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Evolutionary crossroads in developmental biology: hemichordates

Eric Röttinger[1] and Christopher J. Lowe[2,*]

Summary

Hemichordates are a deuterostome phylum, the sister group to echinoderms, and closely related to chordates. They have thus been used to gain insights into the origins of deuterostome and chordate body plans. Developmental studies of this group have a long and distinguished history. Recent improvements in animal husbandry, functional tool development and genomic resources have resulted in novel developmental data from several species in this group. In this Primer, we introduce representative hemichordate species with contrasting modes of development and summarize recent findings that are beginning to yield important insights into deuterostome developmental mechanisms.

Key words: Body plan, Deuterostome, Evolution, Hemichordata

Introduction

Hemichordates are a phylum belonging to the major bilaterian lineage called the deuterostomes (see Glossary, Box 1; Fig. 1A). Hemichordates are exclusively marine organisms and are divided into two major groups (Fig. 1B): the solitary enteropneust worms (the acorn worms) and the colonial and tube-dwelling pterobranchs (Hyman, 1959). Interest in this group of animals has been largely based on their proposed morphological affinities and close phylogenetic relationship to chordates (Brown et al., 2008; Lowe, 2008), making them an informative group with which to gain insights into the early origins of the chordate body plan. Although the composition of phyla that make up the deuterostomes and their phylogenetic relationships have been revised many times (Schaeffer, 1987; Turbeville et al., 1994; Wada and Satoh, 1994; Halanych, 1995; Bourlat et al., 2006; Philippe et al., 2011), zoologists have long proposed potential morphological affinities between hemichordates and chordates, and these similarities have formed the bases for numerous comparative morphological and developmental studies since the 1800s. Hemichordates have thus been influential in the formulation of a range of hypotheses on the origins of chordates (Bateson, 1886; Garstang, 1894; Berrill, 1955; Bone, 1960; Nielsen, 1999; Gerhart et al., 2005; Lacalli, 2005; Brown et al., 2008).

The defining early studies of the developmental biology of enteropneusts were carried out by two pioneers of developmental biology: William Bateson and Thomas Hunt Morgan (Bateson, 1884b; Bateson, 1885; Morgan, 1891; Morgan, 1894). Pterobranchs were first defined as a class in 1877 (Harmer, 1887), but it was not until they were first found in shallow waters that the details of their development were comprehensively described (Stebbing, 1970; Dilly, 1973; Lester, 1988b; Lester, 1988a; Sato et al., 2008). Bateson originally considered enteropneusts to be primitive chordates and therefore informative for understanding the origin of the vertebrates (Bateson, 1886). It was not until much later that the reclassification of hemichordates into their own phylum was widely accepted. Early studies from Metschnikoff (Metschnikoff, 1881) noted close morphological similarities between the early larvae of enteropneusts and echinoderms (see Glossary, Box 1), but the significance of this observation was not fully appreciated until a series of molecular datasets, including Hox gene complements to molecular phylogenetics, robustly supported the sister grouping of hemichordates and echinoderms (Turbeville et al., 1994; Wada and Satoh, 1994; Bromham and Degnan, 1999; Cameron et al., 2000; Furlong and Holland, 2002; Bourlat et al., 2006; Dunn et al., 2008; Swalla and Smith, 2008; Philippe et al., 2011). The profound changes in phylogenetic relationships of the deuterostome phyla are now leading to new testable hypotheses about the early evolution of the lineage and the origins of chordates. An

Box 1. Glossary

Collar. A distinct body region between the proboscis and the trunk that is attached to the proboscis on a medio-dorsal stalk. The ventral mouth opens anterior to the collar.

Deuterostomes. A bilaterian lineage of animals classically defined by the formation of mouth and anus: the blastoporal opening (site of gastrulation) becomes the anus and the mouth forms secondarily, later in development.

Direct development. Development to an adult body plan without an intervening larval stage with a distinct body plan.

Echinoderm. Member of a phylum of marine invertebrates comprising echinoids (sea urchins), asteroids (sea stars), crinoids (sea lillies), holothuroids (sea cucumbers) and ophiuroids (brittle stars). See also McClay (McClay, 2011) for a *Development* Primer on sea urchins.

Enterocoely. An embryonic phenomenon, during which mesodermal coeloms form by out-pocketing of a part of the embryonic gut.

Gonochoric. Having only one, male or female, set of reproductive organs.

Holoblastic cleavage. The cleavage furrow extends through the entire egg or blastomere, resulting in a complete cleavage.

Indirect development. Development to an adult body plan via a distinct larval body plan followed by metamorphosis into an adult.

Lecithotrophic. Having a swimming, non-feeding larva that derives its nutrition from maternally provisioned yolk.

Notochord. An embryonic rod-like structure that is located on the dorsal part of the developing animal and is essential for initiating the differentiation of the adult nervous system.

Proboscis. The highly contractile and expandable anteriormost part of a hemichordate that is used for burrowing and locomotion.

Radial cleavage. After cleavage, the daughter blastomeres are either perpendicular or parallel to each other. This type of cleavage is characteristic of deuterostomes.

Zooid. A single animal that is part of a colonial structure.

[1]Kewalo Marine Laboratory, Pacific Biosciences Research Center (PBRC), University of Hawaii, 41 Ahui Street, Honolulu, HI 96734, USA. [2]Hopkins Marine Station, Department of Biology, Stanford University, 120 Oceanview Boulevard, Pacific Grove, CA 93950, USA.

*Author for correspondence (clowe@stanford.edu)

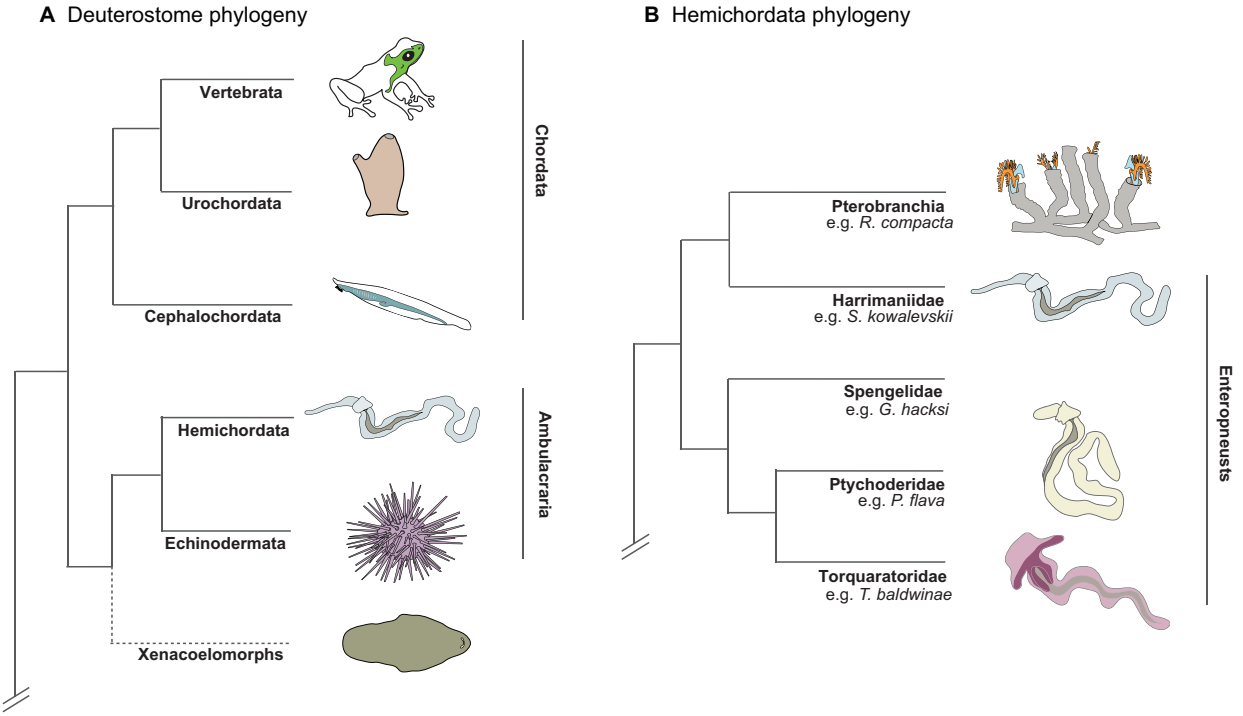

Fig. 1. Deuterostome and hemichordate phylogenies. (**A**) Hemichordates and their sister group the echinoderms make up the Ambulacraria clade and are closely related to chordates. Recent phylogenomic studies have also placed acoelomorphs and xenoturbella, together termed xenacoelomorphs, as a sister group to hemichordates and echinoderms (Philippe et al., 2011), although their position within deuterostomes remains controversial (as indicated by a dashed line). (**B**) Hemichordate phylogeny with limited representation of the pterobranchs and enteropneusts, showing the relationships between lineages containing the most commonly studied hemichordates, *Ptychodera flava* (Ptychoderidae), *Saccoglossus kowalevskii* (Harrimaniidae) and *Rhabdopleura compacta* (Pterobranchia), along with the deep sea family *Torquaratidae* [based on published data (Cameron et al., 2000; Winchell et al., 2002; Cannon et al., 2009; Osborn et al., 2012)].

understanding of these phylogenetic relationships also facilitates the mapping of developmental genetic traits onto the new tree to investigate the evolution of deuterostome developmental mechanisms. New developmental studies in enteropneust and pterobranch hemichordates have begun to focus on mechanisms of early axial patterning and germ layer establishment in order to make comparisons with the abundant developmental data from larval echinoderms (Lapraz et al., 2009; Peter and Davidson, 2010; Angerer et al., 2011) and chordates (Jessell, 2000; Gerhart, 2001; Joubin and Stern, 2001; Kiecker and Lumsden, 2005; De Robertis, 2006; Kimelman, 2006; Dequeant and Pourquie, 2008; Tschopp and Duboule, 2011).

Hemichordates and echinoderms are sister taxa and together form a clade called the Ambulacraria (Metschnikoff, 1881), which is closely related to chordates (Fig. 1A). In many comparative studies of bilaterian development, deuterostomes are represented almost exclusively by chordates. Despite many comprehensive studies of early echinoid (sea urchin) development, echinoderms have largely been excluded from broad body plan comparisons owing to the difficulties of establishing a rigorous basis for comparisons of their penta-radial adult body plan with that of other bilaterians. However, echinoderm larvae are bilaterally symmetric and exhibit compelling patterning similarities with chordates in early endomesoderm specification and axis patterning (Lapraz et al., 2009; Peter and Davidson, 2010; Angerer et al., 2011) facilitating direct body plan comparisons with other phyla. Nonetheless, a phylogenetically denser sampling of deuterostomes is needed to make more rigorous comparisons with protostomes and prebilaterians in order to test hypotheses of early bilaterian evolution and development. Hemichordates share numerous developmental similarities with both chordates and echinoderms and hold great promise for providing insights into the early origins of both chordate and deuterostome development.

In this Primer, we introduce the main hemichordate species used for developmental studies, which represent different lineages and contrasting early life history strategies, and we describe their basic biology and early development. We then highlight significant and recent findings from developmental genetic studies of hemichordates, and conclude by discussing the promises of future work that will make full use of the comprehensive genomic resources and functional tools that have been developed.

Hemichordate phylogeny

Hemichordates, along with echinoderms and chordates, are robustly supported within the deuterostomes (Fig. 1A) on both morphological and molecular criteria (Schaeffer, 1987; Turbeville et al., 1994; Bromham and Degnan, 1999; Cameron et al., 2000; Furlong and Holland, 2002). Recent phylogenomic studies have supported the addition of two phyla to the deuterostomes: Xenoturbellida (Bourlat et al., 2003; Bourlat et al., 2006) and Acoelomorpha (Philippe et al., 2011), together termed xenacoelomorphs, which are small simple worms with no through gut and a simple nervous system. However, both these groups have long and problematic histories of phylogenetic

placement, and their position as the sister group to the hemichordates and echinoderms remains controversial (Hejnol et al., 2009; Lowe and Pani, 2011).

The phylogenetic relationships within hemichordates, and most importantly the placement of pterobranchs, are a critical issue. There is strong support for the placement of pterobranchs as a sister group to the Harrimaniidae, making enteropneusts paraphyletic (Cameron et al., 2000; Cameron, 2005; Cannon et al., 2009) (Fig. 1B). The significance of this finding for developmental biology is that it suggests that studies from enteropneusts might be the most informative for reconstructing ancestral developmental strategies for this phylum (Brown et al., 2008). However, other studies have either placed pterobranchs at the most basal position in the clade (Winchell et al., 2002) or have failed to resolve their position (Osborn et al., 2012). Further studies with additional genes will be required to address this critical question (Cannon et al., 2009).

Enteropneusts are divided into four groups: Harrimaniidae, Spengelidae, Torquaratoridae and Ptychoderidae (Cannon et al., 2009; Osborn et al., 2012). The harrimanids and pterobranchs are largely characterized by direct development (see Glossary, Box 1), whereas the ptychoderids and spengelids exhibit indirect development (see Glossary, Box 1), with a feeding larva and an extended planktonic period. Little is known about the development of the Torquaratoridae as they are deep-water species, although one described species has large eggs suggesting that they might be direct developers (Osborn et al., 2012). It is generally proposed that the echinoderm dipleurula-type larva and hemichordate larvae are homologous (Strathmann and Bonar, 1976; Nielsen, 2001), suggesting that indirect development is a basal developmental strategy for hemichordates, although there are dissenting views (Nezlin, 2000). Most of the research on hemichordate development has been carried out on two species of enteropneust worms: *Ptychodera flava* (Fig. 2A) from the Ptychoderidae and *Saccoglossus kowalevskii* (Fig. 2B) from the Harrimaniidae. However, more recently, pterobranchs (Fig. 2C) as represented by *Rhabdopleura compacta* and other indirect-developing enteropneusts such as *Balanoglossus misakiensis* and *Balanoglossus simodensis* are being developed as valuable additional models, ensuring that, in the future, no one species is over emphasized in comparative analyses (Sato and Holland, 2008; Ikuta et al., 2009; Sato et al., 2009; Miyamoto et al., 2010; Miyamoto and Saito, 2010).

General morphology and habitat

Enteropneusts (acorn worms)

Acorn worms are benthic marine animals that are distributed worldwide. They range in size from a few centimeters up to a meter, and they are found in depths ranging from the intertidal to the deep sea (Holland et al., 2005; Cannon et al., 2009; Osborn et al., 2012). They display a tripartite body organization with an anterior prosome (also called the proboscis; see Glossary, Box 1), a mesosome (or collar; see Glossary, Box 1), and a posterior metasome or trunk (Fig. 2D,E). The dorsoventral (DV) axis is largely defined by the position of the mouth on the ventral side and gill slits on the dorsal side. Most acorn worms use their proboscis to burrow through sand using peristaltic movements, and they feed either by deposit feeding, trapping detritus and sediments in mucus secreted from the proboscis, or by filter feeding (Cameron, 2002; Gonzalez and Cameron, 2009). Their mouth opens on the ventral side, at the base of the proboscis, and the gut runs through to a terminal anus in adults (Fig. 2D,E). The anterior, pharyngeal gut is perforated by paired cartilaginous gill slits that bear a strong morphological and molecular resemblance to those of the basal chordate, amphioxus (Rychel et al., 2006; Rychel and Swalla,

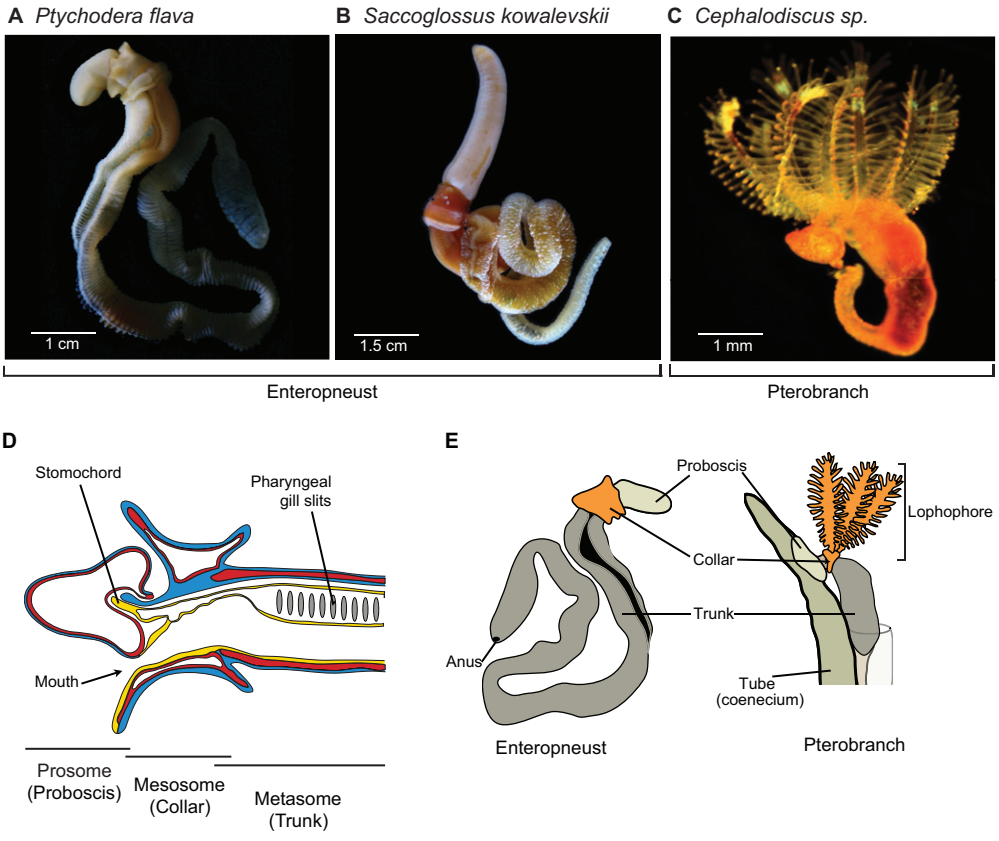

Fig. 2. General hemichordate morphology. (**A**,**B**) Macrophotographs of the enteropneusts *P. flava* (A) and *S. kowalevskii* (B). (**C**) A photomicrograph of a pterobranch zooid of *Cephalodiscus* sp. (image reproduced with kind permission from K. Halanych). (**D**) Schematic representation of a longitudinal section of an enteropneust worm, showing the structure and composition of anterior structures (blue, ectoderm; yellow, endoderm; red, mesoderm). (**E**) The main body structures of an adult enteropneust and a pterobranch.

2007; Gillis et al., 2011). At the very anterior end of the gut, the endoderm projects into the proboscis forming the stomochord (Fig. 2D), which is a supportive structure for the heart/kidney complex (Balser and Ruppert, 1990) and has been proposed to be homologous to the chordate notochord (see Glossary, Box 1) (Bateson, 1886; Morgan, 1894). Currently, there is no molecular support for this homology (Peterson et al., 1999) and it consequently awaits further analysis.

The nervous system of adult enteropneusts comprises a broad epithelial nerve plexus, which is most concentrated in the proboscis, and two nerve cords: one dorsal and one ventral. The dorsal nerve cord is superficial in the trunk, but in the collar is internalized by a process that resembles neurulation in chordates (Bateson, 1884a; Bateson, 1886; Brown et al., 2008; Nomaksteinsky et al., 2009; Kaul and Stach, 2010), which has been used as evidence to suggest that this structure shares homology with the chordate dorsal nerve cord. Unlike chordates, enteropneusts also possess a ventral nerve cord that starts in the anterior trunk and extends posteriorly (Bullock, 1945; Knight-Jones, 1952; Dilly et al., 1970).

Enteropneusts are characterized by separate sexes and in most species oocytes can be clearly seen through the ectoderm. Gametes are free-spawned and fertilization occurs externally.

Pterobranchs

By contrast, pterobranchs are small (several millimeters to centimeters long), generally colonial, filter-feeding animals that live in secreted tubes (collectively, a coenecium) (Sato et al., 2008). Their feeding apparatus – the lophophore – is an extension of the mesosome/collar (Fig. 2E), and their digestive tract is U-shaped, juxtaposing the mouth and the anus near to the tube opening (Dawydoff, 1948). Pterobranchs are mainly found on the surfaces of rocks or shells where they can form dense aggregates. Their colonies are usually hermaphroditic, but individual zooids (Fig. 2C; see Glossary, Box 1) may be gonochoric (see Glossary, Box 1).

Despite rather obvious morphological differences, enteropneusts and pterobranchs share a similar body plan organization and several unifying characters: the stomochord, described previously (Fig. 2D), and a single pair of gill slits in *Cephalodiscus* but not in *Rhabdopleura*. The pterobranch *Rhabdopleura compacta*, which can be found off the south coast of England, is an emerging organism for developmental studies (Stebbing, 1970; Dilly, 1973; Sato et al., 2008). A related species, *Rhabdopleura normani*, is found in shallow waters off Bermuda (Lester, 1988b; Lester, 1988a).

Life cycle and embryology

Saccoglossus kowalevskii

The enteropneust worm *S. kowalevskii* is a direct-developing hemichordate (Fig. 3) that has a broad distribution along the eastern seaboard of the USA (Lowe et al., 2004). Gravid individuals can be collected in spring and late summer. Oocytes are induced to spawn by temperature shock (Colwin and Colwin, 1962); each female will release on average between 200 and 1000 oocytes, which are ~300 μm in diameter and can be fertilized in vitro with a dilute sperm suspension. Early observers carefully described normal development from fertilization through to hatching (Bateson, 1884a; Bateson, 1885; Colwin and Colwin, 1953). Following fertilization, a thick vitelline membrane is raised, similar to that observed in sea urchins, and the embryo undergoes radial, holoblastic cleavage (see Glossary, Box 1) to produce a hollow blastula that is slightly thickened at the vegetal pole (Fig. 4A,A′). Gastrulation occurs by circumferential invagination of the vegetal endomesoderm (Fig. 4B-C′). Following gastrulation, the blastopore closes and the embryo begins to elongate along the anteroposterior (AP) axis (Fig. 4C,C′). At this time, the mesoderm forms by enterocoely (see Glossary, Box 1) following contact with the ectoderm: the proboscis mesoderm forms first, followed by two pairs of lateral enterocoels in the prospective trunk and prospective collar. Two days following fertilization, two circumferential grooves become apparent in the ectoderm, dividing the animal into the prosome, mesosome and metasome (Fig. 4D,D′). Around the same time, the mouth perforates on the ventral side, but the anus does not perforate until later (Fig. 4E,E′). The first pair of gill pores is apparent by day 4, and the animal hatches after ~5 days of development, at which point it resembles a small adult (Fig. 4F,F′). Hatched juveniles are briefly pelagic (see Fig. 3) then begin to burrow in sand and actively feed. Later development primarily involves extension of the trunk and addition of gill slits, and both of these processes appear to continue throughout life.

As an experimental benefit, the rapid pace of *S. kowalevskii* development makes it possible to assess the effects of embryonic experimental manipulations on development and organization of the adult body plan, which can be technically more challenging in animals with prolonged larval development, such as *Ptychodera flava*.

Ptychodera flava

P. flava is an indirect-developing enteropneust that is easily obtained in shallow waters surrounding the Hawaiian Islands and is also common on shallow reef flats through the Indo-Pacific region (Lowe et al., 2004). Developmental work on *P. flava* has been mostly carried out in Hawaii, and sexual reproduction is restricted primarily to the months of December and January (Hadfield, 1975; Tagawa et al., 1998a). During this time, adults spawn hundreds of thousands of oocytes (~120 μm in diameter) into the water column and fertilization occurs externally. The pelagic tornaria larvae feed in the plankton for several months before settling and metamorphosing into benthic juveniles (Fig. 3).

Despite the difference in developmental modes, the early development of *P. flava* and *S. kowalevskii* is similar; cleavage in *P. flava* is radial (see Glossary, Box 1) and holoblastic and gives rise to a hollow blastula (Fig. 4H) (Hadfield, 1975; Tagawa et al., 1998b). The presumptive endomesoderm is located in the vegetal plate of the blastula (Henry et al., 2001), a region that thickens prior to the initiation of gastrulation and formation of the embryonic archenteron (Fig. 4I). Toward the end of gastrulation, the anterior mesoderm forms by enterocoely as the protocoel pinches off from the anterior end of the archenteron and elongates asymmetrically towards the dorsal ectoderm, where it fuses to form the hydropore (a pore that connects the anterior mesoderm to the external environment) (Fig. 4J). A few hours later, the archenteron bends toward the ventral ectoderm where it fuses with the stomodeum to form the mouth (Fig. 4K). The posterior mesoderm forms much later in development, close to metamorphosis, which constitutes a distinct difference from sea urchins and *S. kowalevskii*.

After hatching, the free-swimming tornaria larvae possess a tripartite gut composed of a pharynx, stomach, intestine, a protocoel (anterior mesoderm) and the apical plate (Fig. 4K). The hatched larvae feed and grow in the water column for several months, undergoing progressive morphological modifications (Fig. 4L) prior to metamorphosis (Fig. 4M,N) (Hadfield, 1975; Tagawa et al., 1998b; Nielsen and Hay-Schmidt, 2007). Competent larvae are composed mainly of anterior structures and can be collected by

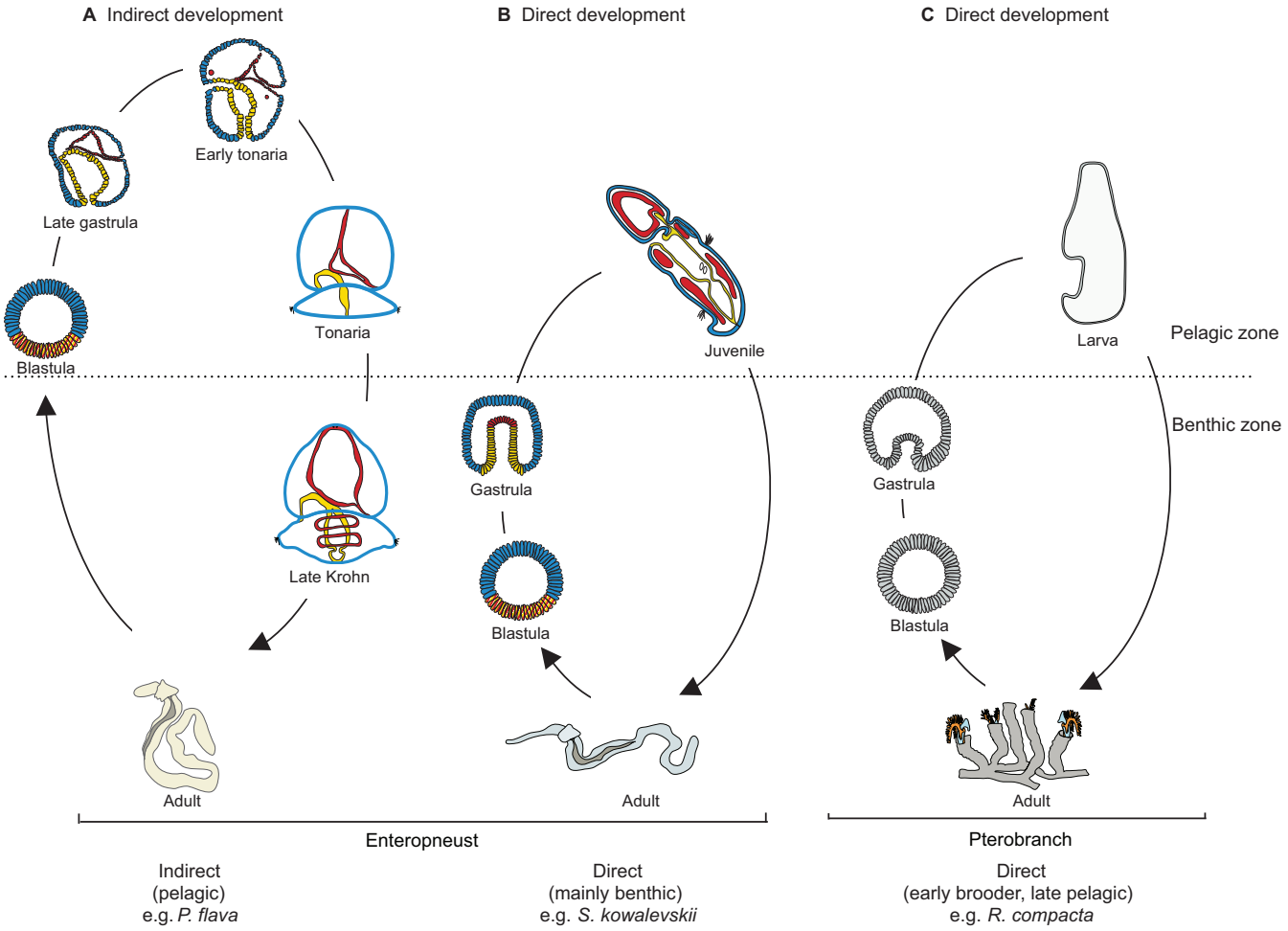

Fig. 3. Hemichordate life cycles. The life cycles of (A) indirect- or (B) direct-developing enteropneust hemichordates and (C) direct-developing pterobranchs. The dashed line indicates the transition from benthos to pelagos. (**A**) Adult ptychoderid enteropneusts (e.g. *P. flava*) spawn eggs and sperm into the water column, where external fertilization occurs. After hatching, the tornaria larva remains in the pelagic zone for several months, undergoing slight morphological modifications before metamorphosing into and settling as a benthic juvenile. (**B**) Fertilization of the direct-developing enteropneust *S. kowalevskii* occurs externally, inducing the formation of a thick vitelline membrane within which early development occurs. Five days after fertilization, the embryos hatch and, after a very brief swimming phase, the juveniles begin to burrow in sand. (**C**) Little is known about fertilization and the developmental stages of breeding pterobranchs (e.g. *R. compacta*), although it is known that ciliated and pigmented larvae develop inside the coenecium. Developmental stages are indicated below each illustration and the internal organization of the germ layers is indicated (blue, ectoderm; yellow, endoderm; red, mesoderm).

plankton tow during the months of April and May in Hawaii. Metamorphosis into the adult form occurs rapidly and can be induced by the collection process. During metamorphosis, the posterior of the larva rapidly proliferates and extends, which contrasts with the early specification of the posterior structures in *S. kowalevskii*.

Another indirect-developing hemichordate, *Balanoglossus simodensis*, has also been successfully reared through metamorphosis under laboratory conditions (Miyamoto and Saito, 2007) and thus might provide an additional species for future comparative studies.

Rhabdopleura compacta

Pterobranchs are not as common as enteropneusts and generally live in cold or deep waters. It was not until their discovery in shallow waters that their development was thoroughly characterized (Stebbing, 1970; Dilly, 1973; Lester, 1988b; Lester, 1988a). Recently, Sato and colleagues have begun to develop the pterobranch *Rhabdopleura compacta* as an important new hemichordate species that is amenable to developmental studies (Sato et al., 2008; Sato and Holland, 2008; Sato et al., 2009). How and when fertilization occurs remains unknown, but developing larvae of this species can be observed year round, with a peak between April and July. Development is direct and lecithotrophic (see Glossary, Box 1) and larvae are ciliated and pigmented. They are initially brooded inside the coenecium (Fig. 2E, Fig. 3C) and then released as swimming larvae.

Experimental approaches in hemichordates

Hemichordates are amenable to many descriptive and experimental techniques, ranging from classic embryology to modern reverse genetics.

In vivo cell labeling (lineage tracing)

Fluorescent, cell-tracing dyes have successfully been injected into individual cells or applied to membranes of cleaving hemichordate embryos to allow researchers to track the fates of single

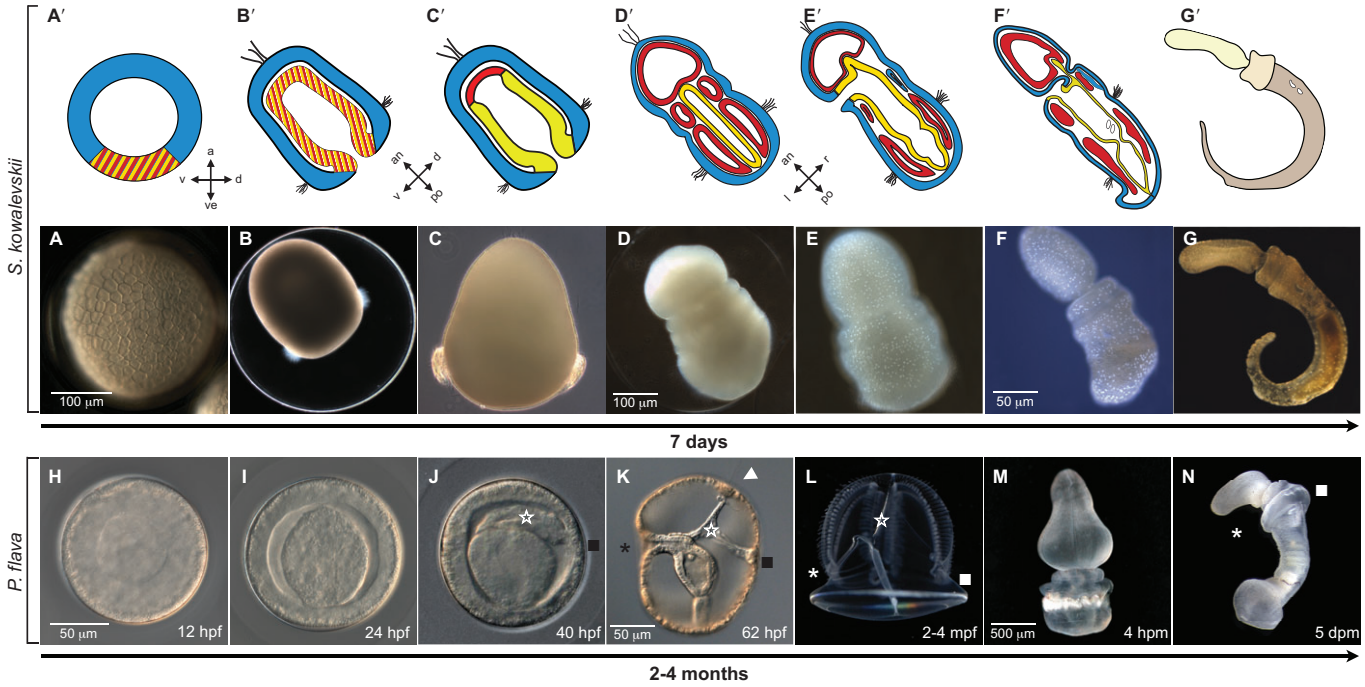

Fig. 4. Embryonic development of the commonly studied hemichordates. (**A-G′**) Direct development of *S. kowalevskii*. Each stage of development (A-G) is also represented schematically (A′-G′), indicating the internal organization of the germ layers (blue, ectoderm; yellow, endoderm; red, mesoderm). Axis orientation is given in A′,B′,D′. B is reproduced with permission (Ettensohn et al., 2004). G is reproduced with permission (Lowe et al., 2003). (**H-N**) Indirect development of *P. flava*. The white square indicates the position of the hydropore, the asterisk indicates the position of the mouth, the white arrowhead indicates the apical plate and the star indicates the protocoel. Below is indicated the timescale of development from egg to juvenile: 7 days for *S. kowalevskii* and 2-4 months for *P. flava*. a, animal; ve, vegetal; an, anterior; po, posterior; v, ventral; d, dorsal; l, left; r, right; hpf/mpf, hours/months post-fertilization; hpm/dpm, hours/days post-metamorphosis.

blastomeres for several weeks or more. These cell-labeling approaches have been successfully carried out in *S. kowalevskii* (Colwin and Colwin, 1951; Darras et al., 2011) and *P. flava* (Henry et al., 2001) (Fig. 5A) and demonstrate that the cleavage patterns, as well as the early fate maps of direct- and indirect-developing hemichordates, are similar to those of indirect-developing echinoids (Colwin and Colwin, 1951; Cameron et al., 1987; Cameron et al., 1989; Cameron and Davidson, 1991; Henry et al., 2001) (Fig. 6). In *S. kowalevskii*, direct injection of lysinated fluorescent dextrans into single cells is possible up to the 132-cell stage; descendants of injected cells can be imaged both in live animals and in fixed specimens at high resolution (Fig. 5B). Clonal descendants of injected cells inside the yolky embryos can be readily imaged in fixed, optically cleared animals even after in situ hybridization. Injections into blastomeres can also be used to target reverse-genetic manipulations to specific regions of the embryo. Other lineage-tracing approaches using light-activated fluorescent molecules have also been used successfully to visualize cell lineages in live animals. Photoactivatable GFP protein (Patterson and Lippincott-Schwartz, 2002) and *kaede* mRNA (Ando et al., 2002) (J. Gray and M. Kirschner, unpublished observations) can be injected into oocytes and fluorescence can be activated in specific regions with a confocal microscope at any point in development (Fig. 5C).

Genetic manipulations

The ability to produce large numbers of synchronously developing embryos by in vitro fertilization facilitates the use of biochemical pathway antagonists (Darras et al., 2011; Röttinger and Martindale, 2011) and recombinant proteins to manipulate large numbers of embryos in both indirect- and direct-developing hemichordate species (Lowe et al., 2006). However, these approaches are limited in indirect-developing species that have feeding larvae owing to the relatively slow larval development and the need to maintain embryos in larger volumes of seawater. In *S. kowalevskii*, specific gene knockdown and overexpression approaches have been developed using microinjection into fertilized oocytes or blastomeres. Overexpression using capped mRNA, as well as gene knockdown by synthetic siRNA, have proven to be successful for numerous genes (Fig. 5D) (Lowe et al., 2006; Darras et al., 2011). Transient transgenic approaches remain to be developed for these organisms, and forward-genetic approaches are unlikely to be practical owing to the long generation times.

Gene expression analyses

In situ hybridizations and immunocytochemistry (Fig. 5E) are now routine in all of the hemichordates discussed here. In *S. kowalevskii*, fluorescent in situ protocols have also been developed, allowing detailed examination of the relative expression patterns of several genes (Pani et al., 2012) (Fig. 5F).

Classical embryology

Early studies established the promise of *S. kowalevskii* for embryological experiments (Colwin and Colwin, 1950). Recently, Darras and colleagues (Darras et al., 2011) have built on this work and combined embryological manipulations with molecular analyses to investigate the inductive capacities of endomesoderm in *S. kowalevskii*. At cleavage stages, blastomeres can be separated

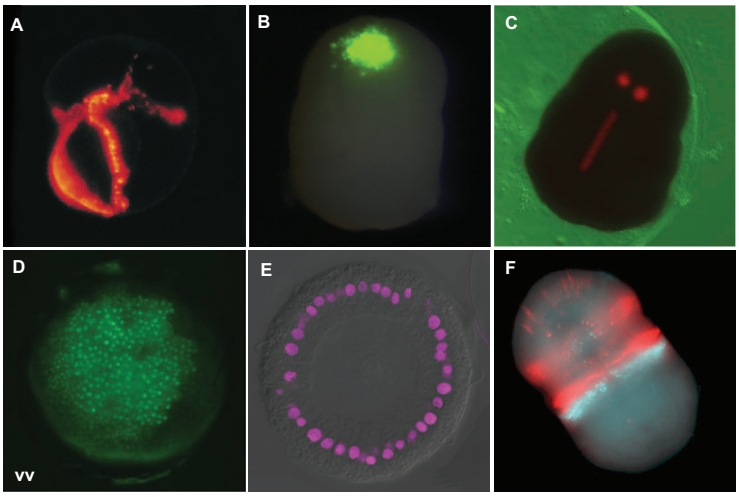

Fig. 5. Experimental techniques available in hemichordates. (**A**) Cell labeling using DiI (red) in *P. flava*. Labeling of a single blastomere at the 8-cell stage gives rise to stained cells in the ventral ectoderm, as well as in the protocoel of a tornaria larva (Henry et al., 2001). (**B**) Cell labeling using fluorescent dextran (green) in *S. kowalevskii*. Microinjection into one blastomere at the 132-cell stage labels the daughter cells in the proboscis (image provided by A. M. Pani). (**C**) In vivo photoconversion of photoactivatable GFP protein for cell-lineage tracing in *S. kowalevskii* (image provided by Rachael Norris). (**D**) Microinjection of mRNA encoding β-catenin:GFP leads to staining of the vegetal pole of *S. kowalevskii* (Darras et al., 2011). vv, vegetal view. (**E**) Anti-histone (magenta) immunocytochemistry in *P. flava* (image provided by Eric Röttinger). (**F**) Double fluorescence in situ hybridization of *pax6* (red) and *engrailed* (cyan) in *S. kowalevskii* (image provided by A. M. Pani).

and reared independently and, at later stages, embryos can be cut and pieces of tissue grafted onto other embryos (Darras et al., 2011; Colwin and Colwin, 1950). When combined with knockdown and overexpression approaches, this is a powerful tool to test for inductive interactions between different tissues, as has been used to great effect in other developmental model organisms.

Key recent findings and their impact on the field

Basic body plan comparisons

Anteroposterior patterning

During AP axis specification, there are close similarities between *S. kowalevskii* and vertebrates in their relative spatial deployment of transcription factors that are involved in ectodermal patterning, including Hox genes, *six3*, *foxG*, *distalless*, *nkx2-1*, *barH*, *engrailed* and *pax2/5/8* (Lowe et al., 2003; Aronowicz and Lowe, 2006; Lemons et al., 2010; Pani et al., 2012). In vertebrates, the expression of many of these genes is restricted to the CNS, whereas in *S. kowalevskii* they are often expressed in circumferential rings in the ectoderm during early development, possibly reflecting the broad distribution of neurons at these stages of development. Epidermal expression of Hox genes is also detected in amphioxus and ascidians, suggesting that this might be an ancestral deuterostome feature that is modified in vertebrates (Holland, 2005; Keys et al., 2005).

These similarities between hemichordate and chordate AP patterning mechanisms provide a molecular basis for establishing general regional homologies between the vertebrate and enteropneust body plans, which has been challenging and contentious based on morphological comparisons alone. Additional investigations of gene expression and function in adult hemichordates might be highly informative in the future. Notably, the embryonic and juvenile proboscis ectoderm shares many patterning similarities with the vertebrate forebrain, the collar shares similarities with the midbrain, and the trunk shares similarities with the hindbrain and spinal cord (Lowe et al., 2003).

More recent work has revealed striking developmental genetic similarities between hemichordate ectodermal and vertebrate late neural plate patterning events. In all vertebrates, local signaling centers in the neural plate, characterized by expression of secreted ligands in predictable AP positions within a conserved transcriptional map, act to divide the brain into discrete regions (Wurst and Bally-Cuif, 2001; Echevarria et al., 2003; Wilson and Houart, 2004; Kiecker and Lumsden, 2005). Although some of the transcriptional signatures associated with vertebrate signaling centers are present in invertebrate chordates (Holland, 2009; Irimia et al., 2010), in most instances the signaling ligands that define the organizing abilities of these centers in vertebrates are not expressed in the corresponding AP positions in the amphioxus and ascidian nervous systems or general ectoderm. These data have been used to support the plausible hypothesis that most vertebrate brain signaling centers were sequentially assembled during chordate evolution. In this scenario, the recruitment of signaling ligands to these centers was the final step achieved in stem vertebrates (Holland, 2009; Irimia et al., 2010). However, ectodermal signaling centers that might be homologous to three of those found in vertebrate brains have now been described in *S. kowalevskii* (Pani et al., 2012), suggesting that these developmental programs predate chordate origins and were first assembled independently of the vertebrate brain. These findings then suggest that the transcriptional similarities and limited complements of signaling ligands in amphioxus and ascidians, rather than representing partially assembled signaling centers, are instead a result of secondary simplification. This work suggests that, although by virtually any morphological criterion vertebrates share many more similarities with amphioxus than with hemichordates, molecular outgroup data from hemichordates are also key for testing hypotheses of the origins of vertebrate developmental mechanisms.

Dorsoventral patterning

Antagonism between bone morphogenetic protein (Bmp), which is the diffusible extracellular ligand of the transforming growth factor β (Tgfβ) family, and its specific antagonist Chordin plays a central role in the establishment of the bilaterian DV axis (Holley and Ferguson, 1997). These proteins have also been proposed to play conserved roles in the specification of bilaterian CNS (Arendt and Nubler-Jung, 1996; De Robertis and Sasai, 1996). Gene expression analyses (Fig. 7) and functional studies show that these proteins are also involved in DV patterning in hemichordates. In *S. kowalevskii*, Bmp genes (Fig. 7A) are expressed on the prospective dorsal side and *chordin* (Fig. 7B) on the ventral side during gastrulation and early development (Lowe et al., 2006). After 3 days of development, neurons are broadly distributed throughout the ectoderm, including the dorsal midline where Bmp genes are expressed. Manipulation of *bmp2/4* levels by overexpression or knockdown results in dorsalized or ventralized embryos,

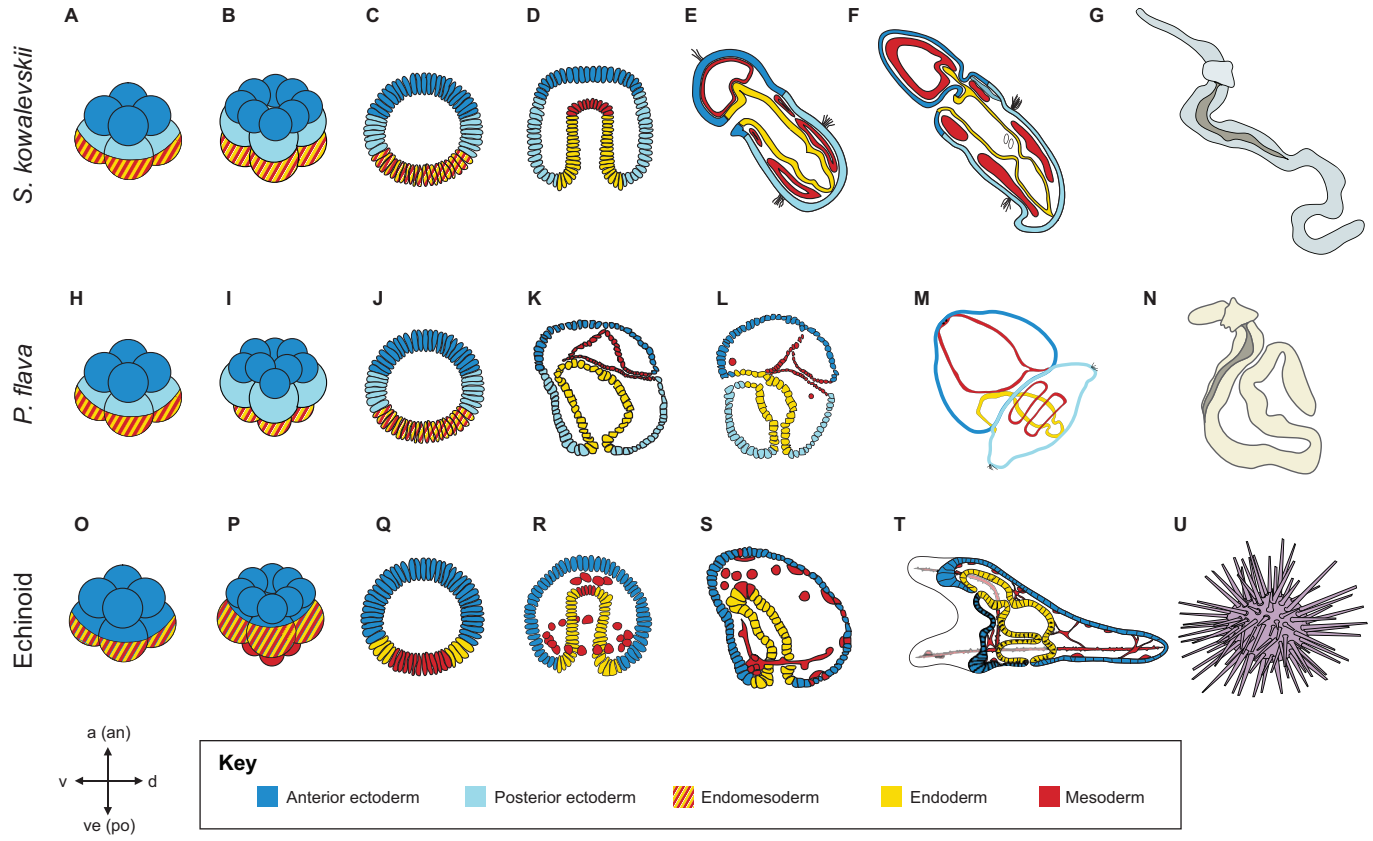

Fig. 6. Comparison of ambulacrarian (hemichordate and echinoderm) fate maps. Schematic representations of fate maps in (**A-G**) the direct-developing hemichordate *S. kowalevskii*, (**H-N**) the indirect-developing hemichordate *P. flava* and (**O-U**) a generic, indirect-developing echinoid. Ectoderm (light and dark blue) arises from the animalmost cells and endomesoderm (orange) is specified in the vegetal pole of all Ambulacraria. However, the timing of endoderm and mesoderm segregation remains unclear in hemichordates. Anterior ectoderm is formed by the animalmost blastomeres (dark blue) and posterior ectoderm arises from the macromeres (light blue) in hemichordates. The terms anterior versus posterior are not normally used in the commonly studied echinoderms and the term ventral is often replaced by oral and dorsal by aboral. See Fig. 4 legend for abbreviations.

respectively, indicating the importance of these genes for DV patterning (Lowe et al., 2006). However, overactivating Bmp signaling (by treatment with recombinant zebrafish Bmp4 protein) does not repress early neural fates, suggesting that, despite playing conserved roles in basic DV patterning, Bmps are not involved in repressing neural fates in all bilaterians.

In *P. flava*, *bmp2/4* and *chordin* are also expressed in the dorsal and ventral ectoderm, respectively (Fig. 7H,I), suggesting that they might play similar DV patterning roles in indirect-developing hemichordates (Harada et al., 2002; Röttinger and Martindale, 2011). Treatment with $NiCl_2$, a potent ventralizing agent used in the manipulation of echinoderms (Hardin et al., 1992), has a strong effect exclusively on patterning of the DV axis of *P. flava* and *S. kowalevskii* embryos. The link between the $NiCl_2$-sensitive ventralizing signal and dorsalizing Bmp signal, as well as the degree of conservation with the molecular mechanism underlying echinoderm DV patterning, remain unclear.

Comparison with a limited number of other genes expressed along the DV axis in Ambulacraria (Fig. 7) reveals both conservation and divergence; *bmp2/4* is expressed on the dorsal side in both hemichordate species studied to date, but is expressed in the ventral ectoderm in echinoids. Despite this spatial difference, in both taxa Bmp signaling is required to specify dorsal fates (Angerer et al., 2000; Duboc et al., 2004; Lowe et al., 2006).

Evolution of a posterior organizer

In chordates, the blastoporal organizer is involved in the initial axial patterning of the AP axis and provides posteriorizing signals to the embryo (Gerhart, 2001; Joubin and Stern, 2001; Holland, 2002). Unraveling the mechanistic basis of organizer function in vertebrates is complex owing to its simultaneous roles in patterning the DV and AP axes: any manipulation of the organizer results in both AP and DV defects, often making it challenging to interpret experimental results. Recent studies in *S. kowalevskii* highlight some of the advantages of this organism for dissecting the genetic mechanisms of early axial patterning. Unlike chordates, the early molecular mechanisms of DV and AP axis specification are mostly independent of one another in *S. kowalevskii*, although many orthologous genes are involved (Lowe et al., 2006). The highly conserved signaling and transcriptional network involved in the early ectodermal patterning of *S. kowalevskii* and chordates raises the possibility that these networks are regulated by homologous morphogens.

A recent study of the early function of β-catenin/Wnt signaling in *S. kowalevskii* has indicated that this pathway plays a key role in specifying endomesoderm and in establishing a posterior organizer (Darras et al., 2011). The role of β-catenin in the specification of the endomesoderm is likely to be broadly conserved in metazoans and has been documented in sea urchins

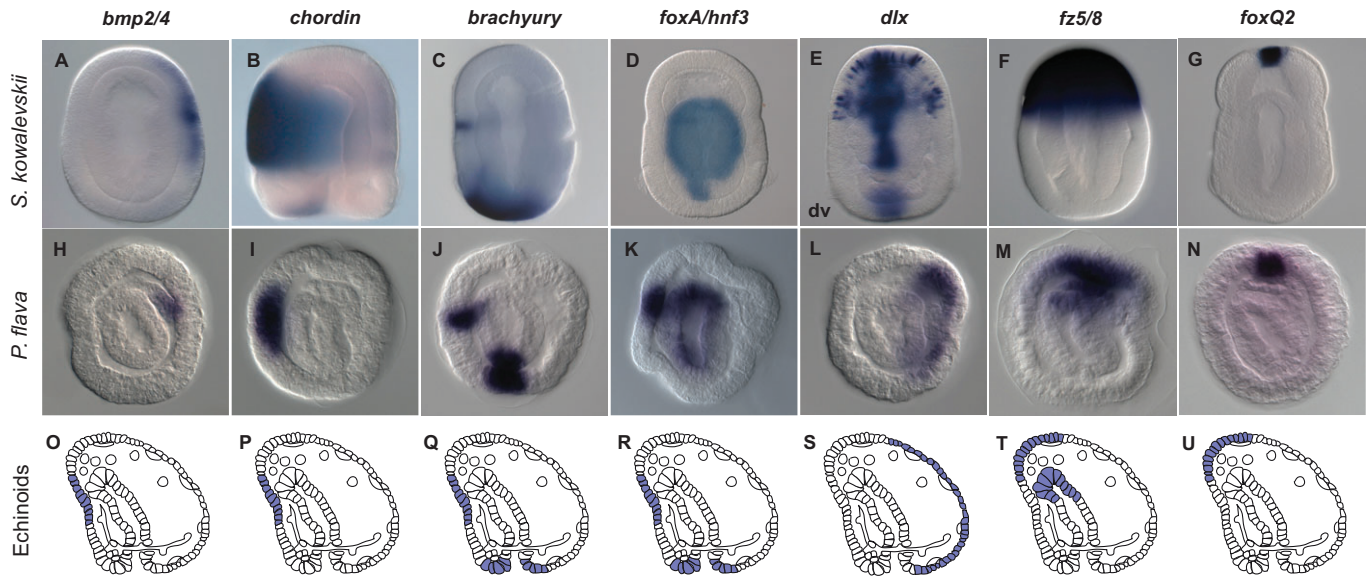

Fig. 7. Comparison of spatial gene expression in hemichordates and echinoderms. Spatial gene expression patterns analyzed by whole-mount in situ hybridization at late gastrula stages in (**A-G**) the direct-developing hemichordate *S. kowalevskii* and (**H-N**) the indirect-developing hemichordate *P. flava*. The corresponding echinoid gene expression patterns at the late gastrula stage are illustrated (**O-U**). A-G provided by J. Gerhart, C. J. Lowe and A. M. Pani; H-N are reproduced with permission (Röttinger and Martindale, 2011); O-U are based on published data: O (Angerer et al., 2000; Harada et al., 2001), P (Duboc et al., 2004; Bradham et al., 2009), Q (Croce et al., 2001), R (Oliveri et al., 2006), S (Howard-Ashby et al., 2006), T (Croce et al., 2006), U (Yaguchi et al., 2008). dv, dorsal view.

(Logan et al., 1999), cnidarians (Wikramanayake et al., 2003; Momose and Houliston, 2007) and nemerteans (Henry et al., 2008). Embryological experiments in *S. kowalevskii* have demonstrated that the establishment of the embryonic posterior domain is dependent on the β-catenin-mediated induction of endomesoderm (Fig. 8A). The animal ectoderm in early blastulae will adopt anterior fates if isolated from posteriorizing signals emanating from the vegetal pole (Colwin and Colwin, 1953; Sive et al., 1989; Darras et al., 2011), although these posteriorizing remain to be characterized. This is very similar to the situation in vertebrates (Sive et al., 1989) and sea urchins (Hörstadius, 1973), and Wnt/β-catenin signaling is involved in this process in both cases (Hörstadius, 1973; Kiecker and Niehrs, 2001; Niehrs, 2010; Angerer et al., 2011).

Morphological homologies with chordates

Classical morphological comparisons have raised hypotheses of homologies between several structures in chordates and hemichordates. Molecular genetic data can help test some of these hypotheses; if the two proposed homologous structures share substantial similarities in the genetic bases of their morphogenesis, then this can add support to hypotheses of morphological homology (Abouheif et al., 1997).

In hemichordates, the dorsal nerve cord has long been compared to the dorsal CNS of chordates (Knight-Jones, 1952), although the relationships between these structures remain uncertain. The hemichordate dorsal nerve cord extends from the proboscis to the anus along the dorsal midline and is superficial along most of its length. However, the collar nerve cord is internalized into a subepithelial, hollow structure through a process that strongly resembles chordate neurulation (Bateson, 1884a; Brown et al., 2008; Kaul and Stach, 2010). Early reports disagreed on the neural composition of this cord, with Bullock (Bullock, 1945) arguing that it was a largely through-conduction tract of axons without associated cell bodies, whereas Knight-Jones (Knight-Jones, 1952) argued for homology with the chordate dorsal nerve cord. Recent analyses clearly show an agglomeration of cell bodies in the hemichordate dorsal collar cord with an associated underlying neuropil (Brown et al., 2008; Nomaksteinsky et al., 2009). However, the internalized portion of the dorsal nerve cord is only a small part of the nervous system, and much less is known about nervous system patterning and organization in the rest of the animal. In particular, there are no available data on the molecular regionalization of the ventral nerve cord, which extends from the posterior collar down the length of the animal, and of the extensive nerve plexus of the proboscis and collar. Further work is required to test whether any region(s) of the hemichordate nervous system show the conserved mediolateral or DV axis patterning mechanisms described in more conventionally centralized nervous systems, such as those of annelids (Denes et al., 2007) and chordates (Jessell, 2000; Holland, 2009).

The dorsolateral gill slits that perforate the pharynx in all enteropneusts and some pterobranchs have close morphological and functional similarities to chordate gill slits (Rychel and Swalla, 2007; Gonzalez and Cameron, 2009). Homology of deuterostome gills has been further supported by reports of stem echinoderm fossils with gills (Jefferies, 1986; Dominguez et al., 2002). Early molecular analyses in hemichordates also demonstrated gill pore expression of the transcription factor *pax1/9*, which has key roles in chordate gill morphogenesis (Holland et al., 1995; Ogasawara et al., 1999; Okai et al., 2000). A recent study also revealed that a suite of transcription factors with conserved roles in the early development of chordate endodermal pharyngeal out-pockets and vertebrate gill slits is also expressed in out-pocketing hemichordate gill pouches (Gillis et al., 2011), providing robust support for the proposed homology of deuterostome gill pouches.

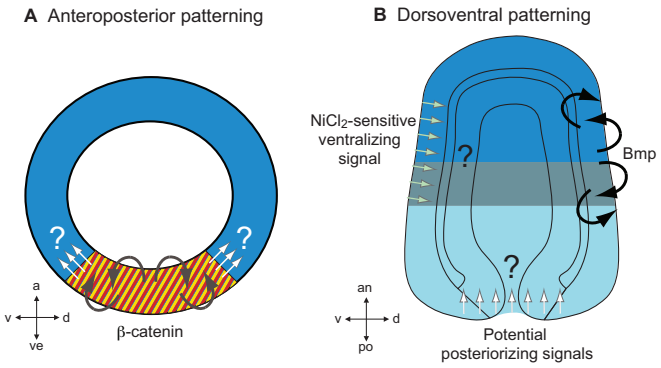

Fig. 8. Current model of hemichordate endomesoderm specification and early axis patterning. (**A**) AP patterning. At the blastula stage, accumulation of β-catenin is required to specify endomesoderm (orange), which in turn signals to the overlying ectoderm (blue) to specify posterior ectoderm via as yet uncharacterized signals (Darras et al., 2011). AP patterning is refined by additional signals at later stages. (**B**) DV patterning. Bmp signaling in the dorsal ectoderm is required to specify dorsal ectoderm and pattern the DV axis (Lowe et al., 2006) and an as yet unidentified (question mark) $NiCl_2$-sensitive signal specifies ventral fates (Röttinger and Martindale, 2011). Dark blue, anterior ectoderm; gray, collar ectoderm; light blue, posterior ectoderm. See Fig. 4 legend for abbreviations.

Limitations and future directions

The most significant limitation with all the hemichordates that have been studied so far is that they have limited reproductive periods. One of the biggest challenges is to extend the experimental period by determining cues that induce gametogenesis. The generation times are not known for any enteropneusts, but are likely to be too long to make forward genetics a practical strategy for functional studies.

Hemichordate developmental research is just beginning to reap the benefits of substantial progress in the development of genomics resources. Development and refinement of new functional approaches, together with the availability of new genomic datasets, provide the essential resources with which to address a wide range of key questions in developmental and evolutionary biology. The genomics resources for *S. kowalevskii* are well developed and include an extensive EST collection for a range of developmental stages and adult tissues (Freeman et al., 2008), and an EST resource is currently being developed for *P. flava* (Röttinger and Martindale, 2011). In addition, the genomes for both species have been sequenced and are currently in the process of being assembled and annotated. A high-quality assembly of the *S. kowalevskii* genome is available in GenBank. Developing methods for transient transgenesis will be the next step in genetic techniques in hemichordates, which will allow functional testing of cis-regulatory element activities.

Hemichordates have already revealed many important developmental insights into early deuterostome and chordate evolution. Previously, reconstructing ancestral developmental strategies of deuterostomes has been challenging owing to the often contrasting development strategies of chordates and echinoderms. For example, mesoderm induction in chordates is mediated by a variety of different signaling ligands that are not involved in specifying larval sea urchin mesoderm. Considering vertebrates, Nodal signaling is important in mesoderm induction in *Xenopus* and zebrafish and in primitive streak formation of mouse (Conlon et al., 1994; Feldman et al., 1998; Agius et al., 2000; Kimelman, 2006), and FGF signaling is key throughout vertebrate development (Amaya et al., 1991; Amaya et al., 1993; Ciruna and Rossant, 2001; Fletcher et al., 2006). Nodal is not involved in mesoderm induction in the invertebrate chordates (Onai et al., 2010) or sea urchins (Duboc et al., 2004; Lapraz et al., 2009), so this developmental role is likely to have evolved in early vertebrates. In ascidians, FGF signals are also important for mesoderm development (Imai et al., 2002; Imai et al., 2003), and in amphioxus FGF signals are important for the development of anterior, but not posterior, somites (Bertrand et al., 2011). In sea urchins, mesoderm is induced by Notch signaling (Sherwood and McClay, 1999; Sweet et al., 2002) without any obvious role of Nodal and only a limited influence by ERK, as one potential cytoplasmic downstream FGF effector in the MAPK pathway (Röttinger et al., 2004). Hemichordate functional developmental data for mesoderm induction will be necessary to reconstruct ancestral deuterostome mechanisms.

Comparative data from within the phylum will facilitate some of the first comprehensive developmental investigations between two species with similar adult body plans but contrasting life history strategies. The difficulties of making broad bilaterian developmental comparisons between species with contrasting life histories are rarely considered, but can be a significant confounding factor in comparisons between groups given the different life histories of many organisms. Furthermore, comparisons of developmental mechanisms between anatomically similar *P. flava* and echinoderm larvae will help to address long-standing questions of whether bilaterian, feeding primary larvae are homologous (Davidson et al., 1995) or whether they have evolved convergently in response to similar selective pressures (Sly et al., 2003).

Hemichordates also have the potential to become compelling models for studying regeneration. The ability to regenerate is widely distributed and occurs to some extent in most of the animal phyla. In vertebrates (i.e. axolotl salamanders), regeneration is limited to regrowth of particular body structures (reviewed by Antos and Tanaka, 2010; Nacu and Tanaka, 2011), whereas certain invertebrates are able to restore large parts of their bodies (Bely and Nyberg, 2010). However, the developmentally simplest and most intensely studied model systems for regenerative medicine are phylogenetically very distant from chordates. The close relationship of hemichordates and chordates suggests that a molecular characterization of regeneration in hemichordates might be very revealing. Enteropneusts have excellent regenerative capacities (Tweedell, 1961; Packard, 1968; Rychel and Swalla, 2008; Humphreys et al., 2010; Miyamoto and Saito, 2010), and impressive anterior and posterior regeneration has been reported in several ptychoderids (Rao, 1955; Rychel and Swalla, 2008; Humphreys et al., 2010) as well as reproduction by fission (Packard, 1968; Miyamoto and Saito, 2010).

Conclusions

Although the potential of hemichordates to improve our understanding of chordate and deuterostome body plan evolution has its historical roots as far back as the early 1800s, the morphological disparities between deuterostome body plans made progress difficult. The recent application of experimental approaches and the availability of genomic resources have enabled molecular developmental studies of germ layer specification and axial patterning in hemichordates. These studies are revealing highly conservative developmental programs that are facilitating unprecedented insights into early deuterostome body plan

evolution. Future work will further test for developmental similarities and differences with chordates and echinoderms to determine ancestral deuterostome strategies. Furthermore, the completed genomes for two species will encourage novel comparative approaches and further functional tool development. The ability to compare the development of both larval and direct-developing species within the same group will provide valuable data that are likely to make a key contribution to furthering our understanding of the evolution of life history and the origins of larvae.

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Competing interests statement
The authors declare no competing financial interests.

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