; V, dorsal; R, right; L, left.

fate in the LRO; ii) at flow stage, FGF is dextralizing; iii) leftward flow of Rspo2 creates the FGF signaling gradient (Fig. 1). What needs to be shown is whether leftward flow indeed accumulates Rspo2 protein at the left side of the LRO.

4. Evolutionary conservation of RSPOs and FGF signaling as symmetry-breaking signal

How conserved may FGF/anti-FGF signaling be in other vertebrates? In all vertebrate models, FGF signaling is involved in LR asymmetry (Boettger et al., 1999; Feistel and Blum, 2008; Fischer et al., 2002; Hamada and Tam, 2020; Meyers and Martin, 1999; Neugebauer et al., 2009; Oki et al., 2010; Schneider et al., 2019; Sivak et al., 2005; Tanaka et al., 2005; Yamauchi et al., 2009). However, a major problem in comparing its effects between and even within model systems is that LR development builds on a number of successive steps acting upstream of leftward flow, many of which depend on FGF signaling, including mesoderm formation, gastrulation, LRO specification, and ciliogenesis. Hence, the difficulty lies in separating indirect effects due to upstream FGF function from those that are associated with asymmetric dextro-sinistral FGF signaling in the LRO. This complexity has resulted in conclusions that appear contradictory since they stem from FGF manipulations where different developmental stages, processes, or regions were affected. To understand the impact of FGF signaling at flow phases, it is therefore essential to rule out earlier indirect effects. *Xenopus* offers the possibility of stage-, site-, and LR-specific experimentation, and employing inhibitory peptides to manipulate Rspo2-FGF antagonism (Lee et al., 2024).

4.1. Rabbit and chick

In chick, experiments with FGFR inhibitors and FGF8 bead implantations identified FGF8 as a dextralizing signal as in *Xenopus* (Boettger et al., 1999; Schlueter and Brand, 2009). Similar experiments in rabbit confirmed FGF8 as a dextralizing signal at flow stages, which blocks the Nodal-Pitx2 cascade (Boettger et al., 1999; Fischer et al., 2002). This has led to the *Release-of-Repression* (RoR) model in rabbit embryo (Feistel and Blum, 2008; Fischer et al., 2002), whereby FGF blocks the Nodal-Pitx2 cascade bilaterally, but ciliary flow unilaterally attenuates this repression on the left side. Our results agree with the RoR model and extend it by demonstrating an endogenous FGF signaling gradient and by providing a mechanism that explains it (Lee et al., 2024). However, chick unlike rabbit does not feature motile cilia. Instead, *Fgf8* expression in Hensen's node (organizer region) is dextrally biased.

4.2. Mouse

In mouse, FGF8 was proposed not as a dextralizing signal, as in frog, chick and rabbit, but as a sinistralizing signal (Meyers and Martin, 1999; Tanaka et al., 2005), apparently contradicting our model. However, the genetic evidence rests on constitutive *Fgf8* mutants that display axial abnormalities. Hence, we suspect that the mouse is not an "outlier" and the discrepancy is rather due to differences in embryonic stages when the analysis was conducted. Indeed, FGF signaling is required to induce the *Tbx6-Dll1-Nodal* cascade in the node during gastrulation and at early-somite stage (Oki et al., 2010). This study indicates that FGF signaling is required for *Nodal* expression in the node, i.e. upstream of leftward flow. In mouse *Rspo2* (Szenker-Ravi et al., 2018) and *Fgfr4* (Weinstein et al., 1998) mutants, LR defects have not been reported but laterality defects could have been missed, either because they may not affect viability or because of gene redundancy. *Rspo2* mutants show hindlimb truncations and their severity is heavily biased to the left hindlimb (Nam et al., 2007). Moreover, human *RSPO2* deficiency causes congenital heart defects (Szenker-Ravi et al., 2018), a condition associated with LR misregulation (Sutherland and Ware, 2009).

4.3. Zebrafish

FGF signaling is important for LR patterning in zebrafish embryos, through its involvement in the activation of Nodal signaling and formation of Kupffer's vesicle (KV), a key organ required for the left-right asymmetric body plan (Hong and Dawid, 2009; Matsui et al., 2011; Neugebauer et al., 2009; Neugebauer and Yost, 2014; Xu et al., 2010). However, its exact role during symmetry breakage is unclear. The difficulty lies again in bypassing early stage FGF requirement. Overwhelmingly, analyses have been conducted with manipulation starting at early cleavage stages or with constitutive Fgf-, and Fgfr mutants where confounding secondary effects of FGF signaling on mesoderm formation and ciliogenesis can occur (Draper et al., 2003). Furthermore, there is functional redundancy among Fgfs in zebrafish acting during early mesoderm development (Draper et al., 2003). In *rspo2* deficient zebrafish embryos, neither LR- nor laterality defects were reported (Tatsumi et al., 2014).

4.4. Sea urchin

A LR axis that employs the *Nodal-Pitx2* cassette is deeply conserved in the animal kingdom and was likely present in the bilaterian ancestor (Grande and Patel, 2009a). Concordantly, sea urchins (*Paracentrotus*

lividus) feature a LR axis and an LRO that employs cilia and the *Nodal-Pitx2* cassette for LR specification (Duboc et al., 2005; Tisler et al., 2016). Sea urchins only have a single *Rspo2-like* gene (e.g. Pliv13037.1 in *P. lividus*) and its role in LR specification has not been investigated. Pharmacological inhibition of FGF signaling in early embryos with SU5402, which primarily inhibits FGFR1 and − 2, abolishes right-sided *nodal* expression altogether. Interestingly, at later stage, SU5402 treatment may lead to randomization and bilateral *nodal* expression (Bessodes et al., 2012), as predicted by our model.

4.5. Phylogeny of R-spondins

The R-spondin family is defined by the presence of two Furin domains followed by a C-terminal Thrombospondin 1 (TSP1) domain. Database searches identify proteins with this architecture in all vertebrates and chordates (e.g. lancelet, *Branchiostoma floridae*), hemichordates (acorn worm, *Saccoglossus kowalevskii*) and echinoderms (e.g. sea urchin, *Strongylocentrotus purpuratus*) (Fig. 2A). In contrast to the widespread distribution of the RSPO family in deuterostomes, in protostomes, RSPO is absent in ecdysozoans, which include well-studied *Drosophila* and *C. elegans*. In contrast, a single RSPO homolog occurs in lophotrochozoans, most prominently in molluscs. This phylum also features a pronounced LR-like body axis asymmetry, which in snails employs the conserved Nodal-Pitx2 cassette for LR patterning (Grande and Patel, 2009b). Indeed, there is evolutionary co-occurrence of RSPO homologs with the Nodal-Pitx cassette (Fig. 2B). Because the Nodal-Pitx cassette occurs in protostomes and deuterostomes, their last common ancestor, *Urbilateria* (De Robertis and Sasai, 1996), is thought to have employed it to specify a primitive LR axis (Blum et al., 2014; Grande and Patel, 2009a). According to this notion, like so many other bilaterian genes (Ball et al., 2004; Kusserow et al., 2005), the Nodal-Pitx cassette was lost in ecdyosozoan ancestors. Similarly, RSPOs phylogeny indicates that it existed before the protostome–deuterostome split and hence in *Urbilateria*. The phylogeny is consistent with early loss in the ecdyosozoan lineage and two separate genome duplications that subsequently produced the extant family of four *R-spondins* present in most vertebrates.

Instead of the Nodal-Pix cassette, ecdyosozoan *Drosophila* employs actin-based molecular motor Myosin 1D (Myo1D) pathway to break LR symmetry of the gut and male genitalia (Géminard et al., 2014; Hamada and Tam, 2020; Hozumi et al., 2006; Spéder et al., 2006). The Hox family transcription factor Abdominal-B (Abd-B) induces *Myo1D* expression in *Drosophila* organ LRO (Coutelis et al., 2013). Unlike RSPO2 and the Nodal-Pitx cassette, which determines sinistral fate, the Abd-B-Myo1D cassette predominantly determines dextral fate of these organs and by regulating the chirality of actomyosin cytoskeleton. Of interest, Myo1D is also required in zebrafish and *Xenopus* LR asymmetry, however, by regulating the LRO morphogenesis (Juan et al., 2018; Tingler et al., 2018).

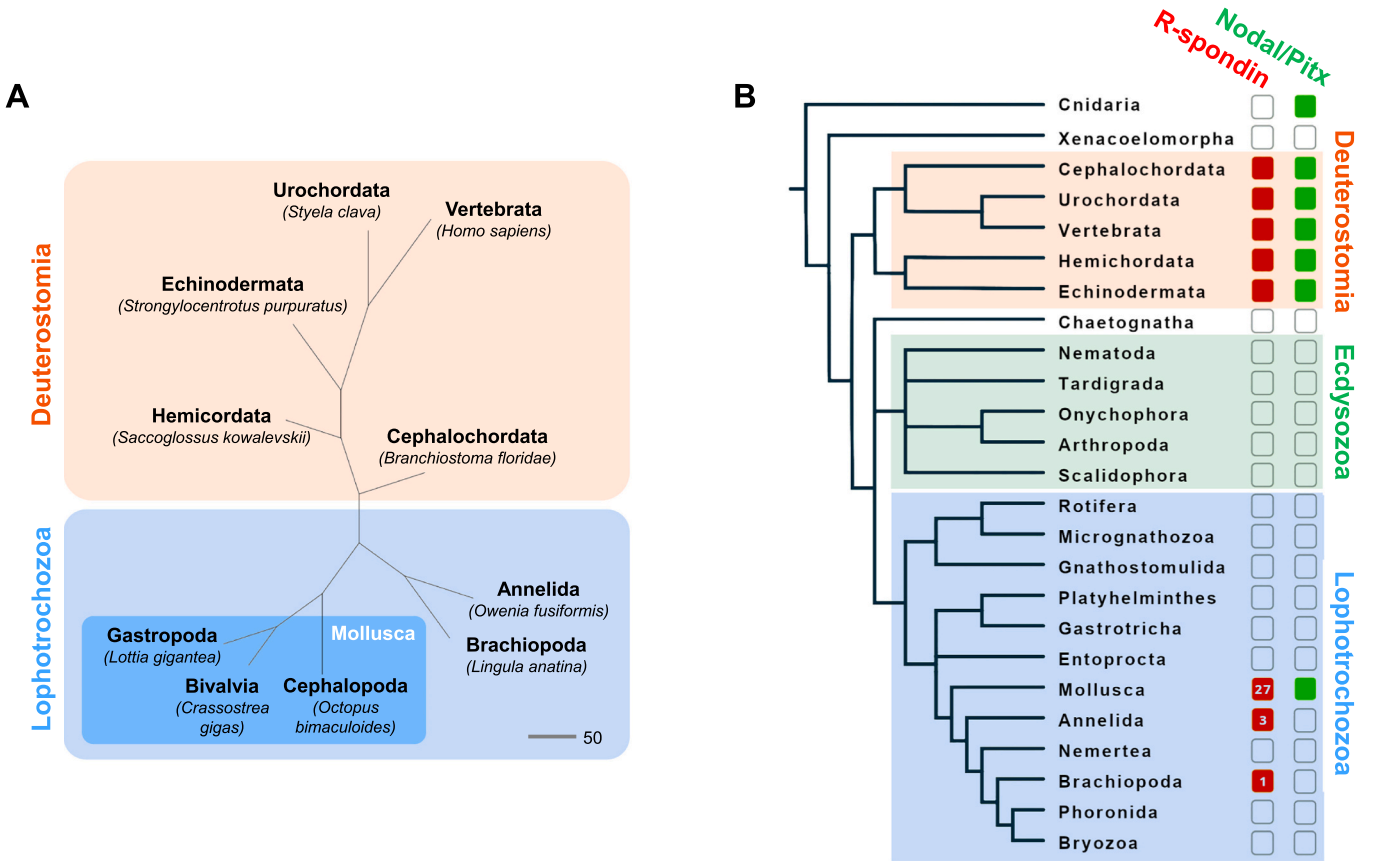


Fig. 2. R-spondin evolution.

(A) R-spondin phylogeny. Unrooted maximum parsimony phylogenetic tree of the R-spondin orthologs showing relatedness and animal subphylum, with representative species in brackets. Scale bar indicates the number of changes per sites. The tree was generated with R library phangorn (doi:https://doi.org/10.1093/bioinformatics/btq706).

(B) R-spondin and Nodal/Pitx co-occurrence in bilaterians. Phylogenetic tree as proposed by Vellutini and Hejnol (Vellutini and Hejnol, 2016). Colors indicate a distinct clade or superphylum. Occurrence of R-spondin orthologous as per this study; occurrence of Nodal/Pitx according to references (Chea et al., 2005; Grande and Patel, 2009b; Kaul-Strehlow and Stach, 2013; Watanabe et al., 2014).

5. A triad of anti-FGF, anti-BMP, and anti-Wnt governs LR, DV, and AP axis formation

A common feature of dorsoventral (DV) and anteroposterior (AP) body axis formation is that the interplay between growth factors and their extracellular antagonists brings about axial specification. Thus, the Chordin-BMP interaction establishes DV patterning while Dkk1-Wnt interaction regulates AP patterning (De Robertis and Tejeda-Munoz, 2022; Hikasa and Sokol, 2013). In both AP and DV patterning, the interaction between the growth factors and their extracellular antagonists creates a signaling gradient along the respective body axis, which provides positional information that is converted into distinct cell fates (Rogers and Schier, 2011). The role of the antagonists is to create the signaling sink and to shape the signaling gradient. Wnt and BMP both employ autoregulatory feedback loops that impart regulation upon size- or other fluctuations such as developmental noise (Ben-Zvi et al., 2008; Hill and Petersen, 2015; Inomata et al., 2013; Leibovich et al., 2018; Reversade and De Robertis, 2005). Moreover, AP/DV body axis patterning are both deeply conserved during evolution, present in the sister group of Bilateria, the cnidaria, where they regulate the oral-aboral axis (Wnt-Dkk1) and orthogonally, the directive axis (Chordin-BMP) (Holstein, 2022).

LR body axis determination on the other hand is assumed to operate via a radically different, mechanosensory mechanism (Djenoune et al., 2023; Katoh et al., 2023). However, it now emerges that LR axis formation after all involves the same principle as AP/DV body axis patterning, with the sinistrodextral vector being determined by an Rspo2-FGF system. Similar to Dkk1 (Mao et al., 2001), Rspo2 acts by inhibiting the growth factor receptor (Dkk1 via LRP6, Rspo2 via Fgfr4) rather than the ligand. Rspo2 action creates an FGF signaling gradient across the midline that regulates the LR axis. Thus, in *Xenopus,* a triad of anti-FGF, anti-BMP, and anti-Wnt signaling governs LR, DV, and AP axis formation (Fig. 3A). Of note, as in BMP and Wnt signaling, where a cocktail of antagonists is involved besides Chordin and Dkk1 that are not discussed here for simplicity, also FGF/LR signaling may involve additional antagonists besides Rspo2.

This triad reveals a simple principle and unity in animal development: Three body axes controlled by three growth factors whose signaling gradients are shaped by extracellular antagonists. This principle stands in contrast to the haphazard, tinkering mode whereby molecular evolution appears to often proceed (Jacob, 1977). Notably, analysis of some of the best-understood model organisms, *Drosophila* and *C. elegans*, indicated that determination of each body axis features a distinct and evolutionary non-conserved mechanism. *Drosophila* does without Wnt/AP signaling axis and instead employs a hierarchy of *bicoid*, gap-, pair-rule-, and segment polarity genes that is not conserved beyond insects (Fonseca et al., 2009; Peel et al., 2005). Nematode development is a prime example for how body axes are patterned without long-range signaling gradients. Instead, in the early *C. elegans* embryo, a series of asymmetric divisions establish the AP, DV and LR body axes (Gonczy and Rose, 2005). This mode of development may be an adaptation in metazoans that are microscopically small. Of note, it would be interesting to know how the largest known nematode, *Placentonema gigantissima*, although it is an unlikely model organism, patterns its body axes: This worm lives as a parasite in the placenta of whales and is up to 8 m long and 2.5 cm wide (Gubanov, 1951).

By comparison to the diversified mechanisms of body axis formation in flies and worms, the triad principle emerging from frogs appears as if fashioned by 'intelligent design'. We suggest that it occurred already in *Urbilateria* (Fig. 3B). This proposition is based on i) the deep conservation of anti-Wnt/AP and anti-BMP/DV patterning predating the deuterostome-protostome split, ii) the likely presence of the Nodal-Pitx cassette specifying sinistral fate in *Urbilateria*, and iii) the suggestive co-occurrence of RSPO with the Nodal-Pitx cassette in animals exhibiting prominent LR asymmetry. Accordingly, we propose that Wnt, BMP, and FGF gradients controlled the AP, DV and LR axes in *Urbilateria*. Supporting this notion, the cnidarian *Hydra* employs both the Nodal-Pitx cassette as well as FGF signaling for bud formation and biradial asymmetry, with the Nodal-Pitx cassette promoting bud formation and FGFR signaling promoting bud detachment (Sudhop et al., 2004; Watanabe et al., 2014).

The absence of BMP, Wnt, and FGF gradient systems as well as of the

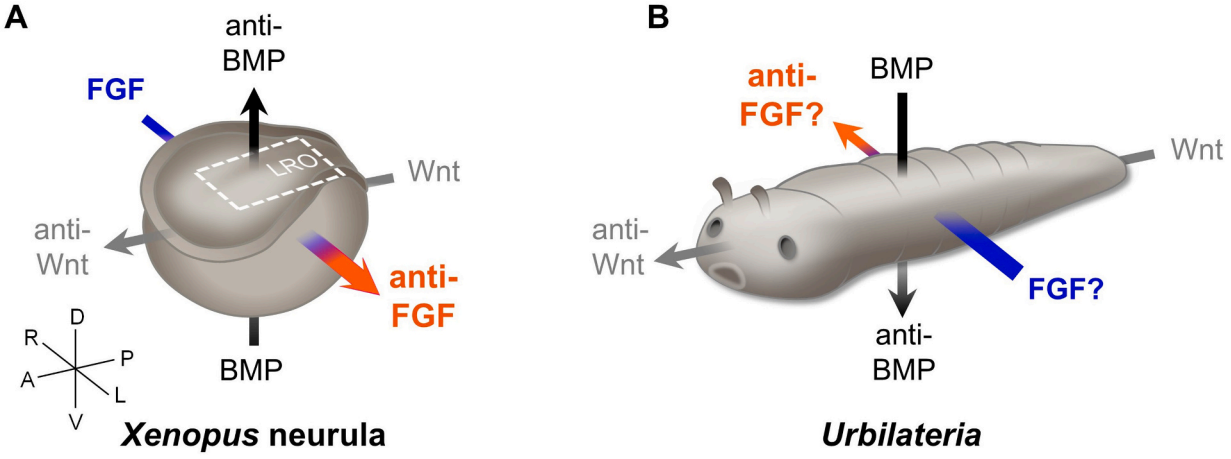


Fig. 3. A triad of FGF, BMP, and Wnt gradients patterns the three body axes.
(A) *Xenopus* embryo. In *Xenopus* AP axis formation, the interaction between posterior Wnt growth factors and anterior Wnt antagonists (e.g. Dkk1, Cerberus) establishes regional identities along the long axis. DV axis formation is driven by a BMP signaling gradient formed perpendicular to the AP axis by the interaction of ventralizing BMP growth factors and their dorsalizing antagonists (e.g. Chordin, Noggin). The LR axis is perpendicular to both the AP and DV axes. FGF signaling specifies dextral fate and is antagonized by the FGFR antagonist Rspo2, which specifies sinistral fate and creates a LR signaling gradient. Collectively, these three signaling gradients serve as a Cartesian coordinate system of positional information during body axis formation. For clarity, the model is a highly simplifying abstraction that projects signals acting continuously during gastrulation and neurulation onto a neurula stage embryo and omits many other signals such as Nodals, Notch, Shh, or Retinoic acid. A, anterior; P, posterior; D, dorsal; V, dorsal; R, right; L, left.
(B) A Cartesian coordinate system for body axis patterning in *Urbilateria*. Speculative model illustrating three perpendicular morphogenetic gradients of Wnt, BMP, and FGF. These gradients controlled AP, DV and LR body axis formation and they were created by growth factor antagonists including Dkk1 and Chordin already present in *Urbilateria*. Of note, *Urbilateria* is predicted to have a ventral nerve cord. Hence, the DV axis is formed by the interaction of dorsalizing BMP and ventralizing Chordin, which is opposite to the situation in *Xenopus*.

Nodal-Pitx cassette in various ecdysozoan phyla can be explained by widespread loss during evolution. Simple metazoans have a higher gene diversity than many evolved taxa (Ball et al., 2004; Kusserow et al., 2005). Likewise, axis formation in cnidarians is more comparable to vertebrates than to flies and worms (Holstein, 2022).

Our hypothesis is testable, e.g. by analyzing if FGF signaling represses the Nodal-Pitx cassette in snail or sea urchin, where it LR patterning well characterized (Duboc et al., 2005; Grande and Patel, 2009b; Tisler et al., 2016). Likewise, the role of RSPO could be tested in invertebrates, including its expression, ability to antagonize FGF signaling (which in vertebrates is specific to Rspo2 but not Rspo1,3,4), and functional involvement in LR patterning.

6. A Cartesian coordinate system for axial patterning

Inspired by classical double gradient models of axis formation such as those from Dalcq and Pasteels (Dalcq and Pasteels, 1938), Yamada (Yamada, 1950), and Toivonen and Saxén (Saxen, 2001) (reviewed in Gilbert (Gilbert, 2024)), we have previously proposed that orthogonal Wnt/AP and BMP/DV signaling gradients serve as a pan-bilaterian Cartesian coordinate system of positional information during body axis formation (Niehrs, 2010). Classic work by D'Arcy Thompson showed that transformations in a Cartesian coordinate system could explain morphological variations and of body shape across species (Thompson, 1917). Thompson used a geometric system of Cartesian coordinates to describe transformations in the size and shape of organs and organisms, suggesting that these transformations occur within a morphological space. By applying transformations within this space, diverse morphologies seen across species could be realized. Thompson noted that body form variations occur as though "the living body is one integral and indivisible whole," consistent with a global regulator, such as morphogenetic gradients (Child, 1941). However, Thompson's work focused on adult shapes and ignored embryonic development, from where adult shape changes ultimately arise as the result of differential cell growth (Briscoe and Kicheva, 2017). Notably, what remained unexplained at the time was the physical nature of the space coordinates since the molecular identity of morphogens was unknown.

The proposition of a Wnt/AP and BMP/DV Cartesian system builds on a large body of work on the roles of Wnt in specifying the AP, and BMP the DV body axis, both of which have been extensively reviewed (e.g. (Bier and De Robertis, 2015; Dale and Wardle, 1999; Holstein, 2022; Tuazon and Mullins, 2015; Umulis et al., 2009; Zakin and De Robertis, 2010)). In brief, during vertebrate gastrulation and early neurulation, Wnt/AP and BMP/DV gradients pattern the entire neural plate (Barth et al., 1999; Kiecker and Niehrs, 2001; Marchant et al., 1998; Nakamura et al., 2023; Wilson et al., 1997), specify mesoderm (Dal-Pra et al., 2006; Dosch et al., 1997; Nordstrom et al., 2002; Stickney et al., 2007), and control morphogenesis and cell migration (Myers et al., 2002; von der Hardt et al., 2007). We now extend this proposition by including an FGF/LR axis (Fig. 3B). Obviously, these signals act not in isolation but in concert with other growth factor cascades in cell specification, e.g. Hedgehog, and Notch (Favarolo and Lopez, 2018; Guzzetta et al., 2020; Krebs et al., 2003).

7. Orthogonal signaling gradients to convey developmental robustness and canalization

If a Cartesian coordinate system of Wnt/AP and BMP/DV and maybe FGF/LR signaling gradients is deeply conserved, what are the features of this morphogenetic program that render it adaptive?

Since Alan Turing's work (Turing, 1990), the autoregulatory system properties and 'evolvability' of morphogen gradients have been widely recognized (Ben-Zvi et al., 2011b; Shilo and Barkai, 2017; Simsek and Ozbudak, 2022; Wartlick et al., 2009). Morphogen gradients create a self-regulatory coordinate system of monotonically changing 'positional information' (Wolpert, 1969) wherein responsive cells receive a space coordinate with respect to the three body axes. This information orchestrates development by regulating growth, polarity and migration, as well as differentiation of cells as an integrated whole. Morphogen gradients not only regulate formation of the body axis during early development but also patterning of individual organs, such as the neural tube in vertebrates or the wing, leg, and eye in *Drosophila* (Miguez et al., 2020; Sagner and Briscoe, 2019; Strigini and Cohen, 1999).

One key benefit of morphogen gradients is that they convey developmental robustness. The term refers to the ability of an organism or embryo to maintain reproducible development despite a plethora of sources for variation that may affect developmental processes, such as genetic heterogeneity, environmental fluctuations (e.g. egg size, temperature, salinity, toxic natural products, humidity, UV light), or stochastic events (Keller, 2002). It has been argued that the capacity to stay "on track" despite the myriad vicissitudes that plague a developing organism—is a key to biological development (Keller, 2002). Developmental robustness is closely related to Waddington's 'canalization', whereby embryonic development follows a stable trajectory despite genetic variations and environmental fluctuations (Waddington, 1942). Waddington visualized this concept through his famous epigenetic landscape model, where development is guided along stable paths (canals) despite potential perturbations.

Indeed, volumes of literature are dedicated to the phenomenon of developmental regulation, the embryos ability to adapt and compensate for alterations in their developmental trajectories following perturbation. Morphogen gradients have long been recognized for their ability to convey regulation and adaptation. Just like trees sway and flex in all directions to absorb wind force without breaking, integrated signaling networks flexibly adjust their activity levels and interactions to withstand external pressures, ensuring their resilience and continued function.

7.1. Developmental robustness & spatiotemporal dynamics

Morphogen gradients exhibit spatiotemporal dynamics that enable precise interpretation. Cells can sense and respond to changes in morphogen concentrations over time, allowing for adaptive cellular behaviors and differentiation patterns in response to perturbations. The underlying mechanisms are positive and negative feedback control, which allow for precise regulation of morphogen distribution and concentration within tissues (Ben-Zvi et al., 2011b; Shilo and Barkai, 2017; Simsek and Ozbudak, 2022; Wartlick et al., 2009). This feedback maintains the stability and robustness of the gradient, ensuring accurate positional information for cell fate specification and tissue patterning. For example, morphogen gradients convey scaling, where embryos maintain consistent proportions and patterns during development, despite changes in overall size, e.g. due to different-sized eggs (Ben-Zvi et al., 2008).

Signaling gradients help explain how organs and organisms cease growth once they attain their characteristic size and shape. In the *Drosophila* wing, the Wg – Dpp double gradient crucially regulates growth and shape (Ben-Zvi et al., 2011a; Fried and Iber, 2014; Hamaratoglu et al., 2011; Kicheva et al., 2014; Schwank and Basler, 2010; Wartlick et al., 2011). Inhibiting either pathway diminishes the wing, while excess Wg or Dpp induces additional growth or duplication. *Drosophila* wing cells perceiving varying Dpp levels stimulate proliferation, while uniform pathway activation inhibits proliferation. Thus, the slope of the Dpp morphogen gradient, not its absolute level, dictates cell proliferation, conveying developmental robustness.

7.2. Developmental robustness through integrated AP-DV-LR body axis patterning

Developmental organizers are signaling hubs that orchestrate development as an integrated whole, a key to developmental robustness and canalization. Besides the Spemann-Mangold organizer (De Robertis,

2009), the mid-hindbrain organizer (Nakamura et al., 2005), as well as the zone of polarizing activity (ZPA) and apical ectodermal ridge (AER) in the vertebrate limb (Anderson and Stern, 2016) duplicate upon transplantation a harmoniously patterned secondary field (twinning). They can regulate when perturbed and their deletion may affect patterning of the entire field they control. That is, experimental perturbation of one axis typically affects the other body axis as well, leading to an integrated, harmonious response (Tuazon and Mullins, 2015). To achieve this, there is ample cross-talk between organizer signals, e.g. Wnt-BMP in AP-DV in axial patterning (Clark and Petersen, 2023; Fuentealba et al., 2007; Kraus et al., 2016) and SHH-BMP-FGF8 in limb patterning (Zuniga and Zeller, 2020). Certain growth factor antagonists embody signal integration in one molecule, for example, Cerberus secreted from the head organizer inhibits BMP, Nodal and Wnt signaling to induce dorsoanterior mesendoderm (Bouwmeester et al., 1996; Piccolo et al., 1999) (Fig. 4). Thus, perturbation affecting one signal is transmitted to others, leading to harmonious adaptation. For a review of body axis signal integration in early zebrafish embryos see (Tuazon and Mullins, 2015).

How is the LRO integrated with the Spemann-Mangold organizer? While the LRO (i.e. posterior gastrocoel roof plate of the early neurula in case of *Xenopus*) forms after axial AP/DV patterning is already well underway, Otto Mangold's Einsteck-experiments showed that this material still has tail-inducing activity (Mangold, 1933). This is because axis determination is a continuous process (Bolkhovitinov et al., 2022; Gont et al., 1993; Hashiguchi and Mullins, 2013; Knezevic et al., 1998) with BMP and Wnt antagonists Chordin and Dkk1 being expressed during neurula stages (Glinka et al., 1998; Sasai et al., 1994). Concordantly, LRO formation depends on the Spemann-Mangold organizer, providing integration of LR with AP-DV patterning (Colleluori and Khokha, 2023; Griffin et al., 2018; Walentek et al., 2012). Moreover, FGF coordinates AP-DV axis formation (Furthauer et al., 2004; Hashiguchi and Mullins, 2013; Kjolby et al., 2019; Pera et al., 2003) as well as LR patterning (Boettger et al., 1999; Feistel and Blum, 2008; Fischer et al., 2002; Hamada and Tam, 2020; Meyers and Martin, 1999; Neugebauer et al., 2009; Oki et al., 2010; Schneider et al., 2019; Sivak et al., 2005; Tanaka et al., 2005; Yamauchi et al., 2009), thus affecting all three body axes. Similarly, Activin/Nodal signaling dose-dependently induces a dorsoventral suite of mesendoderm, which in turn produces the growth factors and antagonists that determine all three body axes (Greenfeld et al., 2021; Hill, 2022; Schier, 2009; Schweickert et al., 2017; Soh et al., 2020; Xu et al., 2014). In mouse, effective leftward flow requires posterior tilting of cilia, which depends on AP axis formation, providing LR-AP axis integration (Nonaka et al., 2005). In *Hydra*, (oral-aboral) Wnt/β-catenin signaling cross-talks with the Nodal cassette (Watanabe et al., 2014). Finally, *Xenopus* Rspo2 affects three signals in one molecule: it amplifies Wnt and inhibits FGF as well as BMP signaling in the LRO, where posteriorizing Wnt, ventralizing BMP, and lateralizing FGF coincide (Kiecker and Niehrs, 2001; Lee et al., 2020; Reis and Sokol, 2020; Reversade et al., 2005; Schneider et al., 2019) (Fig. 4).

In summary, these system properties of orthogonal Wnt-BMP-FGF axial signaling gradients, notably signal integration, may collectively contribute to developmental robustness and canalization for harmonious AP-DV-LR patterning.

8. Conclusion

Reconstructing what the last common ancestor of bilaterians, or *Urbilateria*, looked like and the molecular toolkit that shaped its development is an intriguing question. 100 years after the description of the Spemann-Mangold organizer, elucidation of its molecular nature has led to profound insights regarding the evolution of body axis formation. Comparative molecular embryology supports that *Urbilateria* had an AP and DV body axis that was specified by orthogonal Wnt and BMP signaling gradients, whose shape and polarity were controlled by growth factor antagonists (De Robertis and Tejeda-Munoz, 2022). Recent findings in *Xenopus* revealed a dextrosinistral FGF signaling gradient, the conservation of FGF as dextralizing signal in chick, rabbit and frog, and Rspo2 as sinistralizing FGF antagonist in *Xenopus*. Thus, a triad of anti-FGF, anti-BMP, and anti-Wnt governing LR, DV, and AP axis formation emerges as unifying principle in *Xenopus,* with extensive signaling cross-talk that promotes integrated patterning and canalization. We propose

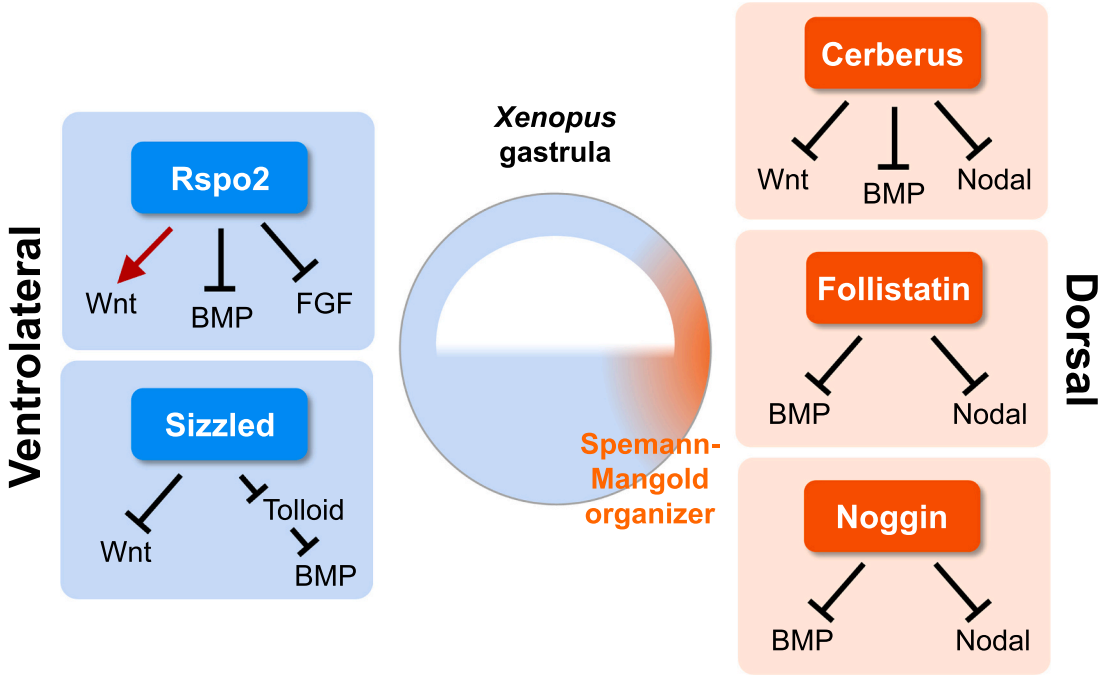


Fig. 4. Multifunctional growth factor antagonists of the Spemann-Mangold organizer.
Multifunctional secreted growth factor antagonists provide signal integration, a feature promoting developmental robustness and harmonious developmental regulation.

that a Cartesian coordinate system of Wnt, BMP, and FGF gradients regulated AP, DV, and LR body axes already in *Urbilateria*.

CRediT authorship contribution statement

Christof Niehrs: Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Ettore Zapparoli:** Writing – review & editing, Investigation, Data curation. **Hyeyoon Lee:** Writing – review & editing, Visualization, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

N/A

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) SFB 1324-B01.

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