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Evo-Devo of *Urbilateria* and its larval forms

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A B S T R A C T

Developmental biology has contributed greatly to evolutionary biology in the past century. With the discovery that vertebrates share Hox genes with *Drosophila* in 1984, it became apparent that all animals evolved from variations of an ancestral embryonic patterning genetic tool-kit. In the dorsal-ventral (D-V) axis, a fundamental experiment was the Spemann-Mangold organizer transplant performed in 1924. Almost a century later, D-V genes have been subjected to saturating molecular screens in *Xenopus* and extensive genetic screens in zebrafish. A network of secreted growth factor antagonists has emerged, and we review here in detail the Chordin/Tolloid/BMP pathway. Chordin establishes a morphogen gradient spanning the entire embryo that was present even in the cnidarian *Nematostella*. This ancient system was present in *Urbilateria*, the last common ancestor of the protostome and deuterostome bilateral animals. We suggest that Urbilateria had a complex life cycle with an adult benthic form on the sea bottom, and also a primary larval pelagic or planktonic phase to disperse the species in the marine milieu. Larvae with two rows of cilia beating in opposite directions to entrap food particles, an apical sensory organ, and a rudimentary eye, are present in many protostome and deuterostome phyla. Although the larval phase has been lost multiple times in evolution, and larvae can adopt traits present in their adult forms, the simplest explanation is that *Urbilateria* had a pelago-benthic life cycle. The use of conserved developmental patterning systems likely placed evolutionary constraints in the animal forms that evolved by natural selection.

1. Introduction

Developmental Biology has been at the forefront of biological research in the last 100 years and has generated important insights into the evolution of animals. The apogee of experimental embryology came in 1924 with the transplantation of the dorsal blastopore lip, which was able to induce a second body axis (Spemann and Mangold, 1924; Spemann, 1938). On a parallel track, developmental genetics originated from the interest of naturalists, such as William Bateson, in understanding how changes of one region of the body into another – called homeotic transformations – might help understand discontinuities in the evolution of species (Bateson, 1894). In 1923, Calvin Bridges and Thomas H. Morgan isolated the *bithorax* homeotic mutant in *Drosophila*, eventually leading to the discovery of collinearity between the location of homeotic genes in the DNA and body segment specification (Bridges and Morgan, 1923; Lewis, 1978). Genetic screens in *Drosophila* and *C. elegans* subsequently identified most of the signaling pathways that regulate embryonic development (for a comprehensive review, see Kimble and Nüsslein-Volhard, 2022).

DNA cloning revolutionized developmental biology and an important event was the discovery of the homeobox (McGinnis and Krumlauf, 1992; Duboule, 1994; Gehring, 1998). A landmark study, in which one of us participated, was the isolation of the first vertebrate Hox gene, now called HoxC6, using *Drosophila* homeobox probes (Carrasco et al., 1984), which was the first development-controlling gene identified in vertebrates. At the time, no one could have predicted that the development of species as different as fruit flies and the frog *Xenopus* would have a common developmental blueprint. We will not review the Hox genes here for they are covered in other articles in this special issue (Morata and Lawrence, 2022; Duboule, 2022; Capecchi, 2022), but cannot resist quoting Steven J. Gould (1991): "The discovery of homeoboxes allowed molecular geneticists to 'go fishing' for vertebrate genes that might be related to the homeotics of *Drosophila* – the key to segmental architecture in insects. Forget all the folk wisdom about the big ones that got away; this has been one of the finest fishing expeditions in human history."

Here we review three main points: First, we analyze saturating molecular screens of genes expressed in Spemann's organizer and in the opposite ventral pole of the *Xenopus* embryo. These studies have revealed

many novel molecular mechanisms, in particular the intricacies of how secreted antagonists of growth factors are utilized to finely regulate the differentiation dorsa-ventral (D-V) tissues. Second, we focus on a D-V network of extracellular signaling proteins consisting of Chordin, Tolloid, Twisted-gastrulation, Crossveinless-2 and Bone Morphogenetic Proteins (BMPs) that patterns the embryo in most animals. Together with the Hox genes in the antero-posterior (A-P) axis, this network has important implications for *Urbilateria*, the last common ancestor of all bilateral animals. Third, we emphasize the life cycle of Urbilateria, which consisted of a marine planktonic larval phase followed by an adult form that settled in the sea-bottom and adopted a bilateral body plan adapted for crawling and burrowing. The main conclusion that emerges from the findings reviewed here is that the immense variety of animals evolved by mutation, duplication, or deletions of an ancestral developmental gene tool-kit that may have channeled the evolutionary outcomes driven by natural selection.

1.1. Saturating molecular screens of D-V genes in Xenopus

The potent inductive activity of Spemann's organizer had been known since 1924, but all efforts to isolate the inducing molecules failed for decades due to lack of adequate technologies. This all changed when cDNA cloning became practical in the 1990s. Around that time, an influential memoir about student experiences in Spemann's laboratory was published (Hamburger, 1988). Several laboratories working with embryos of the model frog *Xenopus laevis* were inspired by this book to reinvestigate the Spemann organizer. In time, this resulted in a complete catalogue of D-V expressed genes and revealed many unexpected molecular mechanisms (Fig. 1).
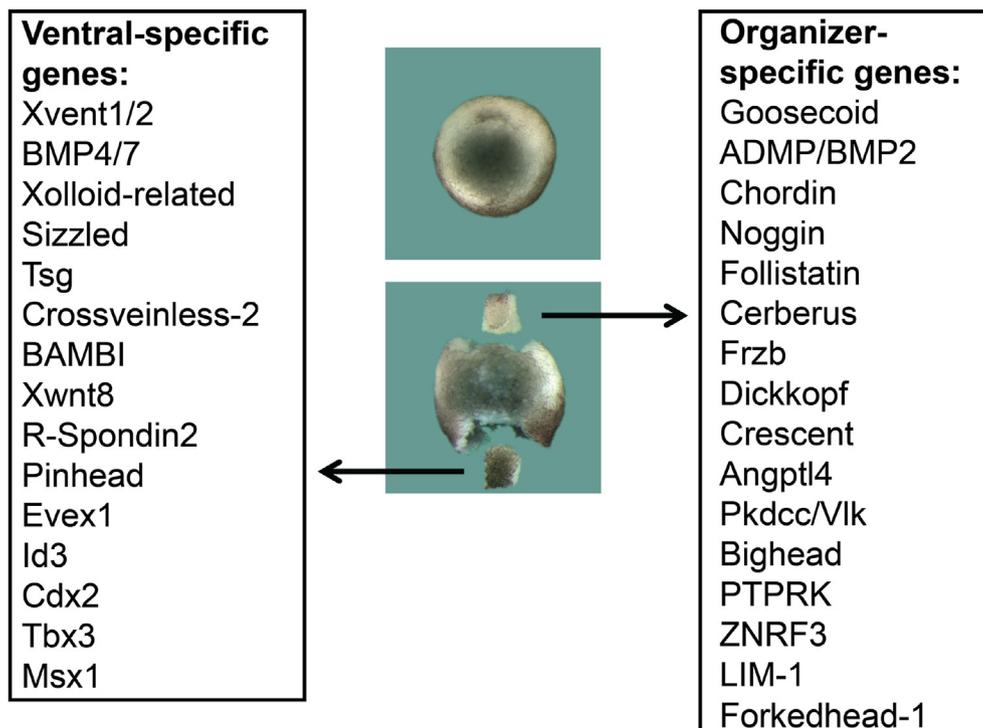
The first dorsal gene to be isolated was *goosecoid*, a homeobox gene that provided the first molecular marker for Spemann's organizer (Cho et al., 1991). Previously, the organizer had to be defined by transplantation. A few months later, the organizer homeobox genes *lim-1* and *forkhead-1* were also cloned (Taira et al., 1992; Dirksen and Jamrich, 1992). The first organizer secreted factor, Noggin, was isolated by Richard Harland using a functional assay involving microinjected pools of synthetic mRNAs (Smith and Harland, 1992). This was followed by the

cloning of Follistatin (Hemmati-Brivanlou et al., 1994) and Chordin (Sasai et al., 1994).

1.2. The Spemann organizer is a source of BMP, Nodal and Wnt antagonists

The main conclusion from these studies was that the Spemann organizer was, unexpectedly, a source of secreted growth factor antagonists. Chordin, Noggin and Follistatin are BMP antagonists (Piccolo et al., 1996; Zimmerman et al., 1996; Fainsod et al., 1997). When these three genes are depleted simultaneously with antisense morpholinos (MOs) the embryo loses all dorsal embryonic structures (Khokha et al., 2005), while the depletion of Chordin alone is sufficient to block the inductive activity of transplanted organizers (Oelgeschläger et al., 2003). Cerberus is a head-inducing protein that antagonizes Nodal, Wnt and BMP signals (Bouwmeester et al., 1996; Piccolo et al., 1999).

The organizer also secretes many Wnt signaling antagonists, of which the most famous is Dickkopf-1 (Dkk1), a molecule that promotes the endocytosis of the Wnt receptor Lrp6 (Glinka et al., 1998). Angiopoietin-like-4 (Angptl4) (Kirsch et al., 2017) as well as Bighead (Ding et al., 2018) also promote LRP6 receptor endocytosis. Other inhibitors bind to Wnt ligands directly, such as Frzb-1 (Leyns et al., 1997) and Crescent (Ploper et al., 2011). Wnt signaling inhibition is very important in the initial induction of the organizer as well as in head and neural plate patterning (Niehrs, 2010, 2022), and this is supported by the plethora of Wnt regulators discovered in the organizer (Fig. 1). A recent addition is the transmembrane protein tyrosine phosphatase receptor-type kappa (PTPRK). The Niehrs laboratory has found that this organizer phosphatase antagonizes Wnt by dephosphorylating the transmembrane E3 Ubiquitin ligase ZNRF3 (zinc and ring finger 3), another organizer-specific protein. Dephosphorylated ZNRF3 is retained in the cell membrane, where it ubiquitinylates the Wnt receptors Frizzled and Lrp6 (Chang et al., 2020). Ubiquitinylation of these receptors leads to their endocytosis and removal from the cell membrane (Nusse and Clevers, 2017; Tejeda-Muñoz et al., 2019; Albrecht et al., 2021). Remakably, PTPRK inhibits Wnt by an indirect mechanism that leads to the endocytosis of Wnt receptors (Chang et al., 2020).

Ventral-specific genes:
Xvent1/2
BMP4/7
Xolloid-related
Sizzled
Tsg
Crossveinless-2
BAMBI
Xwnt8
R-Spondin2
Pinhead
Evex1
Id3
Cdx2
Tbx3
Msx1

Organizer-specific genes:
Goosecoid
ADMP/BMP2
Chordin
Noggin
Follistatin
Cerberus
Frzb
Dickkopf
Crescent
Angptl4
Pkdcc/Vlk
Bighead
PTPRK
ZNRF3
LIM-1
Forkedhead-1

Fig. 1. Molecular screens of dorsal and ventral fragments of the early *Xenopus laevis* gastrula have yielded a plethora of novel genes involved in embryonic patterning. Screenings by multiple laboratories have been saturating and can be compared to the classical saturating genetic screens of zygotic genes that pattern the cuticle in *Drosophila*. The dorsal/ventral ratios of 40,000 protein-coding transcripts are listed in the Supplemental Tables of Ding et al. (2017). The genes mentioned here are not listed in order of enrichment but instead prioritized because they have been the subject of mechanistic studies. The Spemann organizer proved to be a rich source of Wnt, BMP and Nodal secreted inhibitors, revealing an unexpected importance of growth factor antagonists in embryonic patterning.

1.3. Ventral genes oppose the activity of dorsal genes

Using high-throughput methods it has been possible to build a quantitative catalogue of all the genes expressed in the ventral and dorsal dimensions by RNAseq of dissected dorsal and ventral fragments of the *Xenopus* gastrula (Ding et al., 2017) (Fig. 1). The ventral side of the embryo was neglected by embryologists because its transplantation does not result in the formation of ectopic structures as the dorsal organizer does. However, there are hundreds of genes expressed in a ventral to dorsal gradient. It is now clear that the gastrula is an embryonic field, and that for every action of the organizer there is a reaction in the ventral side. Ventral differentiation is activated by BMP4 expression (Fainsod et al., 1994), which in turn activates the *Xvent-1* and *Xvent-2* homeobox genes (related to *Drosophila bar*) (Gawantka et al., 1995). Ventral genes are part of the BMP synexpression group (Niehrs and Pollet, 1999), including Id1 to Id4 (Karaulanov et al., 2004; Ding et al., 2017), BAMBI (Onichtchouk et al., 1999), and many other genes (Fig. 1).

A novel molecular mechanism was recently reported for R-spondin-2 (RSPO2), which was previously considered exclusively a Wnt regulator. RSPO2 is a ventro-lateral secreted protein that forms a ternary complex specifically with the BMP receptor 1A/ALK3 and ubiquitin ZNRF3 transmembrane proteins, causing the endocytosis of BMPR1A (Lee et al., 2020). Thus, R-spondin-2 functions as a BMP feedback inhibitor in the ventral side of the embryo. Another BMP signaling regulator recently identified is Pinhead (Itoh et al., 2021). It is a protein secreted by the ventral marginal zone that binds directly to the dorsal BMP anti-dorsalizing morphogenetic protein (ADMP) (Moos et al., 1995) but, interestingly, not to BMP4. Pinhead cooperates with Chordin in promoting dorsal development, but from the opposite side of the embryo (Itoh et al., 2021). In conclusion, the saturating molecular screens carried out in D-V gene expression patterns in *Xenopus* have uncovered a rich tapestry of novel molecular mechanisms dedicated to the fine regulation of the BMP and Wnt signaling pathways.

1.4. D-V patterning by the chordin/tolloid/BMP pathway

Of all the novel molecules isolated from the Spemann-Mangold organizer, Chordin (Sasai et al., 1994) proved the most informative with respect to embryonic patterning. Chordin is a large protein with four Cysteine-rich von Willebrand factor C (vWFc) domains that function as BMP-binding modules in many proteins (Larraín et al., 2000; Abreu et al., 2002). The *Drosophila* homologue of Chordin is called Short-gastrulation (Sog), and was found to also contain four similar vWFc domains (François et al., 1994; Holley et al., 1995a,b). Having a homologue of Chordin provided a key insight, as genetic experiments in *Drosophila* had shown that the phenotype of *sog* mutants was enhanced by doubling the dosage of *Decapentaplegic* (*Dpp*), the *Drosophila* homologue of BMP2/4 (Ferguson and Anderson, 1992). This genetic hint was sufficient to lead us to the hypothesis that Chordin protein might work in the simplest possible way: by binding and inhibiting BMP in the extracellular space. Biochemical experiments with purified proteins showed that Chordin was indeed able to bind to and inhibit BMPs with high affinity (Piccolo et al., 1996).

Chordin is secreted copiously in the embryo and if it were uniformly secreted it would reach concentrations of 33 nm in the frog gastrula (Lee et al., 2006). Chordin is transcribed on the dorsal side of the embryo, mirroring the regions that have inductive activity after transplantation, and must reach much higher levels on the dorsal side. Since BMPs are secreted only in the picomolar range, a great excess of Chordin exists.

Subsequent work revealed that Chordin is just one element in a morphogenetic biochemical pathway consisting of interacting secreted proteins that encompasses the entire embryo (Fig. 2). The rate-limiting step of this pathway is provided by Tolloid, a Zinc metalloproteinase. We found that Tolloid works by cleaving Chordin at two specific sites, resulting in the release of BMP from inactive BMP/Chordin complexes, reactivating BMP for signaling through its cell surface receptors (Piccolo et al., 1997). In *Xenopus*, Tolloid is called Tolloid-related (Xlr), and there
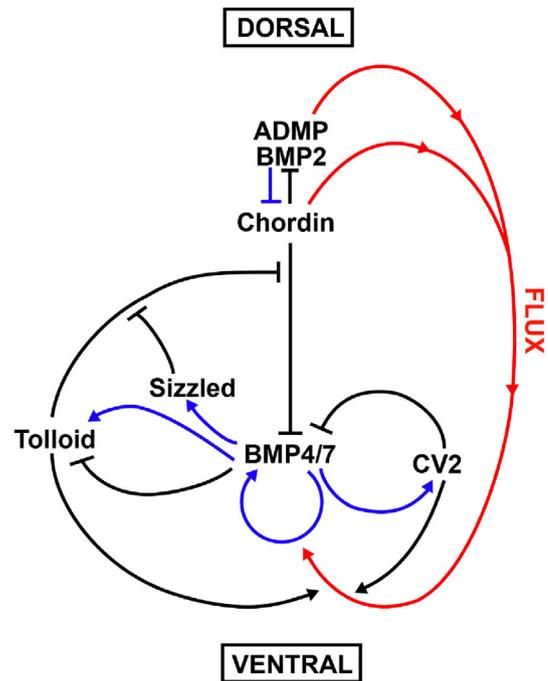
Fig. 2. The Chordin/Tolloid/Sizzled/BMP D-V morphogenetic signaling pathway in *Xenopus*. There are dorsal and ventral BMPs, which are antagonized by copious secretion of Chordin in the dorsal side. Black arrows indicate direct protein-protein interactions determined by biochemical methods. The patterning system is reinforced by transcriptional regulation indicated by blue arrows. In the ventral side BMP signaling is high and drives the transcription of BMP4/7, Crossveinless-2, xTolloid-related (Xlr) metalloproteinase, and Sizzled, a feedback inhibitor of Tolloid. The red arrow indicates the flux or facilitated diffusion of Chordin/BMP towards the ventral side, where Tolloid cleaves Chordin and releases BMPs for peak signaling. The rate limiting step is the Tolloid enzyme and the system is driven by the degradation of Chordin in the ventral side. This highly regulated long-range morphogen pathway ensures that the BMP gradient remains constant even during epiboly when the circular blastopore becomes increasingly smaller in size as it encloses the yolky endoderm, and when the embryo develops at variable temperatures.

is also another homologue called BMP1 which, despite its name, does not encode a BMP growth factor but rather a Tolloid homologue (Dale et al., 2002). *Drosophila* Tolloid was identified in the original Nüsslein-Volhard and Wieschaus genetic screen and was known to have an unusually rich allelic series of mutations that resulted in increased DPP activity. This genetic hint was sufficient for us to design experiments showing that Tolloid worked through the proteolysis of the antagonist Chordin (Piccolo et al., 1997) (Fig. 2).

1.5. Sizzled, Crosveinless-2 and Tsg regulate chordin

In *Xenopus* and zebrafish, Tolloid activity is regulated by a secreted protein called Sizzled (previously called Ogon/Mercedes in zebrafish) that is transcribed in the high-BMP ventral side of the embryo (Miller-Bertoglio et al., 1999; Collavin and Kirschner, 2003). During the course extensive genetic screens in zebrafish, seven zygotic mutations that affected D-V pattern were identified. Five had increased dorsal structures (low BMP) and were mutated in Tolloid (*mini-fin*), BMP2 (*swirl*), BMP7 (*snailhouse*), BMP receptor Alk8 (*lost-a-fin*) and Smad5 (*somitabun*). Only two mutants resulted in ventralized (high BMP) phenotypes (Little and Mullins, 2006; Zinski et al., 2018), which were in *chordin* and *sizzled* (Schulte-Merker et al., 1997; Miller-Bertoglio et al., 1999). In genome-wide transcriptomic analyses comparing dorsal to ventral fragments of *Xenopus* gastrulae (Fig. 1), *chordin* and *sizzled* were the most highly enriched mRNAs, with Chordin a remarkable 9 standard

deviations over the D-V mean dorsally and Sizzled 8 standard deviations on the ventral side, confirming that Chordin and Sizzled are crucial D-V patterning components (Ding et al., 2017).

Microinjection experiments in *Xenopus* showed that *sizzled* mRNA had potent anti-BMP dorsalizing effects in the dorsal half of the gastrula, but none in the ventral half (Lee et al., 2006). In biochemical experiments, we found that Sizzled inhibited degradation of Chordin by Tolloid/Xlr. Enzyme kinetics showed that Sizzled behaved as a competitive enzyme inhibitor (meaning that it binds to the active site of the enzyme) with an inhibition constant of 33 nM, which is in the range of the affinity of Chordin for Tolloid (20 nM) (Lee et al., 2006). Thus, in the embryo the Tolloid metalloproteinase is confronted with the option of binding to Chordin, which it can cleave, or to Sizzled, which is resistant to cleavage. Experiments in zebrafish from the Hibi laboratory independently showed that Sizzled is a Tolloid inhibitor (Muraoka et al., 2006). In zebrafish, Sizzled acts at late gastrula stages during tail formation (Connors et al., 1999, 2006; Tuazon et al., 2020), while in *Xenopus* Sizzled depletion has the same effect as Chordin depletion on D-V patterning (Lee et al., 2006; Inomata et al., 2013).

Sizzled has the structure of sFRPs (secreted frizzled-related proteins), which are proteins that usually act as Wnt inhibitors; however, Sizzled lost its anti-Wnt activity and became dedicated to Tolloid inhibition (Collavin and Kirschner, 2003). On the dorsal side of the embryo, the sFRP protein Crescent is secreted. It is also a potent competitive inhibitor of Tolloid, but has retained its anti-Wnt activity (Ploper et al., 2011). Placental mammals lost the Sizzled and Crescent genes, probably reflecting relaxed requirements for D-V patterning in the absence of yolk in the egg. The *Drosophila* genome lost all sFRP genes (although sFRPs are present in other invertebrates such as nematodes and annelids), indicating that additional ways of achieving regulated Tolloid activity likely exist (see below).

An observation that remained unexplained was that BMP1 was a metalloproteinase, yet was purified from decalcified bone matrix as part of a protein complex in which multiple TGF-β (transforming growth factor-β) superfamily members, designated BMP2 through BMP7, were first isolated (Sampath and Reddi, 1981; Wozney et al., 1988). In enzyme kinetic assays of BMP1 digestion, it was found that BMP4 acted as a non-competitive inhibitor (meaning that it binds to a site different from the active site) (Lee et al., 2009) (Fig. 2). BMP1 is a large protein that, in addition to a protease domain contains three CUB domains, while Xlr/Tolloid contains five of them. CUB domains are an acronym for Complement 1r/s, Uegf (a sea urchin embryonic protein), and BMP1. It was found that purified CUB domains bind directly to BMP4 with an affinity of 20 nM, which is in the physiological range. BMP4 also binds to *Drosophila* Tolloid, and this interaction provides a molecular explanation for the mysterious antimorphic mutations in the protease domain of *Drosophila tolloid*, which have strong anti-Dpp effects (Ferguson and Anderson, 1992; Childs and O'Connor, 1994). CUB domains are present in 56 different human proteins and it is possible that many of them are also BMP or TGF-β regulators (Lee et al., 2009).

Crossveinless-2 (CV2) is a secreted protein with 5 Cysteine-rich vWFc domains similar to the BMP-binding domains of Chordin. Unlike Chordin, CV2 does not diffuse due to a strong heparin sulfate proteoglycan binding site (Serpe et al., 2008). CV2 acts as a local BMP feedback inhibitor in the ventral side (Fig. 2), but also has a high affinity for Chordin/BMP complexes and an even higher affinity for Chordin fragments cleaved by BMP1/Tolloid (Ambrosio et al., 2008). This explains the pro-BMP activity of CV2 by facilitating diffusion of Chordin/BMP complexes to the ventral side where active BMPs are released by Tolloid digestion of Chordin.

Twisted gastrulation (Tsg) is another BMP-binding protein that is required for the proper function of Chordin and CV2. Tsg is transcribed in the high-BMP ventral side, and binds to both BMP and Chordin, as well as CV2 (for simplicity Tsg is not indicated in Fig. 2). The overall effect of Tsg depletion is pro-BMP because it enhances Tolloid cleavage and helps transfer BMP from Chordin to BMP receptors (Oelgeschläger et al., 2000; Zinski et al., 2018).

1.6. The Chordin D-V morphogenetic gradient

The Chordin/Tolloid/CV2/Tsg/BMP morphogenetic pathway has been conserved between *Drosophila* and vertebrates, and in both organisms it has been possible to visualize a Chordin/Sog extracellular gradient (Bier and De Robertis, 2015). Fig. 3A illustrates the Chordin protein gradient at mid-gastrula in *Xenopus* (Plouhinec et al., 2013). It extends over a very long distance (2 mm in this embryo of 1.3 mm diameter and about 10,000 cells), and Chordin protein is concentrated in the narrow region of extracellular matrix (ECM) that separates endoderm and mesoderm from ectoderm. Functional studies have shown that the gradient is driven by the dorsal production of Chordin and its degradation by Tolloid on the ventral side (Plouhinec et al., 2013). From this narrow ECM signaling highway, the facilitated diffusion of Chordin provides positional information to both the ectodermal and mesodermal layers, explaining how D-V tissue differentiation is coordinated between these two germ layers. The Chordin gradient generates a reciprocal gradient of nuclear phospho-Smad1/5/8 resulting from BMP signaling through its cognate receptors (Fig. 3B) (Plouhinec et al., 2013). The phospho-Smad gradient and its regulation by Chordin and BMP1/Tolloid has been studied in considerable detail in the optically transparent zebrafish embryo (Tucker et al., 2008; Tuazon et al., 2020).

BMPs are transcribed at both poles of the embryo, with BMP2 and ADMP at low, and BMP4/7 at high, levels of Smad1/5/8 signaling (Fig. 2). Interestingly, BMPs can diffuse in the embryo both in a D-V and a V-D direction, providing resilience to the D-V patterning system. This is illustrated by the experiment shown in Fig. 4, in which wild-type tissue fragments were transplanted into embryos depleted of BMP signals. Depletion of BMP2/4/7 with antisense MOs results in embryos that have enhanced dorsal structures but still retain D-V patterning (Reversade et al., 2005). However, when BMP2/4/7/ADMP are depleted simultaneously self-regulation is lost, causing the entire ectoderm to become neural tissue (Reversade and De Robertis, 2005). In BMP-depleted embryos the epidermal marker *cytokeratin* is eliminated (Fig. 4A and B) and the pan-neural marker *Sox2* is expressed throughout the ectoderm (Fig. 4D and E), providing an excellent system to test for long-range BMP signaling in the embryo. When lineage-traced wild-type dorsal organizer is transplanted into BMP-depleted embryos, the transplanted tissue gives rise to notochord, and induces epidermis at a distance where Chordin/BMP is digested by Tolloid (Fig. 4C). Similarly, transplantation of wild-type ventral tissue restores epidermis differentiation not only in the graft itself but also at a distance (Fig. 4F). The D-V patterning system needs to be resilient for it determines the allocation of D-V tissues, which occurs only once in the lifetime of the organism.

The Chordin/Tolloid/Sizzled/BMP pathway not only determines cell differentiation but also animal morphology. This is best illustrated by the work of Kinja Ota on the twin-tailed goldfish. These ornamental fish have been kept by breeders for at least 600 years, as they are better adapted to life in a fish bowl. Goldfish have two *chordin* genes, and all twin-tail strains have a stop codon mutation close to the start of *chdA* (Abe et al., 2014). As shown in Fig. 5, mutation in Chordin results not only in twin-tails but also in a much shorter and thicker body shape. This demonstrates that Chordin is a morphogenetic protein. Body shape can also be changed in *Xenopus* by microinjecting Sizzled MO into the two ventral blastomeres at the 4-cell stage, resulting in expanded ventral-posterior tissues, while preserving a normal head region (De Robertis et al., 2017). Sizzled MO increases Tolloid activity, degrading Chordin and increasing BMP activity. In the goldfish, Sizzled antisense MOs result in twin-tailed phenotypes, much in the same way as *chordin* mutation (Abe et al., 2018). In zebrafish, Chordin and Sizzled mutations also result in similar phenotypes, including twin-tails (Schulte-Merker et al., 1997; Miller-Bertoglio et al., 1999). In conclusion, Chordin/Tolloid/BMP establish a facilitated diffusion gradient that controls tissue differentiation (neural plate, neural crest and epidermis in ectoderm; notochord, somite, lateral plate and blood islands in mesoderm) as well as body shape. This helps explain a long-standing evolutionary problem pointed
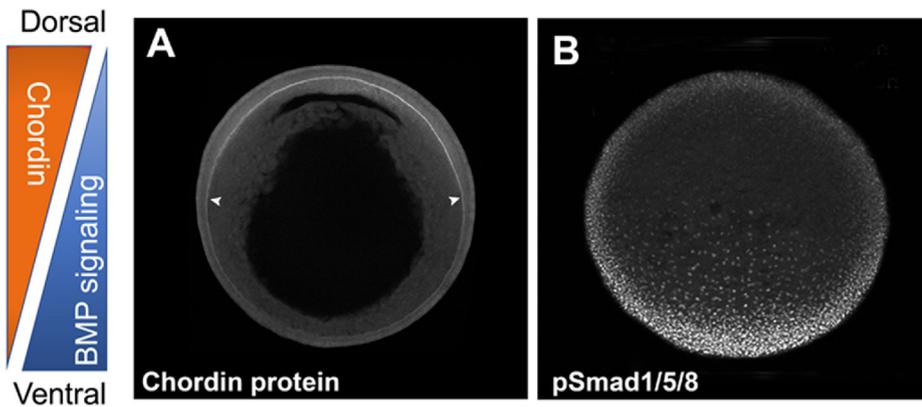
Fig. 3. The Chordin extracellular protein gradient and its reciprocal nuclear phospho-Smad1/5/8 gradient. (A) Optical sections at mid-gastrula stained with an anti-Chordin affinity-purified antibody or (B) an antibody that recognizes the C-tail of the Smad1/5/8 transcription factors only after they have been phosphorylated by BMP receptors. Note that the Chordin gradient extends over a long distance, in this case 2 mm of circumference. Chordin is highly concentrated within the extracellular matrix that separates the ectoderm from endomesoderm (arrowheads). Experimental analyses showed that Tolloid is the key regulator of the BMP gradient. Horizontal optical sections of *Xenopus* mid-gastrula embryos (stage 11). Images reproduced with permission from Plouhinec et al. (2013); copyright 2013 Proceedings of the National Academy of Sciences.
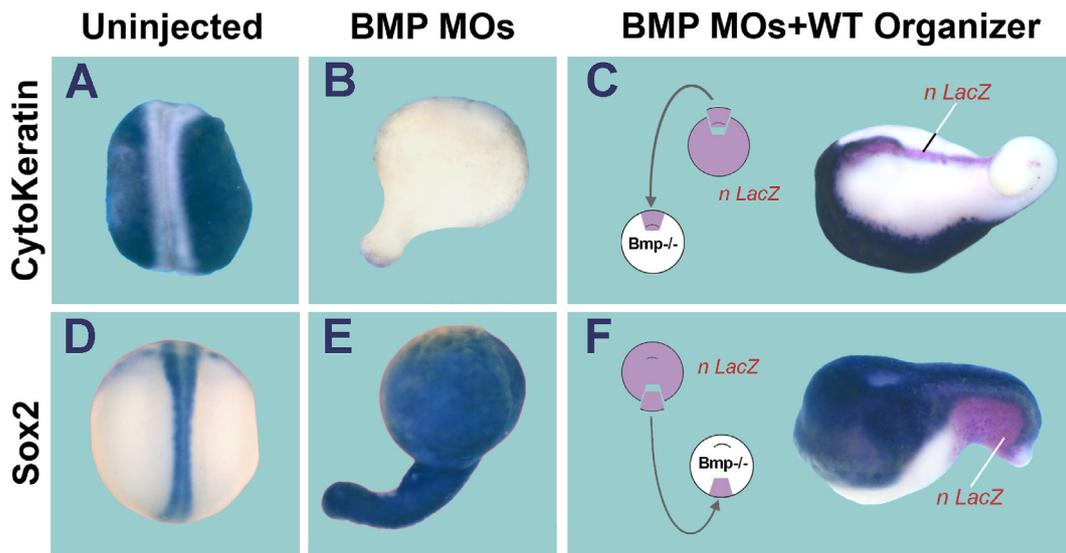
Fig. 4. Transplantation of lineage-traced wild-type *Xenopus* organizer or ventral signaling center into host embryos depleted of BMP2/4/7/ADMP (BMP MOs), in which the entire epidermis becomes neural tissue (marked by Sox2), are able to restore epidermal differentiation (marked by Cytokeratin) at a distance of the graft. (A) Embryo at the neural plate stage showing epidermal differentiation. (B) The combined depletion of BMP2/4/7/ADMP morpholinos eliminates epidermal differentiation and the entire ectoderm becomes neural tissue. (C) A wild-type graft labelled with nuclear LacZ gives rise to notochord (lineage marked by LacZ), yet the organizer-secreted BMPs do not signal because they are blocked by Chordin and are only released in the ventral side by the action of Tolloid after long-range diffusion. (D) Wild-type embryo showing neural tissue marked by the pan-neural marker Sox2. (E) Upon depletion of four BMPs the entire ectoderm becomes neural, showing that neural differentiation is repressed by BMP signaling. (F) Transplantation of wild-type ventral center tissue restored epidermis in the graft and surrounding epidermis repressing neural differentiation and restoring D-V patterning. These experiments show that ventral BMPs, as well as dorsal BMPs, are able to diffuse over long distances in the *Xenopus* embryo. Images reproduced with permission from Reversade and De Robertis, Cell 2005; copyright 2005 Elsevier.

out by D'Arcy Thompson, who noted that the pufferfish and the sunfish were closely related species yet had very different body shapes (Thompson, 1917; De Robertis et al., 2017).

1.7. A conserved developmental tool-kit

Studies in evolution and development – now called Evo-Devo - have been intertwined since their inception. The enormous variety of adult anatomies become simplified when their embryonic development is examined, and common themes in the generation of body forms are more readily apparent. In his insightful book "The Possible and the Actual", François Jacob explained why Evo-Devo is so important: "For it is during embryonic development that the instructions contained in the genetic program of an organism are expressed, that the genotype is converted into phenotype. It is mainly the requirements of embryonic development that, among all possible changes in genotype, screen the actual phenotypes". The main message of this prophetic book was that during

evolution old components are not discarded but, like a tinkerer (*bricoleur* in French) would, used as parts to assemble new objects (Jacob, 1982). We now know that animals share an ancestral took-kit of genes that were used differentially under the guidance of natural selection – through mutation, duplications and deletions – to generate the wonderful variety of animals that surround us on Earth. There is little biochemical novelty in animal evolution because eukaryotic cells are composed of very similar components and organelles; what development does is to re-arrange cells with respect to each other into morphologies that serve new functional needs.

Morphological change depends of ancestral gene networks shared by all animals, such as the Hox genes and the Chordin/Tolloid/BMP system. A powerful way of generating morphological change is to evolve novel transcriptional enhancers that change the region or tissue where a gene is expressed. For example, crustaceans such as shrimp and lobsters evolved thoracic legs into a considerable diversity of specialized feeding appendages called maxillipeds. Multiple maxilliped morphologies

Fig. 5. The twin-tail phenotype in goldfish is caused by a loss-of-function of one of its two Chordin genes. Ornamental twin-tail goldfish have been kept by breeders for centuries as they are better adapted to life in a fishbowl. All twin-tail strains have the same loss-of-function mutation in the *chdA* gene. Note that the Chordin mutation not only affects the tail but also the shape of the body, demonstrating that Chordin generates body morphology. Original photographs courtesy of Prof. Kinya G. Ota, Academia Sinica, Taiwan.

repeatedly correlated with independent shifts in the border of expression of Hox genes (Averof and Patel, 1997).

Genome sequences now provide an open book that records the history of life on Earth. The developmental took-kit involves thousands of conserved genes. The recent sequencing of the sea anemone *Nematostella*, a sister group that separated from the bilateral animals 650 mya, revealed that it has a mega-genome with all of the signaling pathways present in higher animals, plus 2.5% of genes that underwent gene loss in bilateral animals but are present in plants or fungi (Technau et al., 2005; Holstein, 2022). Interestingly, two-thirds of human genes are present in the *Nematostella* genome, while the model protostome organisms *Drosophila* and *C. elegans* lost hundreds of these genes during evolution (Putnam, 2007). Reconstructing the ancestral tool-kit of developmental genes of all animals remains one of the great challenges for Evo-Devo (De Robertis, 2008).

1.8. The urbilaterian ancestor

A crucial problem in evolution is to distinguish between homologous structures related by descent from a common ancestor and convergent evolution that finds similar solutions due to a common functional need. Ernst Haeckel proposed a fundamental homology: that the endoderm and ectoderm of all animals were derived form a marine ancestor that he called the *Gastrea* (Haeckel, 1877). The *Gastrea* was a free-floating (pelagic) hollow ball with a digestive endodermal layer with a mouth opening to the outside. Eventually, *Gastrea* attached to the sea bottom (benthic existence) where food sediment was abundant, and adopted a crawling and burrowing existence that gave rise to the bilateral animals. The *Gastrea*-theory was important because it implied that all animals were monophyletic, i.e., derived from a common ancestor.

Given the extensive conservations of development-controlling genes, we proposed early on that the last common ancestor of all bilateral animals must have been a complex animal that we named *Urbilateria* (De

Robertis and Sasai, 1996). Reviewers objected because this was a word composed of German and Latin roots (ur = primitive, bilateria = bilateral animal), but the name stuck, and our proposal proved very influential. There are 35 animal phyla and 30 are bilateral animals; *Urbilateria* was the last common ancestor of 99% of all animal species that have been described to date. Reconstructing *Urbilateria* became a central problem in Evo-Devo.

Urbilateria had a Hox gene complex consisting of at least 7 Hox genes flanked by up to 8 additional Antennapedia-type homeobox genes forming a Super-Hox complex (Butts et al., 2008). The Hox genes stayed together during evolution due to spatial, temporal, and transcriptional requirements (Darbellay et al., 2019; Duboule, 2022). When the colinear expression of Hox complexes in both the *Drosophila* and mammalian A-P axes was discovered, it became widely accepted that the gene system had to have been present in their last common ancestor. Such an elaborate system, including two regulatory microRNAs within the complex, could not have been assembled multiple times independently by convergent evolution (De Robertis, 2008).

The case for an ancestral D-V patterning system was met with more resistance, despite the conservation of Chordin and Sog. Homology of the D-V body plan was contested at scientific meetings by arguing that genes are used at multiple times and in different cell types during development and that the presence or absence of gene expression did not reflect common patterning. As shown in Fig. 6, work in multiple organisms has now shown that the Chordin/Tolloid/BMP network of extracellular proteins is used to generate a D-V gradient in frogs, sea urchins, beetles, fruit flies and spiders (reviewed in Bier and De Robertis, 2015). The D-V morphogenetic gradient is not generated by an individual gene such as Chordin/Sog, but rather by a complex molecular machinery of mutually interacting proteins such as Tolloid, Tsg, CV2, and BMPs that have been conserved between *Drosophila* and the vertebrates. The leech has lost Chordin, but it has a very different development using teloblast cells that do not require a long-range gradient, utilizing the BMP inhibitor Gremlin instead (Kuo and Weisblat, 2011). In most bilaterians, the Chordin/-Tolloid/BMP pathway constitutes a molecular machine used to establish a facilitated diffusion gradient spanning the entire embryo and must have been inherited from the last common ancestor. This complex D-V gradient-forming mechanism could not have been assembled independently multiple times by convergent evolution and was therefore likely present in *Urbilateria* (Fig. 6).

Work from the laboratory of Ulrich Technau has shown that the Chordin system predates the bilaterians. The sea anemone *Nematostella* has a radial body plan, but its embryos have a directive axis that suggests some degree of bilaterality. Chordin and Dpp are expressed on one side of the blastopore, and a gradient with maximal BMP signaling on the opposite side of the gastrula is generated by shuttling and cleavage of Chordin/BMP complexes by Tolloid (Genikhovich et al., 2015). Importantly, the Chordin-expressing *Nematostella* blastopore has organizer activity, inducing secondary axes after transplantation into a host embryo (Knaus et al., 2016).

Given that genes are readily lost in evolution (De Robertis, 2016), the cnidarian sister group is very helpful in determining whether a gene was also present in the common ancestor. For example, if a vertebrate gene is absent in *Drosophila* but present in *Nematostella* (as is the case for Dkk, sFRPs and Noggin), it should have been present in *Urbilateria* as well. It has been suggested that acoels may be at the base of bilateria, but these are most likely simplified planarians. Acoels lost many genes and retained only a single Antennapedia-like homeobox gene, while *Nematostella*, *Drosophila*, and mammals have complete Hox gene complexes and Chordin/Tolloid/BMP systems (Geinikhovich and Technau, 2017). The evidence suggests that *Urbilateria* was a complex, not a simplified, animal.

1.9. The life cycle of Urbilateria

Animals originated in the ocean and zoologists have long thought that
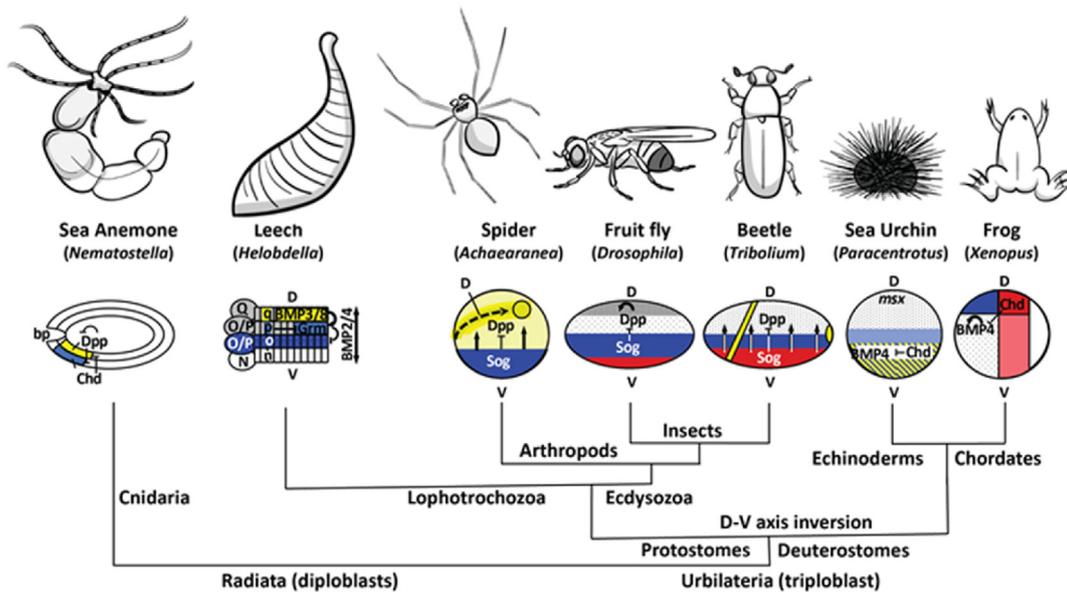
Fig. 6. A Chordin/Tolloid/BMP system of extracellular proteins patterns the entire embryo in the D-V axis in a multitude of embryos. Chordin is called Sog, and BMP4 is Dpp, in insects. Please note that even in the sea anemone *Nematostella*, which predates the divergence of protostomes and deuterostomes from *Urbilateria*, there is a directive axis next to the blastopore of the embryo which expresses Chordin and Dpp, which diffuses and is cleaved by Tolloid at the opposite pole of the embryo generating a BMP gradient. Modified, with permission, from Bier and De Robertis, Science 2015; copyright 2015, American Association for the Advancement of Science Publishing.

a complex life cycle - the pelago-benthic life cycle - is ancestral to all bilaterians (Jägersten, 1972: Nielsen, 2012). *Urbilateria* likely had a pelagic (or planktonic) primary larva followed by a benthic adult in the sea bottom that adopted a crawling and burrowing life in the rich nutritional environment provided by phytoplankton sediments. Planktonic marine larvae are found in 17 animal phyla. Larval forms have been lost repeatedly, sometimes multiple times in the same phylum. The planktonic phase favors the dispersal of the species in the ocean, while some species chose to protect their eggs by increasing their maternally provided yolk. Direct development of yolky eggs into the adult phase limits dispersal in exchange for higher individual local survival (Jägersten, 1972; Nielsen, 2012).

Bilateral animals are classified into protostomes (mouth-first) and deuterostomes (mouth-second) according to their mode of gastrulation. Fig. 7 shows archetypal diagrams of primary larvae of these two great animal branches, emphasizing their similarities. The prototypic protostome larva is the trochophore, characterized by two rings of large cilia that beat in opposite directions, bringing food particles towards a groove in between, which has smaller cilia that transport food particles towards the mouth (Fig. 7) (Strathmann, 1978; Willmer, 1990). Trochophore larvae are found, for example, in marine annelids and mollusks. In the deuterostomes, the dipleurula and tornaria larvae of echinoderms and hemichordates also have two rings of cilia and a through-gut with mouth and anus (Arendt et al., 2001, Jägersten, 1972). A very important homology shared by primary larvae is the apical organ (Fig, 7). It consists of an apical tuft of long motile cilia involved in the perception and integration of environmental signals to control larval ciliary swimming. The apical organ expresses chemosensory receptors and opsins, and sometimes has a rudimentary eye. Sensory-neurosecretory cells from the apical organ release peptides that control larval physiology in cnidarian, annelid, mollusk, flatworms, hemichordates and echinoderm larvae (Arendt et al., 2016). Importantly, the apical organ expresses homologues of the homeobox genes *Six3* (Sine oculis homeobox homologue 3) and *Rx* (Retinal homeobox) from mammals (Arendt et al., 2016).

Marine larvae adopt a variety of morphologies according to their needs to navigate and feed in ocean currents. One important factor affecting this diversity is thought to be the process of adultation, in which characters from the adult bottom-dwelling forms are shifted and
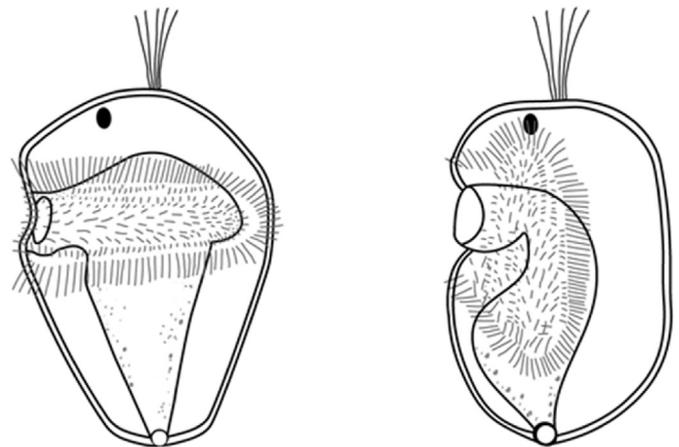
Fig. 7. Idealized diagrams of planktonic larvae of protostomes (trochophore larva of annelids and molluscs on the left) and deuterostomes (tornaria/dipleurula larva of hemichordates and sea cucumbers). Both types of larvae have two large ciliary bands for feeding around the mouth which beat in opposite directions bringing food particles to smaller cilia (called adoral) that direct food into the mouth opening. They also share a sensory apical organ with a tuft of longer cilia and an eye spot (Arendt et al., 2001; Jägersten, 1972; Willmer, 1990).

expressed in the larval stage (Jägersten, 1972). Despite the great variety of morphologies, common ancestral elements such as ciliary bands and apical organs make it very unlikely that primary larvae evolved multiple times by convergent evolution. This suggests that *Urbilateria* had a pelago-benthic life cycle involving larval and adult forms.

### 1.10. The D-V inversion during evolution

The Chordin/Tolloid/Tsg/CV2/BMP is conserved between vertebrates and *Drosophila*, but in an inverted orientation, such that Chordin is dorsal in vertebrates and Sog ventral in fruit flies (De Robertis and Sasai, 1996). This provided molecular support for the idea proposed by Etienne

Geoffroy Saint-Hilaire in a debate that took place in the French Academy of Sciences in 1830, where his opponent was Georges Cuvier (Appel, 1987). This debate was of historical significance, for it took place decades before the publication of the Origin of Species (Darwin, 1859). Geoffroy dissected a lobster and proposed that it had homologous structures (which he called analogous) to those of vertebrates. Lobsters and mammals shared A-P characteristics such as head, thorax, and abdomen, as well as D-V landmarks such as central nervous system (CNS), digestive tract and heart, except that a D-V inversion had occurred. In his own words (Geoffroy Saint-Hilaire, 1822): "I have just found that all the soft organs, that is to say, that the principal organs of life are reproduced in the crustaceans, and consequently in the insects, in the same relationships and with the same arrangement as their analogues among the higher vertebrate animals". He dissected a lobster and inverted it with respect to the ground and noted: "What was my surprise, and I add, my admiration, in perceiving an ordering that placed under my eyes all the organic systems of this lobster in the order in which they are arranged in mammals?" The lobster thus inverted now had the nerve cord dorsal above the digestive tract which was above a ventral heart, as is found in the vertebrates. The inversion of Chordin expression revived this old biological debate (De Robertis and Sasai, 1996).

The diagram in Fig. 8 depicts how the two main branches of bilateral animals, protostomes and deuterostomes, might have evolved from an urbilaterian ancestor. It is proposed that a trochophore-like larva settled for adult life in the sea bottom. The elongated adult bilateral body plan favored a crawling or burrowing existence. *Urbilateria* is shown with an elongated slit blastopore open from the mouth to the anus, a condition known as amphistomy, which occurs in annelid larvae (Arendt et al., 2001). The CNS (in red) is induced in the region surrounding the blastopore. In the protostomes, the slit blastopore closes in the central part, except for the mouth. The nerve cord becomes located ventral to the gut, while the neural tissue anterior to the mouth forms the supraesophageal ganglion with the gut traversing the CNS (Fig. 8). In the deuterostomes, the anus is derived from the blastopore, but a new mouth is perforated secondarily, so that the gut no longer traverses the CNS and remains dorsal to the gut throughout its length (Fig. 8). The region traversed by the gut in protostomes corresponds to the hypothalamus and infundibulum of the mammalian brain (Tessmar-Raible et al., 2007), which is the region where neurosecretory neurons associated with the regulation of food intake are located.

It has been argued that the inversion from protostome to deuterostome body plans occurred when larvae first settled on the sea bottom (Jägersten, 1972). However, it is more likely that urbilaterians enjoyed a long period without competition from other bilateral animals, a garden of Eden existence one might say. During this period before the deuterostome-protostome divergence, many important structures evolved, such as eyes controlled by Pax6, a contractile circulatory system controlled by Tinman and DMEF2, and perhaps appendages regulated by distal-less. Whether urbilaterians were segmented or not remains a matter of debate, but there is evidence that cockroach embryos have oscillatory waves of *Delta* and *hairy* expression and high Wnt expression in posterior mesoderm (Pueyo et al., 2008; Chesebro et al., 2013), as is the case for segmentation in vertebrates (Benazeraf and Pourquie, 2013; Pourquié, 2022).

The protostome and deuterostome adult nervous systems are subepidermal and centralized, suggesting that they both derive from an ancestral anatomically complex CNS (De Robertis, 2008). They both express *netrin* in the midline, which is derived from blastopore closure in the protostomes. In the vertebrate, the floor plate of the CNS is also derived from the blastopore as the late organizer regresses into the tailbud chordoneural hinge (Gont et al., 1993). The CNS induced by the blastopore maintains dorsal-ventral patterning and neuronal identity through a conserved set of homeobox genes that regulate an ancient escape reflex in invertebrates and vertebrates. Msh/Msx regulates the differentiation of Rohon-Beard mechanosensory neurons, Ind/Gbx the formation of interneurons, and Vnd/Nkx2.2 the differentiation of
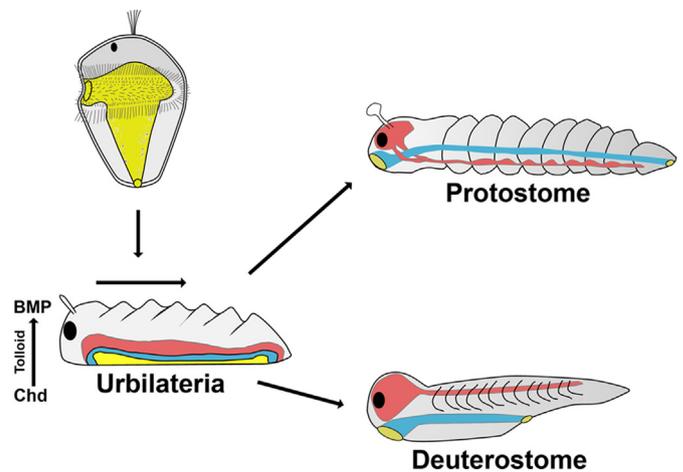
Fig. 8. Urbilateria was the last common ancestor of the deuterostomes and protostomes, and we propose that it had a primary larval form for dispersing in plankton before settling in the sea bottom for adult life. In the diagram shown here the digestive tract of this crawling creature is shown as open lengthwise extending form mouth and to anus (in yellow). This type of gastrulation is called amphistomy, to distinguish it from protostomy (mouth-first) and deuterostomy (mouth-second) type of gastrulation. The CNS is shown in red and is induced in the vicinity of the blastopore. When the ectoderm closes the elongated blastopore ventrally to form a gut tube (in blue), the protostome the nerve cord adopts a ventral subepidermal position, while the brain forms a supraesophageal ganglion that is traversed by the gut in the protostomes. In the deuterostomes a new mouth is formed ventrally and the CNS is not traversed by the gut.

motoneurons (reviewed in Bier and De Robertis, 2015; Arendt et al., 2016).

It has been argued that in deuterostomes the nervous system might have derived from a simple intraepidermal neural network that is insensitive to the D-V BMP gradient, based on experiments on a direct-developing hemichordate (acorn worm) (Lowe et al., 2003, 2006). This has been negated by recent work showing that in embryos of indirect developing hemichordates and echinoderms, a brief treatment with exogenous BMP protein represses neurogenesis and leads to the ancestral Msx-Gbx-Nkx2.2 expression pattern in ectoderm, mimicking the D-V differentiation of the chordate neural plate (Su et al., 2019). Interestingly, these BMP-treated larvae lacked a mouth, indicating that variations in BMP levels may have led to the evolution of a new mouth in the deuterostomes (Su et al., 2019). In addition, based on variations in blastopore closure, it has been proposed that protostomy and deuterostomy may have arisen independently multiple times in evolution (Martindale, 2005). We disagree with this proposal, given the remarkable robustness of the molecular phylogeny grouping of animals into protostomes and deuterostomes (Aguinaldo et al., 1997; De Robertis, 2008).

An exciting proposal by Detlev Arendt is that during central nervous system evolution the primary larva apical organ may have become incorporated into the forebrain, while the rest of the CNS may have derived from a blastopore-induced nervous system. The apical organ, which directs chemosensory and visual sensing in the larva, is specified by Six3 and Rx homeobox proteins, which regulate anterior forebrain development in higher mammals. While the traditional view has been that the apical organ was lost in adult forms, in this new chimeric brain model the apical organ nerve network may have fused with the blastoporal CNS to form the chemosensory and visual part of the brain of the adult forms of urbilaterian ancestors (Arendt et al., 2016).

2. Conclusions

Studies on Hox genes and the conserved D-V network have revolutionized our understanding of animal development. Previously, one

could not have predicted the amazing degree to which the animal gene tool-kit has been conserved in development. Natural selection of random mutations resulted in a wonderful variety of animal forms by modifying ancestral patterning systems. Bilateral animals evolved from marine animal ancestors that had a pelago-benthic life cycle and a very large repertoire of genes to work from.

The deep homologies in developmental gene networks must have channeled the outcomes of evolution. Many body plans that could have been excellent functional solutions might not exist in nature because they could not be generated unless they were compatible with the developmental networks underlying the blueprint of the animal body. The extent to which natural selection of random mutations over long periods of time was constrained by the pre-existing animal genetic structure is a very important problem in evolutionary biology (Gould, 2002). Developmental constraints are not necessarily a negative influence. On the contrary, mutations in developmental genes could have a positive influence, channeling effective adaptive responses to natural selection pressures. For example, in crustaceans the Hox genes have mediated the repeated transformation of legs into feeding appendages, following the channel of least resistance to resolve functional needs (Averof and Patel, 1997). Much of what we traditionally considered convergent evolution might have been caused by the repeated use of homologous embryonic signaling networks.

It will soon be 100 years since the Spemann-Mangold organizer experiment. Experimental embryology was then at the forefront of biological research. Morgan's *Drosophila* developmental genetics opened the way to the identification of genes that specified morphologies. The advent of molecular biology, the great equalizer, revolutionized developmental biology. Studies on many diverse animal genomes are uncovering the ancestral developmental genetic tool-kit and its regulation. As techniques improve, we can expect great advances in our understanding of evolution, which is the central problem of life sciences. It is a wonderful time to be a developmental biologist.

Author contributions

Author contributions: N.T.M and E.M.D.R. analyzed data and wrote the paper.

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Edward M. De Robertis (ederobertis@mednet.ucla.edu).

Materials availability

No custom code, software, or algorithm central to supporting the main claims of the paper were generated in this manuscript.

Declaration of competing interest

The authors declare no competing interests.

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