CHAPTER THREE

Molecular insights into deuterostome evolution from hemichordate developmental biology

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Contents

Abstract

Hemichordates, along with echinoderms and chordates, belong to the lineage of bilaterians called the deuterostomes. Their phylogenetic position as an outgroup to chordates provides an opportunity to investigate the evolutionary origins of the chordate body plan and reconstruct ancestral deuterostome characters. The body plans of the hemichordates and chordates are organizationally divergent making anatomical comparisons very challenging. The developmental underpinnings of animal body plans

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are often more conservative than the body plans they regulate, and offer a novel data set for making comparisons between morphologically divergent body architectures. Here I review the hemichordate developmental data generated over the past 20 years that further test hypotheses of proposed morphological affinities between the two taxa, but also compare the conserved anteroposterior, dorsoventral axial patterning programs and germ layer specification programs. These data provide an opportunity to determine which developmental programs are ancestral deuterostome or bilaterian innovations, and which ones occurred in stem chordates or vertebrates representing developmental novelties of the chordate body plan.

1. Introduction

Hemichordates have long occupied an important place in the study of chordate origins. The link between hemichordates and chordates was recognized by early zoologists and embryologists: Kowalevsky recognized the chordate like gill slits of acorn worms (Kowalevsky, 1866) and Bateson further developed hypotheses on morphological affinities between the two groups (Bateson, 1886). The close relationship between hemichordates and echinoderms was first established by Metchnikoff who recognized the basic organizational similarities in their larval forms (Metschnikoff, 1881), despite the organizational disparity in their adult body plans. Interest in hemichordate biology waned with the rise of genetics and the decline in the influence of embryology (Hall, 2005), and during the next 50 years the phylum received little research attention, and only in the past few decades has there been some headway in resolving some of the promising hypotheses raised by the early investigators.

Hemichordates are divided into 2 classes; the small largely colonial pterobranchs, and the solitary enteropneust worms (Hyman, 1955). Most recent attention has focused on enteropneusts, and this review will focus attention on contemporary developmental biology research from a range of enteropneust species, representing contrasting early life history strategies. There are key anatomical characters that were identified by early investigators as potential morphological homologs of some of the defining features of the chordate body plan. However, the body plans of hemichordates and chordates are sufficiently divergent that establishing unambiguous anatomical homologies has been very challenging, and as I will discuss, molecular work has helped, but not entirely resolved this problem. Hemichordates also offer insights into the evolution of the unique conserved features of the early developmental programs of chordates and, along with echinoderms, offer an outgroup perspective into the evolution of some of the key features of the vertebrate developmental program, including the organizer (Gerhart, 2001), which plays a central role in the early establishment of the vertebrate body plan (Harland & Gerhart, 1997).

Some of the most important work on the development of these marine animals was carried out by embryologists in the late 1800s (Bateson, 1885, 1886; Morgan, 1891, 1894) but with a few exceptions, work on these animals dwindled until late in the next century. Interest in hemichordates, along with other less well known phyla, was reignited with the advent of molecular developmental biology and the emerging field of evo-devo: the availability of new tools for addressing classical zoological and developmental hypotheses, which had languished due to the ambiguous conclusions reached from anatomical comparisons, provided a jolt of new data and reinvigorated interest in animal origins. This has been especially useful in animals with divergent body plans, which have been challenging to make even basic axial comparisons with other bilaterian groups, due to the lack of plausible homologous characters. The molecular underpinnings of embryological development involved in the early establishment of animal body plans can be viewed as another anatomical character—a cryptic one that can only be revealed by molecular manipulations (Slack, Holland, & Graham, 1993). As we move through the fourth decade of contemporary evo-devo, the broader sampling of the molecular underpinnings of animal body plans has taught us that molecular comparisons can be just as fraught with homoplasy as anatomical characters (Abouheif et al., 1997; Hejnol & Lowe, 2015; Martin-Duran et al., 2018; Wagner, 2007). Most comparative developmental data have focused on arthropods and chordates, two groups with highly derived and sophisticated body plans, yet most major bilaterian phyla are characterized by far simpler body plans (Brusca, Moore, & Shuster, 2016). Hemichordates represent one of these simple body plans and have revealed that the relationship between conserved gene regulatory networks and morphological evolution is not nearly as tight as comparisons between arthropods and chordates have suggested (Lowe et al., 2003, 2006; Pani et al., 2012). In the past 20 years, new data have been generated from a range of hemichordate species, representing a broad sampling of the morphological diversity and life history strategies within the group. However, data from Pterobranchs are conspicuously lacking for rounding out a broader picture of evolution within the group. Developmental data have been mostly generated from two species, *Saccoglossus kowalevskii*, a direct-developing species, and *Ptychodera flava*, an indirect-developing species with a long-lived larva that metamorphoses into the adult body plan (Kaul-Strehlow & Röttinger, 2015; Rottinger & Lowe, 2012). Molecular studies from additional species have broadened sampling within enteropneusts, and we are beginning to be able to distinguish between taxon-specific features and ancestral developmental characters for the group. Studies have included research focusing on hypotheses of morphological homology between hemichordates and chordates, as well as others focusing on more classical developmental questions on axis patterning and germ layer specification. Great progress has been made in comparing the hemichordate body plan to that of other bilaterians, and particularly to chordates.

2. The animals

The Hemichordata consists of two major classes, the pterobranchs and the enteropneusts. Enteropneusts are solitary marine worms that burrow in sandy or muddy sediment and feed by a combination of filter and deposit feeding ranging in size from a few millimeters to a meter in length (Cannon, Rychel, Eccleston, Halanych, & Swalla, 2009; Kaul-Strehlow & Röttinger, 2015). Pterobranchs are often small colonial animals connected to each other by a common stalk living as a series of inter-connected zooids (Sato, Bishop, & Holland, 2008).

As someone who has worked on these animals now for 20 years, I can attest to the many challenges of using hemichordates as developmental model species, both at the level of finding, collecting and working with the adult animals, but also challenges around working with their embryos. Research progress on pterobranchs has been slow largely due to their scarcity, with fewer global sites described, but there may be a diversity of these animals still waiting to be discovered (Tassia et al., 2016). Enteropneusts are broadly distributed globally, but more easily found on reef flats in tropical regions and intertidally in more temperate zones. The majority of their diversity and biomass may be found subtidally, as intertidal populations generally require protected bays or inlets. Recent studies have found a surprising deep sea diversity, again suggesting further exploration will uncover additional diversity (Osborn et al., 2012).

The phylogenetic position of hemichordates as sister group to echinoderms within the Ambulacraria is well established (Bromham & Degnan, 1999; Cameron, Garey, & Swalla, 2000; Cannon et al., 2014; Furlong & Holland, 2002) forming the Ambulacraria. Also, their close relationship with chordates mostly well supported by phylogenomic analyses (Fig. 1A).

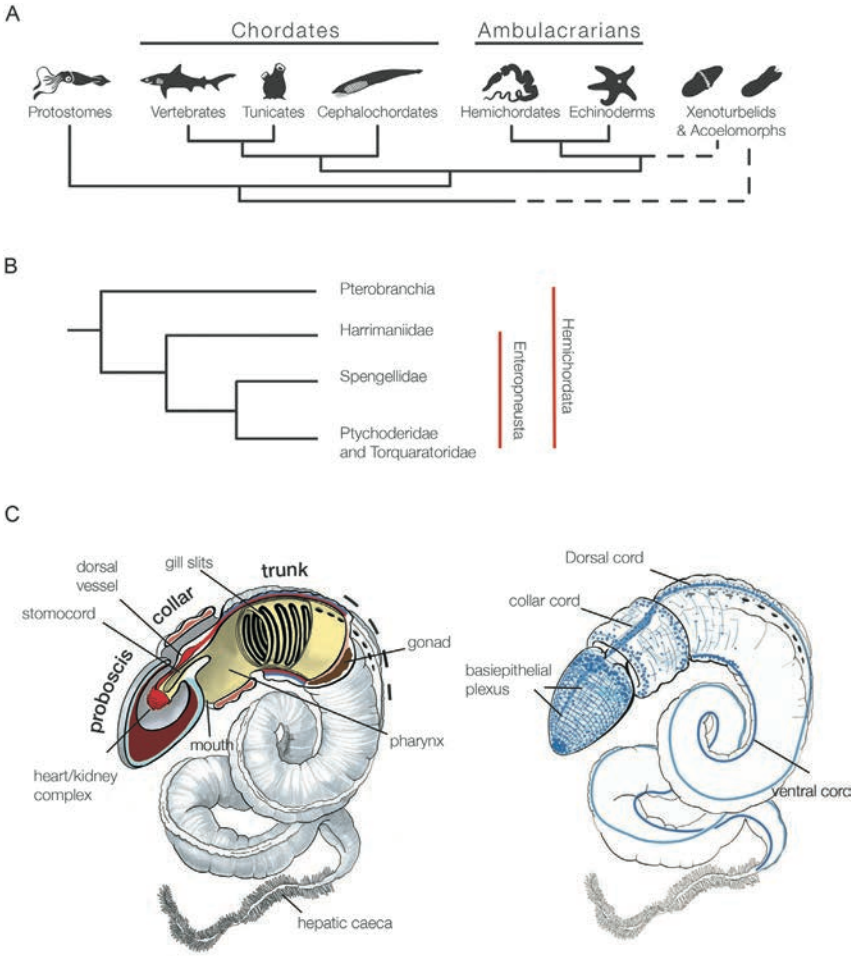

Fig. 1 Hemichordate body plan and phylogenetic relationships. (A) Summary of phylogenetic relationships of deuterostome taxa based on Philippe et al. (2011) and Hejnol et al. (2009). (B) Phylogenetic relationships within hemichordates showing the relationships between pterobranchs and enteropneusts, and the interrelationships between enteropneust families. Based on Cannon et al. (2014). (C) Diagrams of a generalized enteropneust showing the organization of the adult body plan and key anatomical features including the nervous system showing broad distribution of a neural plexus and two nerve cords. *Panel A and C: figure modified from Lowe, C.J., Clarke, D.N., Medeiros, D.M., Rokhsar, D.S., & Gerhart, J. (2015). The deuterostome context of chordate origins.* Nature, *520, 456–465.*

However, their relationship to acoels and Xenoturbellids remains controversial with some studies grouping Xenoturbellids into the deuterostomes, and some grouping acoels and Xenoturbellids as a monophyletic group (Xenacoelomorpha) into deuterostomes (Bourlat et al., 2006; Dunn et al., 2008; Mwinyi et al., 2010; Philippe et al., 2011, 2019) or as basally branching bilaterians (Hejnol et al., 2009; Rouse, Wilson, Carvajal, & Vrijenhoek, 2016; Simakov et al., 2015; Srivastava, Mazza-Curll, van Wolfswinkel, & Reddien, 2014). Nevertheless the core grouping of deuterostome taxa composed of chordates, hemichordates and echinoderms has been stable for some time. However, a recent preprint (Kapli et al., 2020) proposes that support for the classical deuterostome clade is equivocal, and an alternative radical departure to this classical grouping is equally likely; namely a possible closer affinity of chordates to protostomes, leaving the Ambulacraria as a clade separate from chordates and paraphyletic deuterostomes. Such a reorganization would require a reconsideration of how we think about chordate origins, and merits a thorough investigation, but is outside of the scope of this review.

Our understanding of the relationships within hemichordates is now greatly improved with better sampling, and while earlier molecular studies suggested that pterobranchs may nest within enteropneusts (Cameron et al., 2000), a more recent study organizes pterobranchs and enteropneusts into two monophyletic groups (Cannon et al., 2014). Enteropneusts are organized into four families; the Harrimanidae, Spengelidae, Ptychoderidae, with the deep sea Torquaratoridae nested within the Ptychoderidae (Fig. 1B) (Cannon et al., 2014).

3. Body plan and key anatomical features

The body plan of enteropneusts is well adapted to their marine life history as burrowing particulate and filter feeders. The following succinct description of their anatomy is largely to highlight key anatomies that have been important in the debate on the importance of hemichordates in understanding chordate origins. These features are highlighted in Fig. 1C. Other reviews provide a more comprehensive consideration of anatomy and historical background (Gee, 2018; Kaul-Strehlow & Röttinger, 2015; Satoh, 2016). Enteropneusts have a tripartite organization with an anterior proboscis/prosome, middle collar/mesosome, and posterior trunk/metasome (Hyman, 1955). The proboscis is strongly muscular and is involved in both burrowing and feeding. The proboscis, pharynx and gill slits in the anterior trunk are capable of creating feeding currents through ciliary action, passing phytoplankton and diatoms through the ventral mouth into the pharynx, then trapping them in paired pharyngeal gill slits before transporting them ventrally and posteriorly by ciliary action (Cameron, 2002; Gonzalez & Cameron, 2009). Worms also utilize direct particle capture in mucus secreted from the proboscis ectoderm and moved down through the mouth by ciliary action. These ropes of mucus and particles are transferred from the pharynx and into the posterior gut. In the anterior gut, the stomochord (Bateson, 1886; Ruppert, 2005), a supportive structure for the heart/kidney complex (Balser & Ruppert, 1990), projects into the proboscis. The trunk can be very long with the gut running the entire length into a terminal anus. In Harrimaniids, newly hatched juveniles are characterized by a post-anal tail (Bateson, 1885), which is used by the juvenile to move around in the burrow and as a hold fast, but is resorbed as the animals grow, and is not present in other hemichordate groups. The most anterior trunk is defined by the posterior pharynx and paired cartilaginous gill slits (Gillis, Fritzenwanker, & Lowe, 2011; Rychel, Smith, Shimamoto, & Swalla, 2006; Rychel & Swalla, 2007), which give way to the paired gonad, and in some species, a prominent region of hepatic saccules. The nervous system is defined by a basiepithelial plexus throughout the ectoderm and pharynx, but most conspicuous anteriorly in the proboscis and collar (Bullock, 1940; Dilly, Welsch, & Storch, 1970; Hess, 1937; Kaul-Strehlow, Urata, Minokawa, Stach, & Wanninger, 2015; Knight-Jones, 1952; Silèn, 1950). The plexus is most prominent at the base of the proboscis, forming a thick, dense layer of neurites, thickest in the dorsal region. There are regions of potentially centralized elements: two cords running along the dorsal and ventral midlines (Knight-Jones, 1952; Nomaksteinsky et al., 2009; Pickens, 1970). The dorsal cord runs from the most anterior region of the proboscis to the anus. In both the proboscis and trunk, this cord represents a thickening of the pervasive plexus into a superficial cord, with an associated cell bodies or soma, but in the most anterior extent of the dorsal cord that runs the length of the collar, it is internalized by a process that resembles chordate neurulation (Bateson, 1885; Brown, Prendergast, & Swalla, 2008; Bullock, 1945; Kaul & Stach, 2010; Knight-Jones, 1952; Luttrell, Konikoff, Byrne, Bengtsson, & Swalla, 2012; Morgan, 1894). The ventral cord begins at the anterior trunk, connecting to the dorsal cord just posterior to the collar and extends down the entirety of the trunk. Both cords, shown by EM to contain closely packed axons, have associated neural cell bodies, suggestive of an integrative function

(Kaul-Strehlow, Urata, Praher, & Wanninger, 2017; Nomaksteinsky et al., 2009), but their potential role in coordinating neural function remains poorly characterized (Cameron & Mackie, 1996; Pickens, 1970).

4. Life history strategies of species used for developmental studies

There have been a range of species of hemichordate utilized for developmental studies that span much of the phylogenetic diversity of the group (Bateson, 1884, 1885; Brown et al., 2008; Gonzalez, Jiang, & Lowe, 2018; Henry, Tagawa, & Martindale, 2001; Lin, Tung, Yu, & Su, 2016; Miyamoto & Saito, 2007; Morgan, 1894; Sato et al., 2008; Urata & Yamaguchi, 2004) although there are no developmental studies available for any torquaratorid species so far, and molecular studies on pterobranch development are still very limited (Sato et al., 2008; Sato & Holland, 2008). Enteropneusts are split into two developmental categories; direct developers and indirect developers (Fig. 2). Developmental mode falls squarely into a phylogenetic framework with the harrimanid lineage all direct-developers whereas the rest of the class are indirect-developers. The majority of developmental data has been generated from two species; *P. flava* and *S. kowalevskii*. *P. flava* is from the Ptychoderidae, which has a broad Indopacific distribution in sandy reef flats, and is characterized by indirect development (Rottinger & Lowe, 2012). *S. kowalevskii*, from the Harrimaniidae, is characterized by direct development and found intertidally along the majority of the Eastern Seaboard of the USA (Lowe, Tagawa, Humphreys, Kirschner, & Gerhart, 2004). Several other indirect-developing species have been utilized in developmental comparisons; two other ptychoderid species; *Balanoglossus simodensis*, and *Balanoglossus misakiensis* from Japan, and the spengellid *Schizocardium californicum* from the west coast of the USA.

5. Developmental contributions to testing hypotheses of morphological homology

Much of the initial motivation for examining developmental mechanisms in hemichordates was driven largely by the promise of applying new data and techniques to address classical morphological-based hypotheses raised over 100 years ago. Hypotheses of chordate origins have been particularly challenging to test due the large morphological disparity between

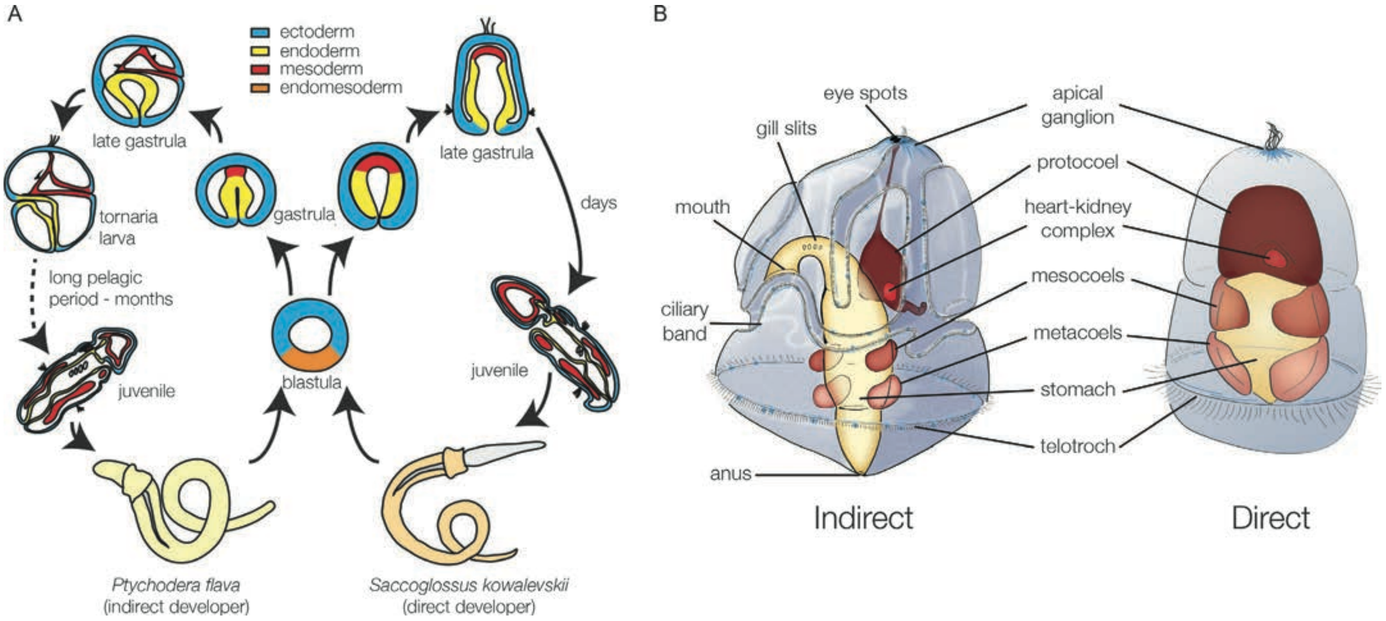

Fig. 2 See figure legend on next page.

chordates and their closest invertebrate outgroups, echinoderms and hemichordates (Lowe, Clarke, Medeiros, Rokhsar, & Gerhart, 2015; Tagawa, 2016). Comparative studies based on morphological comparisons had reached an impasse, and molecular data has the potential to help resolve some of these long-standing hypotheses.

6. Pharyngeal gill slits

The new molecular data has not provided unambiguous support to either accept or refute the proposed anatomical homologies between hemichordate and chordate structures. However, the pharyngeal gill slits are an exception (Fig. 1). Molecular work strongly supports the homology between hemichordate and chordate gill slits (Fritzenwanker, Gerhart, Freeman, & Lowe, 2014; Gillis et al., 2011; Ogasawara, Wada, Peters, & Satoh, 1999; Okai, Tagawa, Humphreys, Satoh, & Ogasawara, 2000; Rychel et al., 2006; Rychel & Swalla, 2007; Simakov et al., 2015). Data supporting homology on morphological characters was already strong: enteropneust gill slits resemble those in amphioxus, both from an anatomical

Fig. 2 Developmental mode of hemichordate species. (A) model of the two contrasting life histories of enteropneust hemichordates. Indirect developers develop into planktonic larvae spending months in the plankton before metamorphosis into benthic juveniles. Direct developers have abbreviated development forming the adult body plan from the embryo. They are defined by a very short pelagic period, and hatch as competent juveniles that can swim or immediately begin to burrow and feed. (B) Detailed models of the two contrasting life histories. The generalized larva is representative of all indirect developing species, late in larval development, many weeks into planktonic development, close to metamorphosis. The model of the direct developer represents 48 h. of development, after gastrulation during embryo elongation, following the formation of the coelomic pouches. Both models are shown as a ventro-lateral tilted view with anterior at the top and posterior at the bottom. *Figure modified from Lowe, C.J., Clarke, D.N., Medeiros, D.M., Rokhsar, D.S., Gerhart, J. (2015). The deuterostome context of chordate origins.* Nature, 520, *456–465. and Simakov, O., Kawashima, T., Marletaz, F., Jenkins, J., Koyanagi, R., Mitros, T., Hisata, K., Bredeson, J., Shoguchi, E., Gyoja, F., Yue, J.X., Chen, Y.C., Freeman, R.M., Jr., Sasaki, A., Hikosaka-Katayama, T., Sato, A., Fujie, M., Baughman, K.W., Levine, J., Gonzalez, P., Cameron, C., Fritzenwanker, J.H., Pani, A.M., Goto, H., Kanda, M., Arakaki, N., Yamasaki, S., Qu, J., Cree, A., Ding, Y., Dinh, H.H., Dugan, S., Holder, M., Jhangiani, S.N., Kovar, C.L., Lee, S.L., Lewis, L.R., Morton, D., Nazareth, L.V., Okwuonu, G., Santibanez, J., Chen, R., Richards, S., Muzny, D.M., Gillis, A., Peshkin, L., Wu, M., Humphreys, T., Su, Y.H., Putnam, N.H., Schmutz, J., Fujiyama, A., Yu, J.K., Tagawa, K., Worley, K.C., Gibbs, R.A., Kirschner, M.W., Lowe, C.J., Satoh, N., Rokhsar, D.S., Gerhart, J. (2015). Hemichordate genomes and deuterostome origins.* Nature, 527, *459–465.*

and functional perspective (Gonzalez & Cameron, 2009; Rychel & Swalla, 2007). In the sister group to hemichordates, the echinoderms, there is no equivalent structure in extant species, yet in certain stem echinoderm fossils there is evidence of gill slits that were subsequently lost in crown groups (Dominguez, Jacobson, & Jefferies, 2002). Genes expression studies of transcription factors with reported roles in chordate pharyngeal and gill pouch patterning including *pax1/9* and *six1/2*, *eya* and three Fox genes *Fox I, FoxC* and *FoxL1* (Fritzenwanker et al., 2014; Gillis et al., 2011; Ogasawara et al., 1999) showed conserved expression domains in *S. kowalevskii* during gill pouch development. Genomic data has now provided added data further strengthening homology: Hemichordates share the same pharyngeal gene cluster that was originally described in chordates (Simakov et al., 2015). This cluster contains 6 genes, 4 of which are transcription factors (*pax1/9, nk2–1, nk2–2 and foxA*) with expression domains in the pharyngeal slits or in the pharyngeal endoderm surrounding them. The genes are in the same order as in the vertebrate clusters, and an ordered cluster is even detected in the genome of an asteroid, making this genomic feature clearly part of a conserved ancestral developmental program for anterior gut patterning in deuterostomes (Baughman et al., 2014; Simakov et al., 2015).

7. Nerve cords and stomochord

Perhaps the most intense interest in hemichordates concerns their potential for providing insights into evolution of the chordate nervous system. The organizational principles of the enteropneust nervous system have long been debated, as there are elements of a pervasive nerve plexus suggestive of local processing (Bullock, 1940; Lowe et al., 2003), but also centralized elements including the collar cord (Kaul & Stach, 2010; Knight-Jones, 1952; Luttrell et al., 2012), which has long been a structure that has fascinated zoologists because of its morphogenesis is similar to the vertebrate nerve cord (Fig. 1). How the nervous system processes information and the role of cord's integration of sensory information will require further neurophysiology experiments (Cameron & Mackie, 1996; Pickens, 1970). Further, if the cords do represent centralized processing centers, are either of them homologous to any region of the chordate dorsal central nervous system? Several groups have either explicitly addressed these questions or have produced data relevant to the question (Formery, Schubert, & Croce, 2019). In Lowe et al. (2003), described in more detail below, classical transcriptional regulators of anteroposterior and mediolateral patterning of the vertebrate central nervous system (CNS) were largely expressed in circumferential domains throughout the ectoderm of *S. kowalevskii*, rather than being expressed coincident with the more centralized elements of the nervous system. They proposed that this may reflect the fundamental organizational principle of hemichordates nervous system as a plexus (Lowe et al., 2003). Later work further tested this hypothesis and examined the expression of a range of marker genes conserved in fruit flies and/or vertebrates for neural progenitor cells (*soxB1* and *msi*), differentiating neurons (*elav, prox, bruA* and *dclk*), and mature neurons (*syt1, gad, th, tph, vmat* and 5-HT) (Cunningham & Casey, 2014). The initial distribution of neurons is broadly spread throughout the ectoderm but this was transitory, and before hatching the developing nerve cells of the trunk become more aligned with the midlines (Cunningham & Casey, 2014) as the cords begin to form. Neural markers such as Gaba, 5HT, VaChT rather than exclusively patterning genes were also examined in adult *P. flava* (Nomaksteinsky et al., 2009). They reveal a clear aggregation of neurons at the base of the proboscis, concentrated dorsally, and also along the dorsal cord and ventral cord, with far fewer neurons in the general ectoderm. This pattern was also observed in the post-metamorphic juveniles in another indirect-developing species, *B. misakiensis* (Kaul-Strehlow et al., 2017). The cords are organized with a concentration of cell bodies and a clear neuropil. Cholinergic and GABA-ergic cell bodies were identified in the cords and proboscis stem, with only sparse expression of the cholinergic marker VaChT outside of these domains (Nomaksteinsky et al., 2009). Nomaksteinsky and colleagues propose that the cords and proboscis stem represent a bonafide CNS, with the remaining sparser distribution of neurons throughout the ectoderm representing a peripheral nervous system. The authors remark on the close similarities between the internalized collar cord and the dorsal cord of vertebrates, but in conclusion do not make a strong statement of homology of any part of the enteropneust CNS to chordates, and raise the possibility of deep homology of the patterning module rather than specific neuroanatomies between hemichordates and chordates.

Further studies have focused more specifically on the potential homology of the collar cord to the chordate dorsal nervous system (Kaul-Strehlow et al., 2017; Luttrell et al., 2012; Miyamoto & Wada, 2013). The striking morphological similarities between hemichordate collar cord and vertebrate neural tube, and the process of neurulation were first noted by classical zoologists (Bateson, 1884; Bullock, 1945; Dawydoff, 1948; Knight-Jones, 1952; Morgan, 1894) and have been recently reinvestigated by several groups

(Kaul & Stach, 2010; Luttrell et al., 2012; Miyamoto & Wada, 2013). Miyamoto and Wada (2013) investigated the developmental basis of neurulation in *B. misakiensis* and demonstrate that the stomochord and dorsal endoderm that underlie the region of collar ectoderm that gives rise to collar cord, are a broad source of hedgehog (hh) ligand, which has been shown to be a key determinant in the D/V dorsoventral patterning of the vertebrate neural tube (Echelard et al., 1993). They also demonstrate that there is some spatial pattern in the D/V aspect of the collar cord using conserved markers of neural tube D/V patterning, such as *bmp2/4*, *dlx* and *pax3/7.* However, this D/V patterning is not nearly as extensive as that found in vertebrates, representing more dorsal than ventral markers. In *S. kowalevskii*, *bmp2/4* and *dlx* are similarly localized along the midline, with an additional dorsal neural tube marker, *tbx2/3*, but their expression extends along the entire length of the dorsal midline rather than restricted to the collar cord. In addition, classical markers of the vertebrate floor plate and ventral neural tube, such as *netrin*, *sim*, and the motor neuron marker *mnx,* are expressed ventrally, making comparisons quite complex as markers are split across both cords (Lowe et al., 2006). Kaul-Strehlow et al. (2017) again investigated the expression of mediolateral patterning genes in post metamorphic juveniles of *B. simodensis,* which showed little evidence of conserved spatial domains in the collar cord, and they concluded that the collar cord may represent an independent acquisition in the enteropneust lineage.

So far, the evidence in support of homology of any region of the hemichordate nervous system to the CNS of vertebrates is decidedly mixed, and while molecular patterning data do not explicitly rule out homology, it is also equally plausible that cords evolved independently in enteropneusts. Similar data from a variety of protostome groups also show a poor correspondence of mediolateral patterning data and cord formation, and suggest patterning similarities between model species may represent convergence rather than homology, with neuroanatomies evolving independently (Martin-Duran et al., 2018). Despite the extensive developmental patterning data available, clearly more work is needed to decipher the neural organization of hemichordates. There are few contemporary studies of the neural organization of hemichordates with a limited array of cross reactive antisera (Gonzalez et al., 2018; Kaul-Strehlow et al., 2015; Miyamoto, Nakajima, Wada, & Saito, 2010), and ultrastructure (Kaul & Stach, 2010; Kaul-Strehlow et al., 2015; Kaul-Strehlow & Stach, 2013), but a better understanding of neural organization, distribution, cell type composition and how it is regulated by conserved networks of regulatory genes is badly needed.

The origin of the chordate notochord has a long history in the classical comparative literature (Annona, Holland, & D'Aniello, 2015) and the hemichordate stomochord (Fig. 1) has long been proposed as a potential homolog of this key feature of the chordate body plan. The idea was originally proposed by Bateson (Bateson, 1886) and supported by other investigators (Balser & Ruppert, 1990; Miyamoto & Wada, 2013). However, an analysis and survey of structural and patterning genes during stomochord formation fail to provide support for the homology of the two structures (Satoh et al., 2014). Rather, expression of FoxE quite specifically in the stomochord (Fritzenwanker et al., 2014) provides evidence that this structure may have more affinities with the chordate endostyle. FoxE (TTF2), a thyroid specific transcription factor involved in vertebrate thyroid gland development (Damante, Tell, & Di Lauro, 2001) and is also expressed in the club-shaped gland of amphioxus larvae that eventually gives rise to the endostyle in adults (Yu, Holland, Jamrich, Blitz, & Hollan, 2002), as well as in the endostyle of ascidians (Hiruta, Mazet, Yasui, Zhang, & Ogasawara, 2005).

8. Insights from comparative axial patterning

While most interest in the developmental biology of hemichordates has focused on testing morphological homology, extensive patterning data now provide insights into body plan evolution at the level of axial patterning, not only for providing insights into the origin and evolution of chordates, but also to our understanding of how conserved axial patterning relates to the evolution of morphology. While it remains challenging to use developmental data to reconstruct ancestral deuterostome anatomical characters (Lowe et al., 2015), patterning data provides a suite of characters often more conserved than the morphologies they regulate, so offering an opportunity to infer ancestral deuterostome developmental modules. Using this approach, we can begin to test what aspects of the vertebrate axial programs evolved in stem chordates or stem vertebrates, and are therefore associated with the assembly of the features of the chordate body plan, and which ones are deep deuterostome or bilaterian innovations. There are now both descriptive and functional developmental data on early axial patterning for both the larval and adult body plans. Below I discuss the evolutionary implications of these studies for both body plan evolution and chordate origins.

9. Anteroposterior patterning

For the most part, comparative anteroposterior (A/P) and dorsoventral (D/V) patterning has been examined across phyla during ectodermal patterning, largely focused on central nervous system development. Particularly striking has been the close patterning similarities between arthropods, the errant annelid, *Platynereis dumerilii* and vertebrates (Arendt & Nubler-Jung, 1996; De Robertis, 2008; Reichert & Simeone, 2001). Suites of transcription factors with conserved relative spatial register and similar patterning roles have been described in all three groups during CNS development leading to the hypothesis of an ancestral complex CNS in the common ancestor of bilaterians (Denes et al., 2007; Hirth et al., 2003; Tomer, Denes, Tessmar-Raible, & Arendt, 2010; Vergara et al., 2017). A comprehensive examination of the expression of conserved orthologous transcription factors with conserved roles in CNS patterning during early development in *S. kowalevskii* shows the same exquisite conservation of pattern along the A/P axis, with the same relative spatial register as we find in the other models with complex central nervous system organization (Fig. 3A). However, the major difference is that in most cases, the expression domains wrap around entire ectoderm rather than being restricted to either the dorsal or ventral side (Lowe et al., 2003), as is the case for the many expression domains restricted to the neural plate of vertebrates. A subset of these genes has also been examined in two other hemichordate species with expression domains that are very similar (Gonzalez, Uhlinger, & Lowe, 2017; Kaul-Strehlow et al., 2017). For example, vertebrate forebrain patterning genes such as *six3*, *foxG*, *retinal homeobox* and *nk2–1* are restricted to the developing proboscis ectoderm, those involved in midbrain patterning such as *otx*, *pax6* and *barH* down into the collar/mesosome, and hindbrain, and trunk patterning genes such as *engrailed (en)*, *gbx*, and Hox genes are expressed in the metasome/trunk. Hox genes show some divergence from this pattern, with certain members of the cluster associating more with the midlines and nerve cord development than the entire ectoderm (Aronowicz & Lowe, 2006; Gonzalez et al., 2017). This general pattern has now been shown in three difference species: most comprehensively in the direct developer *S.kowalevskii (*Aronowicz & Lowe, 2006*;* Lowe et al., *2003)*, but also in representative species from the two remaining enteropneust families; the ptychoderid *B.simodensis* (Kaul-Strehlow et al., 2017) and the Spengellid, *S.californicum (*Gonzalez et al., 2017*)* and thus

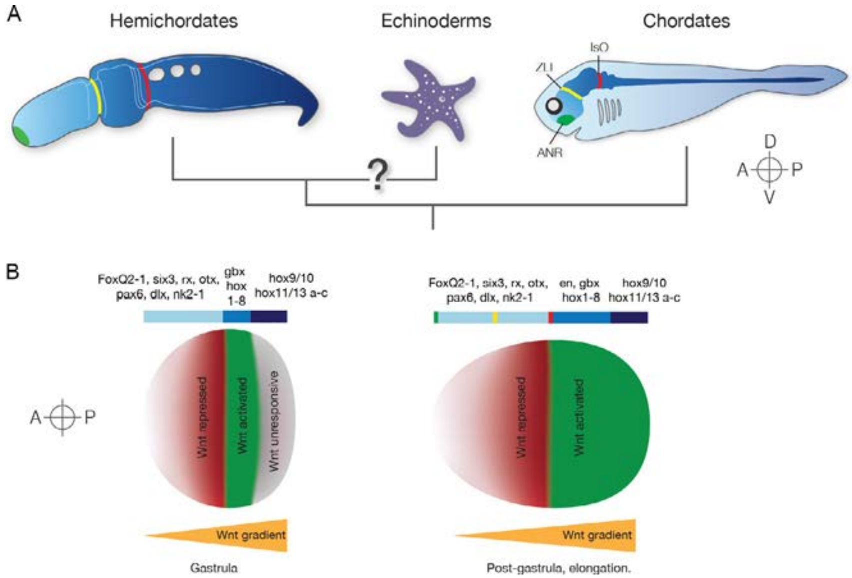

Fig. 3 Summary of ectodermal anteroposterior axial patterning of enteropneusts and chordates. (A) Comparison of A/P patterning in a generalized enteropneust and vertebrate. The blue gradient represents the similarities in gene expression of homologous transcription factors involved in specifying the main regional compartments of the vertebrate CNS and enteropneust ectoderm. The green yellow and red stripes represent homologous local signaling centers positioned at the same relative position in the CNS of vertebrates and general ectoderm of hemichordates. (B) Summary of the role of cWnt signaling during early development of *S. kowalevskii*. The blue rectangles represent the regional expression of groups of conserved transcription factors along the A/P axis of the embryos. Two developmental stages are shown and represent a surface view of the ectoderm at late gastrula and post gastrula stage. The red territory represents the anterior embryonic territories that are repressed by the action of cWnt. The green territory represents the territory dependent on cWnt signaling for gene activation. The gray blastoporal territory in the posterior of the gastrula represents a region not responsive to cWnt during blastula and gastrulation. *Panel A is modified from Lowe, C.J., Clarke, D.N., Medeiros, D.M., Rokhsar, D.S., Gerhart, J. (2015). The deuterostome context of chordate origins.* Nature, 520, *456–465.*

represents the ground state of enteropneusts (Kaul-Strehlow et al., 2017). Expression domains of the majority of these genes are in the general ectoderm rather than uniquely associated with the neural cords or regions of neural concentration at the base of the proboscis. This suggests a general role in providing spatial patterning information throughout the ectoderm, to both nervous system and epidermal tissue. A general role in ectodermal patterning for many of the A/P patterning genes may be a conserved feature of this suite of genes. Holland et al. (2013) point out that many of the transcription factors with distinct expression territories in *S. kowalevskii* that were investigated in Lowe et al. (2003) also have expression domains in the ectoderm outside of the CNS of chordates.

Interest has largely focused on the adult body plan of enteropneusts, but the same patterning network is also responsible for patterning the larval A/P axis and has been examined explicitly in *S. californicum* and to some extent also in *P. flava* (Gonzalez et al., 2017; Rottinger, DuBuc, Amiel, & Martindale, 2015; Rottinger & Martindale, 2011; Su et al., 2019). The metamorphosis from larval to adult body plans in enteropneusts is dramatic, resulting in a rapid change in body plan from a planktonic feeding larva, constructed of a gel-filled body with thin epithelia, using ciliary bands for feeding and swimming, to a benthic, muscular burrowing worm over the course of 48 h. (Gonzalez et al., 2018; Miyamoto et al., 2010; Morgan, 1891). Despite these major morphological transformations of the body regions between the two life history stages, the underlying A/P patterning network seems largely conserved (Gonzalez et al., 2017). The transcription factors with conserved roles in anterior patterning, markers of the proboscis and collar in the adult, are deployed in similar relative expression domains to their distribution in the adult as the larval body plan begins to form during embryogenesis and beyond. The most terminal markers, the posterior class Hox genes, are also expressed around the blastopore of embryos and eventually, the anus, following embryogenesis. However, the anterior/mid trunk markers, the medial and anterior class Hox genes, are not activated during the establishment of the larval body plans, and are only transcribed late in larval development when the adult body plan is beginning to form, closer to metamorphosis (Gonzalez et al., 2017). A similar distribution of patterning genes is also described in echinoderm larvae (Yankura, Martik, Jennings, & Hinman, 2010) suggesting that ambulacrarian larvae are a trunkless body plan and are best described as swimming heads (Lacalli, 2005). I will further discuss the importance of considering life history in comparative studies in a later section.

10. Origin of CNS signaling centers

In *S. kowalevskii* embryos the A/P patterning similarities with vertebrates can be extended even further. The vertebrate brain and central nervous system develop by successive refinement of the neurectoderm into specialized territories and domains. Signaling centers act as secondary organizers that mediate regional patterning in the CNS, and are defined by the local secretion of specific ligands (Echevarria, Vieira, Gimeno, & Martinez, 2003; Kiecker & Lumsden, 2012). The three main centers are the anterior neural ridge (ANR), the zona limitans intrathalamica (ZLI) and the midbrain hindbrain organizer or isthmus (Iso), which are conserved throughout vertebrates (Fig. 3A) but are either absent or divergent in invertebrate chordates (Albuixech-Crespo et al., 2017; Scholpp & Lumsden, 2010). However, some investigators (Holland et al., 2013) still contest the absence of local neural organizers in cephalochordates. This would suggest that local neural signaling centers may have been a stem vertebrate innovation that was associated with the evolution of a more complex CNS. Surprisingly, in *S. kowalevskii*, these same signaling center regulatory programs are established at equivalent regions of the A/P transcriptional network following gastrulation, as the embryo begins to elongate and establish the major divisions of the body plan; an ANR-like domain at the anterior tip of the proboscis/prosome expressing *fgf8/17/18* and *secreted frizzled 1/2/5*, a ZLI-like domain at the boundary between the developing proboscis and the collar expressing *hh*, and an Iso-like domain expressing *fgf8/17/18/Wnt1* (Fig. 3A). Functional evidence supports similarities in the regulatory program both upstream and downstream of these localized domains of ligand expression, suggesting a conservation of the regulatory program in the absence of any morphological homology (Pani et al., 2012). Although this interpretation has been challenged (Holland et al., 2013), suggesting that differences in gene expression domains between hemichordates and vertebrates are more consequential than acknowledged. For example, *irx* and *fezF*, two important transcription factors with abutting domains in the anterior neural plate of chordates, including amphioxus (Irimia et al., 2010), are important in the establishment of the ZLI in vertebrates, are not expressed in the same pattern in *S. kowalevskii*, leading the authors to question the ZLI comparison. However, a follow up study of cis-regulation of the defining ligand of the ZLI, *hh*, strengthens the evidence of the homology of the ZLI regulatory program between hemichordates and vertebrates (Yao et al., 2016). Comparative sequence analysis is generally used to test for conservation of regulatory sequences between taxa, yet it is clear that functionally conserved enhancers do not always display strong sequence conservation (Fisher, Grice, Vinton, Bessling, & McCallion, 2006; Hare, Peterson, Iyer, Meier, & Eisen, 2008), and genes with similar spatiotemporal expression domains may share conserved regulatory logic. The defining ligand of the ZLI, Sonic Hedgehog (shh), has many distinct expression territories within the vertebrate CNS under the regulatory control of distinct enhancers (Epstein, McMahon, & Joyner, 1999; Jeong et al., 2008; Jeong, El-Jaick, Roessler, Muenke, & Epstein, 2006; Jeong & Epstein, 2003). Two enhancers have been identified that are involved in expression of Shh in mouse at the ZLI—SBE1 and SBE5. SBE 1 is an intronic enhancer in intron 2 of the Shh locus and involved in expression in the ventral forebrain and ZLI (Epstein et al., 1999). Surprisingly, an analysis of intron 2 of Hh in *S. kowalevskii* revealed a putative homologous enhancer with similar transcription factor binding motifs to SBE1, but in a scrambled order relative to the mouse enhancer. Recent progress in the development of transgenic approaches in *S. kowalevskii* (Minor et al., 2019) facilitated testing this enhancer for activity *in vivo*. Injection of a GFP transgene of this hemichordate intronic enhancer demonstrated activity in the putative hemichordate ZLI-like domain, at the boundary between the proboscis and collar, supportive of a conserved function. Most surprising was that cross species enhancer swaps demonstrated that the regulatory logic of the hemichordate SBE1-like enhancer was sufficient to drive reporter expression in mouse in largely the same spatiotemporal domains as the endogenous mouse SBE1 element. Conversely, the mouse enhancer drove identical expression to the endogenous hemichordate SBE1 enhancer during early hemichordate development. So, despite the fundamental body plan differences and 600 million years of independent evolution that separates vertebrates and hemichordates, the cis-regulatory logic of an enhancer, along with its trans regulators for a key developmental ligand, is conserved and involved in patterning very different ectodermal structures. This enhancer is not present in either amphioxus or tunicates indicating secondary loss from an ancestral state, and outlines the importance of outgroup comparisons for determining which gene regulatory innovations are deuterostome-specific, predating the origins of the chordate body plan, and which ones are stem chordate, or stem vertebrate innovations, and potentially instrumental in the evolution of specific features of the chordate body plan. This work clearly demonstrates the challenges of using gene regulatory networks for reconstructing ancestral morphological characters. Given how diverse the body plans are that these homologous programs regulate, our ability to reconstruct morphological characters from these data is limited.

11. Early establishment of A/P pattern

From a wide range of vertebrate studies, the early establishment of A/P pattern in the embryo is now well characterized. A number of signaling pathways are implicated, with minor differences between animal species, yet largely conserved roles throughout the groups are largely conserved (Arnold & Robertson, 2009; Schier, 2001; Schoenwolf, 2000). One of the most important signaling pathways for A/P specification is the canonical Wnt (cWnt) cascade. It has the strongest effect on head patterning, repressing the most anterior neural fates of forebrain and midbrain, but also promoting more posterior, hindbrain fates in a dose-dependent manner (Glinka et al., 1998; Kiecker & Niehrs, 2001; Leyns, Bouwmeester, Kim, Piccolo, & De Robertis, 1997; McGrew, Hoppler, & Moon, 1997; Nordstrom, Jessell, & Edlund, 2002; Piccolo et al., 1999). Wnt antagonists, such as Dkk and secreted frizzled, are localized anteriorly in the neural plate, prechordal plate and organizer, to protect the anterior neural territory from the posteriorizing effects of Wnt ligands secreted from the posterior neural plate and underlying mesoderm (Glinka et al., 1998; Houart et al., 2002; Kiecker & Niehrs, 2001; Piccolo et al., 1999). This has led to the hypothesis that a simple cWnt gradient, low in the anterior and high in the posterior, is largely responsible for the patterning of the ectoderm, and has been proposed as a fundamental conserved mechanism of A/P patterning (Kiecker & Niehrs, 2001; Niehrs, 2010; Nordstrom et al., 2002). This model is strongly supported from comparative vertebrate studies focused on forebrain, midbrain and hindbrain, but surprisingly little data has been generated from the most posterior regions of the neuraxis, and recent work has challenged the importance of Wnt in posterior specification (Polevoy et al., 2019).

In the invertebrate chordates, the role of cWnt in A/P patterning is likely broadly conserved for the most part. However, in both amphioxus and ascidians there are distinct differences in details, with cWnt largely involved in the specification of the far anterior and posterior of the A/P axis (Feinberg, Roure, Piron, & Darras, 2019; Onai et al., 2009) suggesting that their neuraxes are not fully patterned by cWnt. In echinoderms, nothing is known about the role of Wnt in adult patterning; but in larvae, cWnt also plays a conserved role in anterior patterning (Range, 2018; Range, Angerer, & Angerer, 2013; Range & Wei, 2016), even though the lack of a larval trunk territory (Yankura et al., 2010) makes a full comparison of axial patterning with vertebrates difficult. The role of cWnt patterning in protostomes has been most comprehensively examined in planarians where it is involved in the establishment of the A/P axis during homeostasis and regeneration, and regulates the developmental decision between head and tail (Gurley, Rink, & Sanchez Alvarado, 2008; Petersen & Reddien, 2008). In the polychaete *Platynereis dumerilli*, cWnt also plays a key role in head suppression, and in arthropods there are several examples of cWnt involved in posterior growth (Angelini & Kaufman, 2005; Marlow et al., 2014; Martin & Kimelman, 2009; Miyawaki et al., 2004), and some evidence from the beetle Tribolium that Wnt may also be involved in head patterning (Fu et al., 2012). It is therefore broadly accepted that cWnt is a conserved element of A/P patterning (Petersen & Reddien, 2009).

The close transcriptional similarities between vertebrates and hemichordates during ectodermal A/P patterning raise the possibility that the upstream regulation of this gene regulatory network is also conserved. A recent study of cWnt signaling in *S. kowalevskii* again exhibits some striking similarities with vertebrate patterning (Darras et al., 2018). The 13 Wnt ligands, four frizzled receptors, and a range of Wnt antagonists show strongly localized patterns along the animal/vegetal and A/P axes during embryogenesis. The ligands are mostly concentrated in the posterior ectoderm from blastula through gastrula stages, and throughout almost all ectoderm with the exclusion of the most anterior territory. Like the expression of transcription factors along the A/P axis, ligands, receptors and antagonists are expressed in ectodermal rings. Unlike sea urchins, we do not observe any ligand expression in the endomesoderm precursors (Robert, Lhomond, Schubert, & Croce, 2014). Wnt antagonists Sfrps and Dkk1/2/4 are localized anteriorly in the ectoderm, in a manner very similar to their disposition in other bilaterian species, including vertebrates (Glinka et al., 1998; Range, 2018). Manipulation of the levels of cWnt signaling during the most critical stage of A/P patterning, mid-blastula through gastrulation, results in major transformations of the fate of the A/P axis. Over-expression of Wnt3 by RNA injection, or constitutive activation of cWnt signaling by the application of a Gsk3ß antagonist, resulted in the truncation of anterior fates in a dose dependent manner: both proboscis and collar fates were lost, with the anterior trunk marker engrailed (en) expressed at the far anterior tip of experimental embryos, and a remaining trunk territory that seemed relatively normal. The reciprocal experiment of lowering cWnt activity by over-expression of Wnt antagonists resulted in the complementary phenotype of a greatly enlarged proboscis with reduced trunk. However, in neither experiment was the axis completely anteriorized or posteriorized, very similar to the phenotypes in vertebrate experiments (Kiecker & Niehrs, 2001). A QPCR analysis following GSK3ß inhibitor treatments through gastrulation, resulting in the constitutive activation of cWnt signaling, demonstrated a clear molecular read out that cWnt repressed anterior fates in a dose dependent manner down to the posterior collar, and promoted anterior and mid trunk fates. Notably, GSK3ß inhibitor treatments through gastrula stage had no effect on the most posterior fates defined by the expression of posterior Hox at this early stage of A/P specification. It was only after gastrulation, as the embryo started to elongate during an extended period of posterior growth, that posterior Hox became entrained into cWnt regulation in a regulatory loop with Brachyury that bares a strong resemblance to the mechanisms of posterior growth in vertebrates (Fritzenwanker, Uhlinger, Gerhart, Silva, & Lowe, 2019). The targets of cWnt signaling during embryogenesis in *S. kowalevskii* are strikingly similar to those described in vertebrates; repression of markers of forebrain-midbrain/proboscis-collar markers and promotion of hindbrain/anterior trunk markers (Fig. 3B). The lack of early cWnt sensitivity of posterior Hox during embryogenesis during the initial establishment of posterior fates challenges the simple model of cWnt function in early A/P patterning (Niehrs, 2010). The possibility that this may be a conserved feature of deuterostomes is supported by work in Xenopus reporting similar findings and deserves further investigation in other species (Polevoy et al., 2019).

The exquisite conservation of a cWnt regulated transcriptional network involved in ectodermal A/P regionalization between such contrasting body plans again reveals the rather loose coupling of gene regulatory networks with morphological evolution, and demonstrates how tight regulatory conservation seems to be permissive rather than restrictive in morphological output over macroevolutionary time frames. This type of data is extremely valuable for making broad comparisons across groups and establishing regional rather than anatomical homologies. This is particularly useful in comparisons where morphological disparity is large, making even basic regional comparisons challenging. From both the transcriptional and cWnt patterning data, the proboscis of hemichordates and the forebrain of vertebrates share fundamental patterning similarities as does the midbrain and collar, and the hindbrain-spinal cord/trunk. This does not imply any type of morphological homology, but rather reflects a common ancestry from a homologous ancestral territory; a deep homology (Shubin, Tabin, & Carroll, 2009). However, these data establish a good regional framework for investigating anatomical homology. For example, if the collar cord is homologous to any region of the vertebrate CNS, then it would not be the hindbrain or spinal cord, but rather the midbrain, as the collar transcriptional profile most closely resembles this region of the vertebrate CNS (Kaul-Strehlow et al., 2017). Also, the first gill slit in both vertebrates and hemichordates develops in a similar transcriptional regional territory along the A/P axis, further supporting homology.

12. Dorsoventral patterning

The D/V axis has featured prominently in comparative body patterning (Arendt & Nubler-Jung, 1994; De Robertis, 2008; De Robertis & Sasai, 1996). Details of axis patterning have been well characterized in both vertebrates and arthropods, and it is very well established that the CNS of arthropods and chordates are established by a homologous signaling system. However, how these close patterning similarities relate to the homology of anatomies patterned by this conservative regulatory system is less clear (Arendt, 2018; Lowe et al., 2006; Martin-Duran et al., 2018). The CNS of both arthropods and chordates forms in a region of the ectoderm that is protected from the neural-repressive effects of Decapentaplegic/Bmp by Short gastrulation(sog)/Chordin (Holley & Ferguson, 1997). In arthropods, the CNS forms on the ventral side, and in chordates on the dorsal side. The distribution of the Bmp/Chordin axis defines the position of the CNS in both of these distantly related groups, so this molecular axis is inverted in chordates in relation to arthropods, and gives support to an old classical hypothesis that chordates evolved by D/V axis inversion of an ancestor with a CNS orientated on the ventral side (Geoffroy-St. Hilaire, 1822). The phylogenetic position of hemichordates, as a group closely related to chordates, with two nerve cords; one on the dorsal and the other on the ventral side makes this group particularly interesting for the investigation of the role of a Bmp/Chordin axis during early development. This axis has now been investigated in both indirect and direct-developing species with some common and contrasting findings (Lowe et al., 2006; Su et al., 2019). In *S. kowalevskii* (Lowe et al., *2006*), *bmp2/4* is expressed throughout embryogenesis and into juvenile development on the dorsal side in the ectoderm, overlapping the region where the dorsal cord forms. Chordin is expressed early in gastrulation very broadly in the ectoderm, but excluded from the dorsal side, and becomes increasingly localized to the ventral side at later stages. This orientation of a Bmp/Chordin axis resembles that of protostomes rather than chordates, where ventral is defined as the side of the body apposed to the substratum and the side on which the mouth opens. Manipulation of Bmp signaling through embryogenesis has a profound effect on the D/V axis in all three germ layers. Knockdown of *bmp2/4* results in ventralization of the body plan. There is an expansion of the mouth territory dorsally to the extent that at later stages the proboscis becomes detached from the rest of the body. Ventral molecular markers expand and dorsal markers are repressed. Following *bmp2/4* over expression, treated embryos exhibit a reciprocal dorsalized phenotype. Interestingly, A/P organization seems largely unaffected without any major changes suggesting that D/V and A/P patterning may be mostly uncoupled in this species. Strikingly, following over-expression of *bmp2/4*, neural fates are not repressed as they are in both arthropods and chordates. The role of a Bmp/Chordin axis in *S. kowalevskii* is largely to provide broad D/V patterning information across all three germ layers, including the ectoderm, similar to other groups. The inversion of the molecular axis between protostomes plus hemichordates and chordates suggests that some sort of axis inversion occurred in stem chordates. There are some clear anatomical patterns related to this axis that seem well conserved; on the Bmp side, there are gill slits, a contractile heart-like dorsal vessel and coelomic mesoderm; on the chordin side there is the majority of the striated muscle. This anatomical relationship to the Bmp/Chordin axis is conserved between hemichordates and chordates and was inherited from a common ancestor (Lowe et al., 2015). However, there are some striking differences in two of the main morphological features polarized along the D/V axis. First, the functional data clearly demonstrated that the hemichordate mouth is sensitive to bmp levels—it expanded in the absence of Bmp and was repressed in excess Bmp. This is in contrast to chordates where the mouth forms in the Bmp territory and suggests a novel relationship of the chordate mouth to Bmp signaling. Second, the nervous system shows a very different relationship to the Bmp/Chordin axis. *Bmp2/4* in *S. kowalevskii* is expressed along the dorsal midline, coincident with the forming dorsal cord Thus, if either of the neural cords is homologous to the chordate dorsal cord, then an inversion scenario would suggest that this would be the ventral cord rather that the dorsal cord. However, as previously discussed, we do not see strong molecular support for homology of either cord. In addition, manipulation of the levels of Bmp in *S. kowalevskii* seemed to play more of a patterning role in the ectoderm rather than a role in segregation of a discrete neurectoderm. Clearly this critical component of bilaterian D/V patterning can support a range of neural architectures while maintaining a primary role in D/V patterning. D/V axis inversion can therefore be considered independent from issues of nervous system centralization and the complexities of making direct comparisons between hemichordate and chordate nervous systems.

Data from indirect-developing species share some patterning similarities, but also some distinctive differences with *S. kowalevskii* (Su et al., 2019). First, it is important to note that embryogenesis gives rise to a larval body plan rather than an adult body plan, so the morphological output of the embryonic patterning is very distinct between indirect and direct developers. Nothing is known about how the adult nervous system is patterned during metamorphosis, as the larval nervous system is replaced by the adult nervous system. The larval nervous system is organized with an apical territory and a more posterior territory tracking the ciliary bands (Dautov & Nezlin, 1992; Gonzalez et al., 2018; Miyamoto et al., 2010; Nakajima, Humphreys, Kaneko, & Tagawa, 2004). Manipulating Bmp signaling during early embryogenesis has a similar patterning effect on the D/V as was observed in *S. kowalevskii*; Bmp protein treatments dorsalized embryos, and Bmp inhibition with an inhibitor resulted in ventralization. Several of the same markers were used in this study, and the response was remarkably similar between these contrasting life histories. The response of the mouth to Bmp manipulations was also the same; Bmp inhibition resulted in a dorsal expansion of the mouth, and Bmp protein treatments blocked mouth formation. However, unlike in *S. kowalevskii*, the authors argue that Bmp represses neural fates; the apical neural territory that gives rise to the ciliated apical organ and the larval eye spots is lost when Bmp is over expressed, as is the more posterior ciliary band territory and associated neurons. The authors argue that their data supports a conserved role of BMP in neural suppression, in line with other bilaterian model species. However, transitory treatments with Bmp protein through gastrulation results in the repositioning of the ciliary band territory more ventrally rather than suppression of neural fates altogether, raising the possibility that loss of neural territories is perhaps due to a patterning effect. Nevertheless, in both cases Bmp activity during embryogenesis is on the dorsal and not the ventral side. Data from both life history strategies strongly support a clear molecular inversion between hemichordates and chordates in D/V organization, yet the large differences in D/V composition of the two groups does not allow for a clear step by step reconstruction of how this inversion may have occured.

13. Endomesoderm specification

The specification of endomesoderm and the formation of a separate endoderm and mesoderm has been studied quite extensively in deuterostome taxa. The canonical Wnt pathway again emerges with a critical and conserved developmental role in the specification of the animal/vegetal axis. In this case, β-catenin plays a key role in the specification of the animal vegetal axis and in endomesoderm induction in a wide variety of animal taxa, from cnidarians (Wikramanayake et al., 2003), to nemerteans (Henry, Perry, Wever, Seaver, & Martindale, 2008), echinoderms (Logan, Miller, Ferkowicz, & McClay, 1999; Miyawaki et al., 2003; Wikramanayake, Huang, & Klein, 1998) and chordates (Imai, Takada, Satoh, & Satou, 2000). This role is proposed to have deep ancestry in metazoan development (Wikramanayake et al., 2003). Work in *S. kowalevskii* has furthered supported this hypothesis and established the importance of β-catenin in endomesoderm specification (Darras, Gerhart, Terasaki, Kirschner, & Lowe, 2011). Embryological experiments in the 1950s on *S. kowalevskii* (Colwin & Colwin, 1950, 1953) established the timing and importance of specification of the endomesoderm in the vegetal pole for normal embryonic patterning. The presence of the vegetal pole is critical for the correct patterning of the overlying animal territory that gives rise of the embryonic ectoderm. Removal of the vegetal pole at the 4 or 8 cell stage, and culture of animal explants results in the failure of any posterior fates to form, and the explants take on apical fates, with long stiff cilia typical of the apical tuft (Darras et al., 2011). This also indicates that the endomesoderm is a source of early posteriorizing factors starting at blastula stage. Based on data from indirect-developing echinoderms (Logan et al., 1999; Wikramanayake et al., 1998) and direct-developing hemichordates, the role of β-catenin in endomesoderm specification can be confidently reconstructed as an ancestral feature of deuterostomes.

14. Mesoderm specification

The mesoderm of adult enteropneusts is divided into three major territories; the most anterior territory, called the protocoel, forms all the muscle and mesoderm of the proboscis/prosome and forms as a single anterior coelom. The mesoderm of the collar/mesosome and trunk/metasome derive from two pairs of coeloms called the mesocoels and metacoels, respectively

(Hyman, 1955) (Fig. 2B). This tripartite coelomic organization is shared with other invertebrate deuterostomes including cephalochordates and echinoderms (Bateson, 1884; Conklin, 1932; Remane, 1963), and a tripartite coelomic arrangement is proposed to be ancestral for deuterostomes. In both indirect and direct-developing species, the protocoel forms at the end of gastrulation from the tip of the archenteron. However, the timing of mesocoel and metacoel formation is different between life history strategies (Fig. 2). In *S. kowalevskii*, these two pairs of posterior coeloms form immediately following gastrulation by enterocoely, as the embryo begins to elongate (Bateson, 1884; Green, Norris, Terasaki, & Lowe, 2013), whereas their formation in indirect-developing species occurs long after embryogenesis, very late in larval development, close to the onset of metamorphosis (Gonzalez et al., 2018; Lin et al., 2016; Morgan, 1891, 1894; Urata & Yamaguchi, 2004).

A molecular perspective into the evolution of mesoderm in deuterostomes is becoming much clearer. Work in a variety of vertebrate model species has determined the importance of both Nodal and FGF signaling in this process (Kimelman, 2006), with FGF signaling being important both for induction and maintenance of mesodermal fate (Fletcher, Baker, & Harland, 2006; Schulte-Merker & Smith, 1995). In amphioxus, FGF signaling has been demonstrated to play an important role in the formation of primary somites (Bertrand et al., 2011). In ascidians it is involved in the specification of the notochord, mesenchyme, and secondary muscle development (Darras & Nishida, 2001; Kim & Nishida, 2001; Yasuo & Hudson, 2007). Outside of chordates, in echinoderms, FGF signaling has been implicated in myogenesis and skeletal morphogenesis (Andrikou, Pai, Su, & Arnone, 2015), so it is clear that FGF is a promising ligand for understanding the evolution of deuterostome mesoderm.

Like vertebrates, hemichordate mesoderm is formed, not by the inheritance of a molecular determinant, but through induction by extrinsic factors. The nature of the molecular signals for mesoderm specification in enteropneusts has now been established in two species with contrasting developmental modes. This was first characterized in *S. kowalevskii* *(*Green et al., *2013)*. Fgf8/17/18, the ancestral ligand that gave rise to FGF 8, 17 and 18 in vertebrates, is expressed in the ectoderm overlying the regions of the endomesoderm that form the coeloms, in the right spatiotemporal pattern to be a candidate for an inducing signal. In addition, one of the two FGF receptors, FGFRB, is expressed in the archenteron in the regions where mesoderm is forming. Both ligand and receptor localization suggested that the ectoderm plays a role in signaling to the underlying endomesoderm to trigger the formation of mesoderm. Functional analysis by siRNA knockdown or RNA over expression supported this: knockdown of either the *fgf8/17/18* ligand or the receptor *fgfRB* resulted in the loss of mesodermal compartments. Over expression of *fgf8/17/18* had the opposite effect, with an over proliferation of muscle and loss of endoderm. Embryological explant experiments combined with siRNA knockdown established that the formation of mesoderm from endomesoderm requires direct contact from the ectoderm, the expression of ligand in the ectoderm, and FGFRB in the endomesoderm.

Studies in *P. flava* also establishes the importance of FGF signaling the early formation of the larval anterior mesoderm and muscle differentiation, with results very similar to those described in *S. kowalevskii* using FGF signaling inhibitors (Fan, Ting, Yu, & Su, 2018). Posterior coelomic compartments form late in larval development, close to metamorphosis, and although the details of the molecular determinants of their development are unknown, they may also be under the regulation of FGF. The formation of the adult body plan through metamorphosis of *P. flava* is accompanied by a burst of muscle differentiation, and Fan et al. (2018) report that FGF signaling may play a role in this transition. The process of metamorphosis is thought to be partly triggered by the settlement of larvae onto sand. Autoclaved sand is less effective at triggering metamorphosis than untreated sand suggesting that some bioactive material in the sand is also a critical component (Lin et al., 2016). The addition of bFGF protein resulted in modest increase in the rate of metamorphosis but only in the presence of sand; bFGF alone was not effective at promoting the transition to adult body plan. Inhibition of metamorphosis by the use of FGF signaling inhibitors showed variable results depending on the inhibitor used, so a definitive role of FGF remains to be further tested. However, very little is known about the process of metamorphosis and the complex integration of environmental and molecular cues, so this is an exciting new set of observations.

A separate study in the role of Nodal-like signaling in *P. flava* has argued for a role of Nodal signaling in mesoderm induction (Rottinger et al., 2015). There is a single Nodal gene and it has two expression domains; in the vegetal pole at the boundary of ectoderm and endomesoderm at early blastula, and in the ventral ectoderm following gastrulation. Early embryonic treatment with a Nodal-like signaling inhibitor resulted in the loss of anterior mesoderm (Rottinger et al., 2015). Further experiments are required to investigate this observation to determine whether this is a direct or indirect effect, and comparisons in *S. kowalevskii* will also be important to determine whether this is a conserved feature of enteropneusts or unique to indirect developers.

In summary, data from a wide range of species now suggests that the role of β-catenin and FGF signaling in the specification of endomesoderm and mesoderm are largely conserved in deuterostomes.

15. Life history considerations

In the grand hypotheses of chordate origins, the significance of life history has loomed large (Gee, 1996). Many hypotheses have focused on how modifications to an ancestral adult body plan gave rise to chordates, whereas probably the best known of classical chordate origins hypotheses have derived chordates by transformation of an ancestral larval body plan (Gee, 1996). One of the most influential figures in chordate origins is Walter Garstang who developed the Auricularian hypothesis, which was refined over a series of publications starting in 1894 through 1928 (Garstang, 1894, 1928). This hypothesis argued that the earliest chordates were simple and resembled a tunicate tadpole that evolved by transformation of the larval body plan of an ancestral deuterostome animal. This ancestral animal would have had a sessile adult and a larva that resembled the dipleurula larva of echinoderms and hemichordates. There are many elements to this elegant hypothesis that were refined over a series of papers (Holland, 2011), but the most significant element of the hypothesis was the derivation of the chordate central nervous system from a dorsal migration and fusion of the larval ciliary bands, and eventual transformation into the CNS of a chordate tadpole. This hypothesis was later championed and modified by Romer and Berrill (Berrill, 1955; Romer, 1972), and an inversion scenario was incorporated to fit with contemporary developmental data (Nielsen, 1999).

One major challenge to the hypothesis came from molecular phylogenetics: a key aspect of the hypotheses was that the tunicates represent the most basal branch of chordates. We now know that tunicates are in fact the sister group to vertebrates, and cephalochordates are the most basally branching chordate group (Delsuc, Brinkmann, Chourrout, & Philippe, 2006), making some key elements of the hypothesis untenable. Observations from paleontology have also challenged the hypothesis: a key component of the Auricularian hypothesis was the presence of a sessile deuterostome ancestral adult, which in later iterations by Berrill proposed to be similar to hemichordate pterobranchs. The latest fossil data suggest that the ancestral body plan of hemichordates was likely to be motile, combining elements of both enteropneust and pterobranch body plans (Nanglu, Caron, & Cameron, 2020; Nanglu, Caron, Conway Morris, & Cameron, 2016), weakening one of the elements of the hypothesis. Similar conclusions have been reached from the synthesis of molecular genetics and phylogenetics (Brown et al., 2008). Molecular genetic data have generally not been very supportive of the idea of larval origins of the chordate body plan. First, work from several species (as previously described), has revealed that it is the adult body plan with the closest patterning similarities to the chordate body plan along the A/P axis, not the larva. Patterning data from ambulacrarian larvae reveal that the larval body plan represents a truncated A/P axis, and essentially a "swimming head" from a patterning perspective (Gonzalez et al., 2017; Lacalli, 2005). A larval origins scenario would require a wholesale heterochronic shift of most of the adult patterning program earlier into the larval body plan. Based on current data, it seems more plausible that the chordate body plan evolved from an adult rather than larval life history stage. However, recent work in *P. flava* (Su et al., 2019) has revived this idea based on a modified version of the Garstang/Romer hypothesis as proposed by Nielsen (Nielsen, 1999), with ciliary bands of a dipleurula-like ancestor converging on the ventral side of the larva before reorienting to the dorsal side through a D/V axis inversion. As described in the previous section on D/V patterning, the authors experimentally manipulated the levels of Bmp signaling in both enteropneust and echinoid larvae. These data demonstrate that the position of the ciliary band responds to the levels of Bmp, and transitory Bmp treatments result in ventral relocation of the band and the formation of a ventral territory of neurogenic ectoderm. They also show that genes involved in mediolateral patterning of the vertebrate neural plate also line up in a similar fashion in the ectoderm of ambulacrarian larvae and respond similarly to Bmp signaling. Based on these similarities, they argue that tinkering with Bmp signaling in an ancestral larval body plan may have resulted in the initiation of a Garstang-like transformation of an ancestral larval body plan, which could have been the first phase in the path toward a chordate body plan. However, the discussion of the data fails to consider the evidence in favor of the alternative scenario, adult origins of the chordate body plan. Haag (2005) lays out similar problems of larval origins from the perspective of echinoderm body plans, and argues that it is the adult rather than the larva that merits close comparisons with chordates. Currently, when considering the entirety of the patterning data from enteropneusts, it is strongly supportive of adult origins rather than larval origins of the chordate body.

The comparisons of embryogenesis across phyla rarely consider the implications of comparisons of non-equivalent life history stages, but this could clearly have a significant impact on evolutionary comparisons. Where larval and adult body plans have significant differences in their basic organization, we may expect marked differences between the early embryogenesis of indirect and direct developers. For example, the nervous system organization of larval and adult enteropneusts is completely different. Most of the larval nervous system is lost at metamorphosis, and the formation and patterning of the adult nervous system occurs *de novo*, late in larval development and through metamorphosis (Gonzalez et al., 2018; Miyamoto et al., 2010). We have little understanding of the morphogenesis of adult structures by remodeling of an existing larval body plan, but the developmental programs responsible for adult nerve cord morphogenesis are likely to be distinct from those involved in the morphogenesis of the larval nervous system in the apical organ and ciliary bands. In another example, work from *S. kowalevskii* demonstrates that the early action of Wnt signaling through embryogenesis results in the activation of anterior and medial Hox genes during gastrulation that are involved in regulating trunk morphogenesis (Darras et al., 2018). However, gene expression analysis in the indirect developing species *S. californicum* clearly shows no expression of the orthologous Hox genes until closer to metamorphosis as the adult body plan is beginning to form, indicating a lack of trunk in larvae (Gonzalez et al., 2017). The difference in Hox activity between life history strategies may be a result of differences in Wnt activity during embryogenesis, and we should certainly expect to find significant differences in embryonic programs between indorevt and direct developers related to such striking patterning differences. These examples demonstrate that when species are chosen to represent developmental mechanisms of a clade of animals, it is important to consider life history strategy as a significant source of within phylum developmental variation.

16. Summary and future directions

The past 20 years has seen much progress in hemichordate biology. There has been progress on many fronts; from molecular phylogenetics, genomics, developmental biology to paleontology and anatomy. A synthesis of insights from these disparate fields allows a tentative glimpse into the early evolution of deuterostomes. The ability to reconstruct detailed morphological characters remains challenging, as the large organizational and morphological disparity between hemichordates and chordates that was daunting for comparative morphologists remains a major issue, even with the new types of data generated over the last two decades. However, there is building consensus that early deuterostomes are likely to have been benthic worms that used a set of pharyngeal gills for feeding. Molecular, genomic and paleontological data have strongly supported the homology of pharyngeal gill slits (Cannon et al., 2014; Caron, Morris, & Cameron, 2013; Gillis et al., 2011; Nanglu et al., 2016, 2020; Ogasawara et al., 1999; Rychel et al., 2006; Simakov et al., 2015). The details of the morphological characters of this benthic worm are still difficult to reconstruct. However, it is clear from the strong axial conservation between hemichordates and chordates that this worm had a well patterned A/P axis (Aronowicz & Lowe, 2006; Darras et al., 2018; Kaul-Strehlow et al., 2017; Lowe et al., 2003; Pani et al., 2012), defined by an anterior ectodermal region that likely utilized a conserved transcription factor gene regulatory network to partition the entire ectoderm into major divisions of the body plan. The entire extent of the A/P axis would have been regulated by a cWnt gradient, with a well-defined "head" territory negatively regulated by cWnt, and trunk fates promoted by cWnt, and a posteriorly-situated Wnt/brachyury loop involved in trunk elongation. The loose coupling of A/P patterning to any specific neural architecture means that the details of the nervous system are difficult to reconstruct, but it is likely that there was some degree of centralized processing (Nomaksteinsky et al., 2009). However, given the likely feeding mode of mucociliary suspension/particle feeding, this ancestral nervous system was unlikely complex. The D/V axis, like all bilaterians, was patterned by a Bmp/Chordin axis; Bmp expressed dorsally and chordin on the ventral side. Some sort of axis inversion would have occurred in stem chordates. Gill slits, a dorsal heart-like contractile dorsal vessel and coelomic mesoderm would have been associated with the Bmp side, and striated muscle more associated with the ventral side (Lowe et al., 2006). The mouth was ventral and negatively regulated by Bmp. The relationship of the nervous system to the Bmp/chordin axis also suggests that this patterning mechanism can support a range of neural conformations over macroevolutionary time periods. So again, reconstruction of ancestral deuterostome neural characters along this axis is challenging. The similarities of early embryogenesis between hemichordates, cephalochordates and echinoderms suggest that we can confidently reconstruct several aspects of the early specification of germ layers. β-catenin is central in patterning the animal vegetal axis and the early specification of the endomesoderm (Darras et al., 2011; Wikramanayake et al., 1998, 2003), and mesoderm would have formed by enterocoely and was regulated at least in part by FGF signaling (Andrikou et al., 2015; Bertrand et al., 2011; Fan et al., 2018; Green et al., 2013).

Future work in hemichordates must carry out some basic characterizations of pterobranch development and develop some genomic resources. There are few contemporary studies on these animals and they are necessary to provide a broader understanding of the development of the clade (Sato et al., 2008; Sato & Holland, 2008; Stach, Gruhl, & Kaul-Strehlow, 2012). Recent work on regeneration in hemichordates demonstrates their enormous potential for providing insights into the limited regenerative capacities of vertebrates. Many hemichordate species are capable of anterior regeneration as well as posterior regeneration of the trunk and tail, rapidly regrowing the anterior region of the body plan that shares so many patterning similarities with vertebrate brains (Arimoto & Tagawa, 2015, 2018; Luttrell, Gotting, Ross, Alvarado, & Swalla, 2016; Rychel & Swalla, 2009; Yoshimura, Morino, & Wada, 2019). A molecular characterization of these processes will provide some valuable comparative information to deuterostome regenerative biology. The proliferation of available hemichordates genomes will make a genomics approach to understanding hemichordate biology increasingly important. Improvements in genome sequencing and assembly are facilitating the rapid production of high quality, chromosomal level assemblies. A high-quality assembly of the genome of *S. californicum* is now available, and improvements to the original *P. flava* genome are in the works. Single cell technologies have enormous potential to provide insights into cell type characterizations in the nervous system and provide a basis for more detailed comparisons with other bilaterians. Several groups have ongoing projects using this technology. Better genomes are now facilitating improved functional approaches to understanding the biology of hemichordates. So far, these have been limited to *S. kowalevskii*, but have been technically challenging in indirect-developing species. Therefore, generating new experimental approaches by adopting and modifying emerging technologies from model species will be critical to allow more mechanistic insights into the development of hemichordates and the origins of vertebrates.

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