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Evolutionary crossroads in developmental biology: amphioxus

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Summary

The phylogenetic position of amphioxus, together with its relatively simple and evolutionarily conserved morphology and genome structure, has led to its use as a model for studies of vertebrate evolution. In particular, the recent development of technical approaches, as well as access to the complete amphioxus genome sequence, has provided the community with tools with which to study the invertebrate-chordate to vertebrate transition. Here, we present this animal model, discussing its life cycle, the model species studied and the experimental techniques that it is amenable to. We also summarize the major findings made using amphioxus that have informed us about the evolution of vertebrate traits.

Key words: Cephalochordates, Vertebrate evolution, *Branchiostoma*, Amphioxus, Lancelet

Introduction

Amphioxus (also called lancelets or cephalochordates) form one of the three chordate subphyla, along with urochordates (see Glossary, Box 1) and vertebrates (Schubert et al., 2006) (Fig. 1A). They form a small group comprising about 35 species (Poss and Boschung, 1996). The number of genera within the cephalochordate subphylum has long been debated, but recent molecular phylogenetic studies show that cephalochordates are divided into three genera (Kon et al., 2007) (Fig. 1B): *Branchiostoma*, *Epigonichthys* and *Asymmetron*.

Described for the first time in 1774 (Pallas, 1774), lancelets were classified as molluscs and were called *Limax lanceolatus*. Later, in 1834, they were renamed as *Branchiostoma lubricus* (Costa, 1834) and classified as animals closely related to vertebrates. The first to use the name Amphioxus was William Yarrell, who named them *Amphioxus lanceolatus* and for the first time described their notochord (see Glossary, Box 1), the defining morphological trait of chordates (Yarrell, 1836). After these initial studies, and until the beginning of the 20th century, amphioxus was considered to be a vertebrate. A consensus was then reached whereby amphioxus were considered to be the closest living relatives of vertebrates, with urochordates representing the most basally divergent chordate lineage. These evolutionary relationships were based on morphological characteristics and on some molecular studies of rRNA genes (Winchell et al., 2002). However, in 2006, based on large molecular data set analyses, it was established that cephalochordates represent the most basally divergent lineage of chordates, being the sister group of urochordates and vertebrates (Bourlat et al., 2006; Delsuc et al., 2006) (Fig. 1A).

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The adult anatomy of amphioxus is vertebrate-like, but simpler. Amphioxus possess typical chordate characters, such as a dorsal hollow neural tube and notochord, a ventral gut and a perforated pharynx with gill slits, segmented axial muscles and gonads, a post-anal tail, a pronephric kidney, and homologues of the thyroid gland and adenohypophysis (the endostyle and pre-oral pit, respectively) (Fig. 2A). However, they lack typical vertebrate-specific structures, such as paired sensory organs (image-forming eyes or ears), paired appendages, neural crest cells and placodes (see Glossary, Box 1) (Schubert et al., 2006). This simplicity can also be expanded to the amphioxus genome structure. Indeed, two rounds of whole-genome duplication occurred specifically in the vertebrate lineage. This

Box 1. Glossary

Benthic. Living on or closely to the bottom of the sea or a lake.
Cirri. Thin external processes around the amphioxus mouth, which function as the first filter during feeding by eliminating unwanted large or noxious particulates.
Co-opted. When an existing gene, organ or structure is recruited for a new function during evolution.
Cyclostomes. Sister group of gnathostomes, comprising hagfishes and lampreys.
Gill bars. The cartilaginous structures on each side of the pharynx localized between the gill slits in amphioxus.
Gnathostomes. Vertebrates with articulated jaws.
Holoblastic segmentation. Cleavage of whole embryo or zygote that can be equal, resulting in sister cells of similar sizes, or unequal, giving rise to sister cells of different sizes. Different amphioxus species show different holoblastic segmentation.
Notochord. An elongated skeletal structure present in all chordate embryos found dorsal to the gut and ventral to the nerve cord. In most adult chordates, it disappears or becomes highly modified; in adult amphioxus, it persists as a skeletal rod.
Oligolecithal. Small eggs with low vitellus (i.e. egg yolk) content.
Organizer. An embryonic signalling centre located at the dorsal lip of the blastopore that plays a crucial role in the organization and formation of the main body axes of a developing embryo.
Orthologue. Homologous genes that are derived by speciation from an ancestral gene.
Paralogue. Homologous genes that are derived by gene duplication from an ancestral gene.
Placode. An area of thickening in the embryonic non-neural ectoderm in which some specific organs or structures later form.
Planktonic. Living, floating or drifting in the ocean or in bodies of fresh water.
Polyploidization. A process that produces a numerical change in a whole set of chromosomes in a given organism.
Ripe. The state of adult animals that are capable of releasing gametes.
Synapomorphy. A character shared by two or more taxa and their closest ancestor.
Urochordates (also known as tunicates). A sister group of vertebrates, characterized by a thick secreted covering layer (a 'tunic') and the presence of a notochord in the larva.

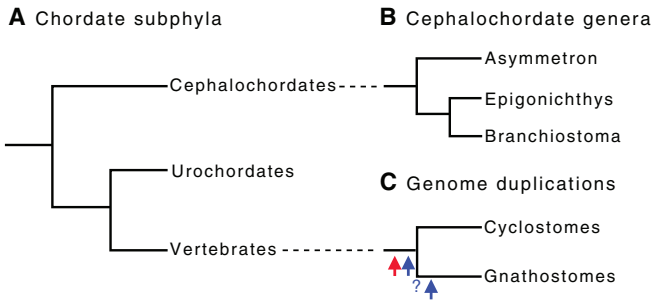

Fig. 1. Phylogenetic relationships between chordate subphyla. (**A**) The chordate phylum can be split into three subphyla: cephalochordates (amphioxus), which have a basal position; vertebrates; and urochordates. (**B**) The cephalochordate subphylum is divided into three genera: *Branchiostoma*, *Epigonichthys* and *Asymmetron*. (**C**) Comparisons of amphioxus, *Ciona intestinalis* and vertebrate genomes confirm that two rounds of whole-genome duplication occurred specifically in the vertebrate lineage. The arrows indicate the evolutionary points at which the two complete genome duplications occurred. The first duplication event (red arrow) is thought to have taken place at the base of the vertebrate lineage, before the cyclostome-gnathostome split, whereas the exact timing of the second genome duplication (blue arrows) is still a matter for debate.

hypothesis (the 2R hypothesis) (Ohno, 1970) has been widely accepted, although different proposals have been made, and are still debated, concerning the number and timing of the duplications (Fig. 1C) (Friedman and Hughes, 2001; Kuraku et al., 2009; Skrabanek and Wolfe, 1998). These two genome duplications have been convincingly supported by the complete genome sequencing of several chordate species (Dehal and Boore, 2005). The consequences of these duplications are that for each amphioxus gene, a group of one to four paralogues (see Glossary, Box 1) can be found in the vertebrate genome (secondary gene losses account for the variations in the precise number of paralogues). Moreover, the 'pre-duplicated' amphioxus genome possesses one representative of almost all the members of all the gene families that presumably existed in the ancestor of chordates, in contrast to the situation in the two other chordate subphyla, urochordates and vertebrates, which have specifically lost different members of these gene families (e.g. the homeobox, tyrosine kinase or nuclear receptor families) (Bertrand et al., 2011a; D'Aniello et al., 2008; Takatori et al., 2008).

Thus, the morphological and genomic simplicity of amphioxus, together with its key phylogenetic position make it an invaluable animal model for understanding the invertebrate-chordate to vertebrate evolutionary transition.

Habitat and life cycle

All amphioxus species are filter-feeding marine animals that burrow in sand, gravel or shell deposits in tropical or temperate waters around the world. Studies of amphioxus habitat have been performed for several species and have revealed some common features, e.g. the importance of sediment type for their distribution. A general preference for coarse sand and gravel is observed for many species (Desdevises et al., 2011; Gosselck, 1975; Webb, 1958; Webb and Hill, 1958b), but some exceptions to this rule exist. Another shared feature is that almost all amphioxus species are found in shallow waters close to the sea shore (from 0.5 m to 40 m deep) (Desdevises et al., 2011; Gosselck, 1975). But, again, there is an exception as one species (*Asymmetron inferum*) has been found at a depth of 229 m (Kon et al., 2007). Other factors, such as temperature and salinity changes, have also been shown to be very important in the life cycle of some amphioxus species (Webb, 1956a; Webb, 1956b; Webb and Hill, 1958a). Thus, the migration of amphioxus populations between winter and summer (Webb, 1971), as a result of temperature changes, or the restriction of amphioxus larvae to waters of high salinity and temperature, have been reported (Webb and Hill, 1958a). Finally, a negative correlation between the presence of amphioxus and organic matter content has been noted for some species (da Silva et al., 2008; Webb and Hill, 1958a).

Concerning the spawning behaviour, all known amphioxus species spawn after sunset during their breeding season. They swim up to the water surface, lay their gametes in the water column and then sink gently back to the sand bottom. The duration and structure of the spawning season, however, may vary between different species. Thus, the spawning seasons of *B. belcheri* and *B. lanceolatum* span 2-3 months in spring (Fuentes et al., 2007; Fuentes et al., 2004; Wu et al., 1994), whereas *B. floridae* spawns from early May to September (Stokes and Holland, 1996). This 'single spawning season' trait is not a general behaviour; *A. lucayanum* spawns during two separate 3-month periods during the year (one in spring and one in autumn) (Holland, 2011). The spawning behaviour of different amphioxus species also varies, from a few contiguous spawning days per year for *B. belcheri* (Wu et al., 1994), to approximately weekly spawning during the breeding season for *B. floridae* (Stokes and Holland, 1996). Non-biotic variables that could explain these differences, other than the global water temperature, which delimits the spawning season, or day length, have not been found, except for *A. lucayanum* (Holland, 2011). Individuals of this species seem to spawn predominantly 1 day before the new moon (Holland, 2011). Finally, in all amphioxus species, once the gametes are released and fecundation occurs, embryos remain planktonic (see Glossary, Box 1) until metamorphosis. They then migrate to the sand where they become benthic (see Glossary, Box 1).

Amphioxus embryogenesis was first described by Kowalevsky (Kowalevsky, 1867), who divided it into an early phase of development resembling that of invertebrate deuterostomes (i.e. a hollow blastula invaginates to form a gastrula in a similar manner to the sea urchin) and a later phase that is vertebrate-like (with the formation of a notochord, a dorsal hollow nerve chord, segmented axial muscles, etc.) (Fig. 3). The small (~100 μm) oligolecithal (see Glossary, Box 1) eggs of amphioxus follow a pattern of radial holoblastic segmentation (see Glossary, Box 1). The transparent embryos then develop rapidly (supplementary material Movie 1), and, depending on the species, form larvae after 2-3 days. The free-living planktonic larvae show an asymmetric body plan: the mouth forms on the left side, the gill slits form on the right-ventral side and the right series of somites form half a segment posterior to the left ones. During metamorphosis, which occurs 2-3 weeks to 2-3 months after fertilization, depending on the species, much of this asymmetry disappears, although the axial muscles retain their asymmetry. An interesting difference between *Branchiostoma* and *Asymmetron* species is that while *Branchiostoma* adults show two rows of symmetric gonads ventrolaterally, *Asymmetron* develops gonads on only the right side of the body. A higher growth rate has been observed for species living in warmer waters (Desdevises et al., 2011; Stokes, 1996). The lifespan in the wild also varies between different species, from an estimated 2-3 years for *B. floridae* and *B. belcheri* (Chen et al., 2008; Chin, 1941; Futch and Dwinell, 1977)

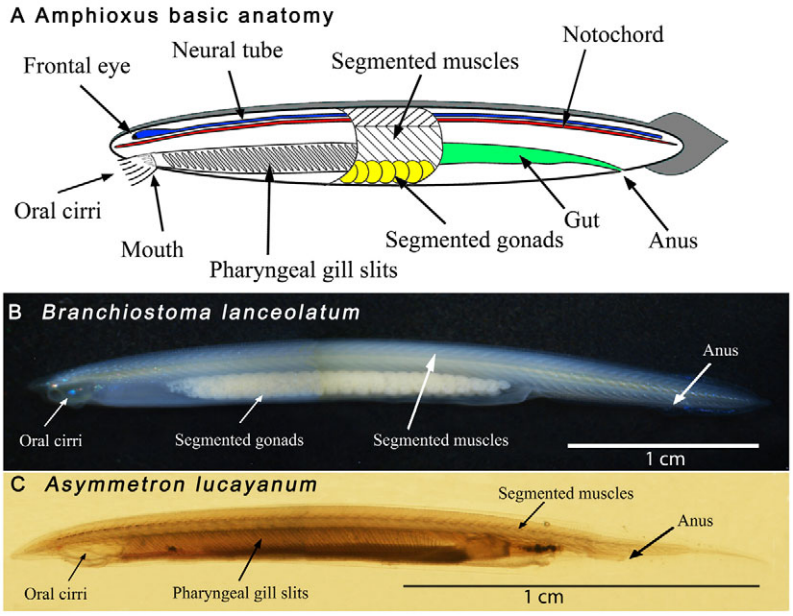

Fig. 2. Amphioxus possess typical chordate characteristics. (**A**) Schematic view of amphioxus basic anatomy. The most important morphological characteristics are depicted. These include the dorsal hollow neural tube (blue), the dorsal notochord (red), the ventral gut (green) and segmented gonads (yellow). (**B**) *Branchiostoma lanceolatum* adult individual collected from Racou Beach in France; the oral cirri, the segmented gonads and muscles, and the anus are labelled. (**C**) *Asymmetron lucayanum* adult individual collected in Eilat in Israel; the oral cirri, pharyngeal gill slits, segmented muscles and anus are labelled. In all three images, anterior is towards the left and dorsal is towards the top.

to 4-5 years for *B. senegalense* (Gosselck and Spittler, 1979) and up to 8 years for *B. lanceolatum* (Courtney, 1975; Desdevises et al., 2011).

Amphioxus model species

Although most of the early descriptions of amphioxus anatomy and embryonic development were performed using *Branchiostoma lanceolatum*, current studies use amphioxus embryos from four different species. Three of these four species belong to the genus *Branchiostoma* (*B. belcheri*, *B. floridae* and *B. lanceolatum*) and one to the genus *Asymmetron* (*A. lucayanum*).

Branchiostoma floridae

In the late 1980s, Nicholas D. Holland and Linda Z. Holland, working with animals from Tampa Bay in Florida, managed for the first time to induce the spawning of ripe (see Glossary, Box 1) animals using a non-lethal electric shock (Holland and Holland, 1989). This method provides synchronously developing embryos and opened up studies of the amphioxus model to evolutionary developmental biologists. *B. floridae* has since become a leading model animal, and it is currently the only amphioxus species to have had its genome sequenced (Putnam et al., 2008). However, electric shock can induce spawning only on natural spawning days during the spawning season, which therefore hampers laboratory work.

Branchiostoma belcheri

B. belcheri was first described by Gray in 1847 (Gray, 1847), and different synonymous names have been used for animals collected at different locations (reviewed by Poss and Boschung, 1996). However, two recent studies have established that the original *B. belcheri* description contains at least three different cryptic species that can cohabit at the same location: *B. belcheri*, *B. tsingtauense* and *B. japonicum* (Xu et al., 2005; Zhang et al., 2006). Here, we refer to this collection of species as *B. belcheri*. Although *B. belcheri* was used for developmental studies in the 1950s and 1960s (Tung et al., 1958; Tung et al., 1962a; Tung et al., 1962b), it was first used for evolutionary developmental biology studies in the 1990s (Terazawa and Satoh, 1997; Yasui et al., 1998). *B. belcheri* is the only amphioxus species for which a complete life cycle in captivity has been achieved (Yasui et al., 2007; Zhang et al., 2007) (see Box 2), but spawning induction has not been developed for this species. Thus, *B. belcheri* embryos are obtained naturally from adult animals that are kept in large tanks (Hirakow and Kajita, 1990); during the spawning season, females swim up from the sand and spontaneously lay eggs, which can be recovered and cultured in large Petri dishes containing naturally inseminated seawater. This method has been used to provide synchronously developing embryos.

Branchiostoma lanceolatum

B. lanceolatum (Fig. 2B), which has been collected from Racou Beach in France, is the only species for which induction of spawning is possible on a daily basis (from different individuals). A simple method, consisting of a temperature shock, leads to spontaneous spawning after sunset 36 hours after induction (Fuentes et al., 2007; Fuentes et al., 2004). By changing the day/night cycle, this method also allows embryos to be harvested during the day, rather than at sunset. This approach is also used in laboratories inland, where the animals are kept ripe in artificial sea water (Theodosiou et al., 2011). The development of aquaculture techniques for *B. lanceolatum* now allows individual animals to be kept for long periods of time and to develop gonads in captivity (Fuentes et al., 2007; Somorjai et al., 2008b) (see Box 2).

Asymmetron lucayanum

A. lucayanum (Fig. 2C) embryos have been obtained recently from Bimini in The Bahamas (Holland, 2011; Holland and Holland, 2010); however, a spawning induction method has not yet been developed for this species. Embryos are obtained during spawning seasons by keeping ripe adults in a dish that is continuously exposed to artificial light during the late afternoon of collection, and then placing them in the dark at 21:00 h. Although the use of *A. lucayanum* as a model for evolutionary developmental biology

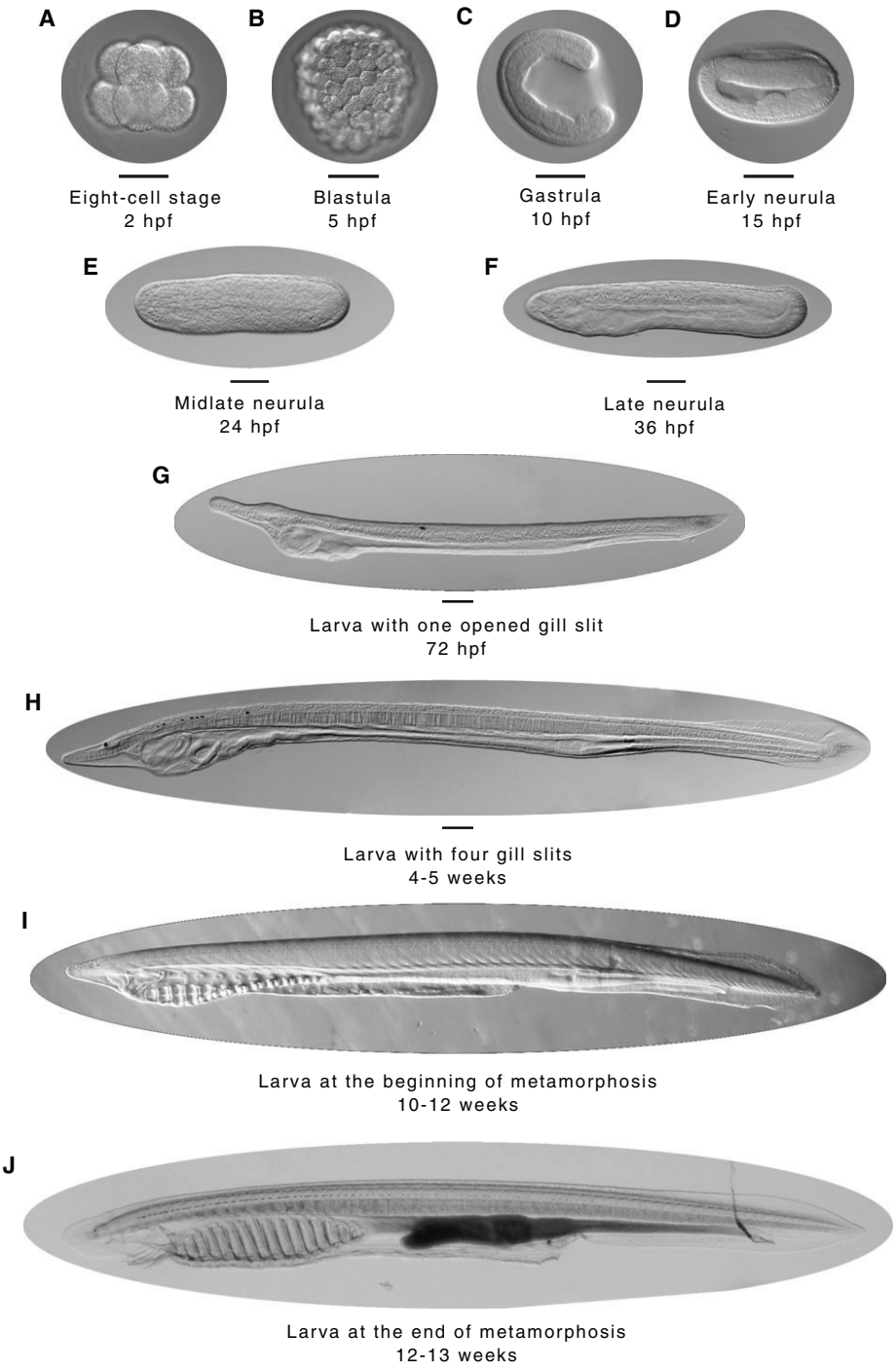

Fig. 3. The amphioxus life cycle. The most representative embryonic and larval stages of the *B. lanceolatum* life cycle are presented; the developmental timing for each stage at 19°C is also indicated. (**A-C**) The early stages of development, before neurulation, are similar to those observed in other invertebrate deuterostomes such as the sea urchin. (**D-G**) By contrast, the later stages of development, after neurulation, are similar to those seen in vertebrates. (**H-J**) The asymmetric larvae (H,I) become partially symmetric after metamorphosis (J), although the axial muscles keep their asymmetry. Scale bars: 50 μm.

studies is currently limited, its life cycle, with two spawning seasons per year, and its divergence from the *Branchiostoma* genus will probably make it an invaluable animal model in the future.

Experimental techniques

The first experimental techniques developed for amphioxus were in toto in situ hybridization (Fig. 4A-G), which takes advantage of the transparency of embryos to study gene expression patterns, and immunostaining (Fig. 4H-J), which is used for protein localization (Holland et al., 1992). Functional studies based on the activation or inhibition of different signalling pathways, and subsequent analysis of the phenotype, have also been undertaken. The most common approach has been the use of small molecules to inhibit or activate different pathways (Fig. 4K,L). Some examples of these treatments include: retinoic acid (RA) and its antagonist BMS009 for the study of the role of RA signalling (Escriva et al., 2002a; Holland and Holland, 1996; Schubert et al., 2004; Schubert et al., 2005); LiCl for studying the Wnt/β-catenin pathway (Holland et al., 2005; Onai et al., 2009); SU5402 for studying fibroblast growth factor (FGF) signalling (Bertrand et al., 2011b); U0126 for examining mitogen-activated protein kinase (MAPK) signalling (Bertrand et al., 2011b); BMP4 to upregulate the bone morphogenetic protein (BMP) signalling pathway (Yu et al., 2007); and activin and SB505124 for the activation and repression, respectively, of Nodal signalling (Onai et al., 2010).

Box 2. Aquaculture of amphioxus

The possibility of obtaining embryos – using induced spawning – on a daily basis prompted several laboratories to develop amphioxus aquaculture facilities. Aquaculture techniques, which use natural and artificial sea water both in marine stations and in inland laboratories (Fuentes et al., 2007; Fuentes et al., 2004; Somorjai et al., 2008b; Theodosiou et al., 2011), have been developed for two of the four experimental amphioxus species (*B. belcheri* and *B. lanceolatum*). Currently, *B. lanceolatum* is the only amphioxus species that can be induced to spawn on any given day during the breeding season, and aquaculture techniques have allowed the long-term culture of individual *B. lanceolatum* in small volumes of water (Somorjai et al., 2008b). Inland laboratories are now also able to maintain and induce spawning of ripe *B. lanceolatum* in artificial sea water (Theodosiou et al., 2011). However, although embryos can be cultured until the adult stage in captivity (Desdevises et al., 2011), a complete life cycle of *B. lanceolatum* has not yet been achieved in the laboratory, and only short-term cultures exist. By contrast, long-term cultures of *B. belcheri* have been developed, both in the absence and presence of sand as the burying substratum (Yasui et al., 2007; Zhang et al., 2007). Moreover, when maintained in natural seawater with sand, *B. belcheri* has been successfully raised in phase with the natural life cycle observed in the wild through two generations in the laboratory (Zhang et al., 2007).

Methods for manipulating gene expression have also been developed in amphioxus (Holland and Yu, 2004). Thus, microinjection of unfertilized eggs has been successfully employed in *B. floridae*, using both morpholinos and mRNA (Onai et al., 2010; Schubert et al., 2005) to knock down and to overexpress a given gene, respectively. Currently, to our knowledge, only mRNA injection is efficient in *B. lanceolatum*, and microinjecting *B. belcheri* or *A. lucayanum* has not been performed successfully. Unfertilized egg injection has also been used to generate transient transgenics, using plasmids that contain a reporter gene, the expression of which is under the control of cis-regulatory elements or a promoter sequence (Fig. 4M) (Holland et al., 2008a; Yu et al., 2004). However, in contrast to pharmacological treatments, the manipulation of gene expression using microinjection techniques still requires optimization, and is thus used less frequently.

Key recent findings

Evolution of the chordate genome

Amphioxus genome sequence data have been crucial for answering two major questions in evolutionary biology. First, what are the phylogenetic relationships within chordates? Second, did the genome of vertebrates undergo several duplications, as postulated in the 2R hypothesis (Ohno, 1970)?

As previously discussed, the classical phylogenetic view placed cephalochordates as the sister group of vertebrates and placed urochordates at the base of the chordate lineage. This classification was based on morphological characteristics that are shared by amphioxus and vertebrates, but are absent in urochordates (Schaeffer, 1987), and also on some phylogenetic studies (Winchell et al., 2002). However, in 2006, using large sequence data sets, it was shown that urochordates are, in fact, the sister group of vertebrates, and cephalochordates are the most basally divergent group among chordates (Bourlat et al., 2006; Delsuc et al., 2006). This new phylogenetic framework has important implications because it shows that: (1) urochordates are extremely derived animals; and (2) the ancestor of chordates was probably an amphioxus-like animal, as various fossils suggest (Holland and Chen, 2001).

The answer to the second question, regarding the genome duplications, has been much debated since Ohno proposed – in the 2R hypothesis – that two rounds of complete genome duplication (polyploidization, see Glossary, Box 1) occurred at the base of the vertebrate lineage (Ohno, 1970). For many years, the lack of sufficient sequence data stimulated many discussions, not only about the validity of this hypothesis, but also about the number and timing of the genome duplications (Skrabanek and Wolfe, 1998). The demonstration of the 2R hypothesis finally came from analyses using complete genome sequences of species throughout the chordate lineage (Dehal and Boore, 2005), and was corroborated when the first amphioxus genome sequence became available (Putnam et al., 2008). At this point, there is still an unanswered question concerning the exact timing of such genome duplications within the vertebrate evolutionary history. Did they occur before the split between cyclostomes (see Glossary, Box 1) and gnathostomes (see Glossary, Box 1), or did one duplication occur before and one after this evolutionary branchpoint (Fig. 1C)? The answer to this question cannot be obtained using uniquely phylogenetic approaches (Escriva et al., 2002b; Kuraku et al., 2009) and awaits the publication of complete cyclostome genome sequences.

Hox clusters and the evolution of cis-regulatory elements

The amphioxus genome contains a single homeobox (Hox) cluster, which was partially characterized in 1994 (Garcia-Fernandez and Holland, 1994). Since then, it has been viewed as ‘archetypal’ for the chordate lineage. This Hox cluster has been maintained intact, is ordered, contains 15 Hox genes and represents the most prototypical deuterostome Hox cluster known (Amemiya et al., 2008). The most anterior 14 Hox genes are the orthologues (see Glossary, Box 1) of the Hox genes present in the ancestor of all chordates, whereas the last gene, *Hox15*, may be the result of an amphioxus-specific duplication. Conservation between amphioxus and vertebrates has been observed not only at the level of cluster structure and gene expression (Wada et al., 1999), but also at the gene regulatory level. The cis-regulatory activity of the amphioxus Hox cluster has been studied in transgenic mice and in chick embryos, and these studies have shown that the mechanisms controlling Hox gene expression in the neural tube, including a dependence on RA signalling, have been conserved during half a billion years of evolution (Manzanares et al., 2000). Moreover, amphioxus Hox cluster cis-elements can drive localized reporter gene expression in vertebrate neural crest cells, in derivatives of neurogenic placodes and in branchial arches, despite the fact that cephalochordates lack these structures (Manzanares et al., 2000). This implies that the cis-regulatory elements of the Hox gene cluster are conserved among chordates and that these elements were co-opted (see Glossary, Box 1) for the regulation of Hox gene expression in new structures during the evolution of the vertebrate lineage.

The conservation of non-coding cis-regulatory sequences has also been highlighted in other contexts. For example, genome-scale comparisons between mammals and teleosts have revealed a large number of conserved non-coding elements (CNEs) that act as tissue-specific enhancers (Woolfe et al., 2005). The only invertebrate in which conservation of some of these CNEs have been found is the amphioxus (Holland et al., 2008a; Hufton et al., 2009; Putnam et al., 2008). Studies using *lacZ* reporter constructs

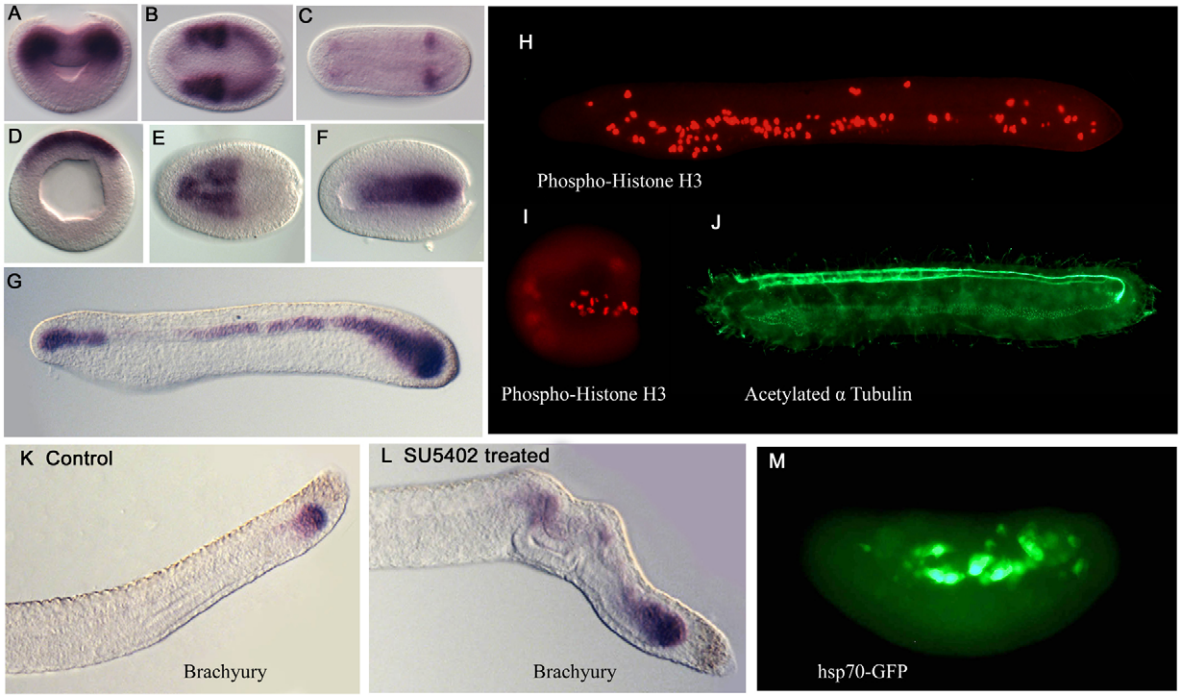

Fig. 4. Several experimental approaches are available for developmental studies of amphioxus. (**A-G**) Examples of gene expression patterns studied by whole-mount in situ hybridization. Expression patterns of *Delta* (A-C), *Neurogenin* (D,E), *Netrin* (F) and *Brachyury2* (G) are shown for the gastrula (A,D), neurula (B,C,E,F) and late neurula (G) stages (blastoporal views are shown in A and D; dorsal views are shown in B,C,E,F; a lateral view is shown in G). (**H-J**) Examples of immunostaining with antibodies against phosphorylated histone H3 (H,I) and acetylated tubulin (J) in embryos at the gastrula (I) and late neurula (H,J) stages. (**K**,**L**) Pharmacological treatment of amphioxus neurula with the fibroblast growth factor signalling inhibitor SU5402. In the control larva (K), *Brachyury2* expression is restricted to the tailbud, whereas SU5402-treated larva (L) exhibit *Brachyury2* expression throughout the entire notochord, which elongated during the treatment period. (**M**) Transient transgenic amphioxus obtained by microinjecting unfertilized eggs with a reporter plasmid (p339_hsp70-GFP) using I-*Sce*I meganuclease. All images show experiments using *B. lanceolatum* embryos.

for these CNEs have shown that there is at least partial conservation of tissue-specific expression in both amphioxus and mouse, indicating that both the sequences and regulatory functions of these enhancers have been conserved throughout the last 500 million years (Holland et al., 2008a; Putnam et al., 2008).

Axial patterning

The evolution of embryonic patterning through the three spatial body axes – anteroposterior (AP), dorsoventral (DV) and left-right (LR) – had major implications in the evolution of the metazoan body shape. Recent studies using amphioxus as a model, in particular those that have focused on the establishment of the AP and DV axes, have deciphered the most probable ancestral state of axial patterning in chordates and how this state might have evolved in this lineage.

In vertebrates, the AP and DV axes are induced during gastrulation by a signalling centre, the ‘organizer’ (see Glossary, Box 1) (De Robertis et al., 2000). In the 1960s, graft approaches already suggested the presence of a region in the amphioxus gastrula that exhibited clear organizer properties (Tung et al., 1962a; Yan, 1999). This territory was able to induce the formation of a secondary axis in amphioxus, as does the vertebrate organizer (reviewed by Yan, 1999). This finding was recently confirmed through gene expression analyses and functional studies. Indeed, it has been shown that molecular mechanisms controlling both DV and AP patterning of the early gastrula (Yu et al., 2007) are conserved between vertebrates and amphioxus (Fig. 5A). In particular, a conserved role for the BMP signalling pathway in the specification of the DV axis, and a conserved functional opposition between BMP and Nodal signals have been demonstrated (Kozmikova et al., 2011; Onai et al., 2010). Given that tunicates show divergent expression of organizer genes and have a development that is determinate and largely based on fixed cell lineage, instead of an inductive vertebrate-like development (Gerhart, 2001), it seems likely that the organizer was present in the chordate ancestor and that it was specifically lost in tunicates.

The co-linear expression of Hox genes is instrumental for establishing and specifying different structures in different germ layers along the AP axis in vertebrates (Kmita and Duboule, 2003). The expression of Hox genes is also co-linear in amphioxus (Wada et al., 1999), and a direct role for RA in the control of axial patterning in amphioxus through the control of Hox gene expression has also been described (Escriva et al., 2002a; Holland and Holland, 1996; Schubert et al., 2004; Schubert et al., 2005). This has major implications because the presence of migratory neural crest cells (NCCs) in vertebrates hampered the demonstration of a direct role for RA/Hox in specific germ layers. The lack of NCCs in amphioxus, together with a role for RA/Hox in the patterning of non-neural ectoderm and of endoderm (Schubert et al., 2004; Schubert et al., 2005), implies an ancestral patterning of germ layers through a RA/Hox code, on top of which a NCC contribution was added in vertebrates. Moreover, it has

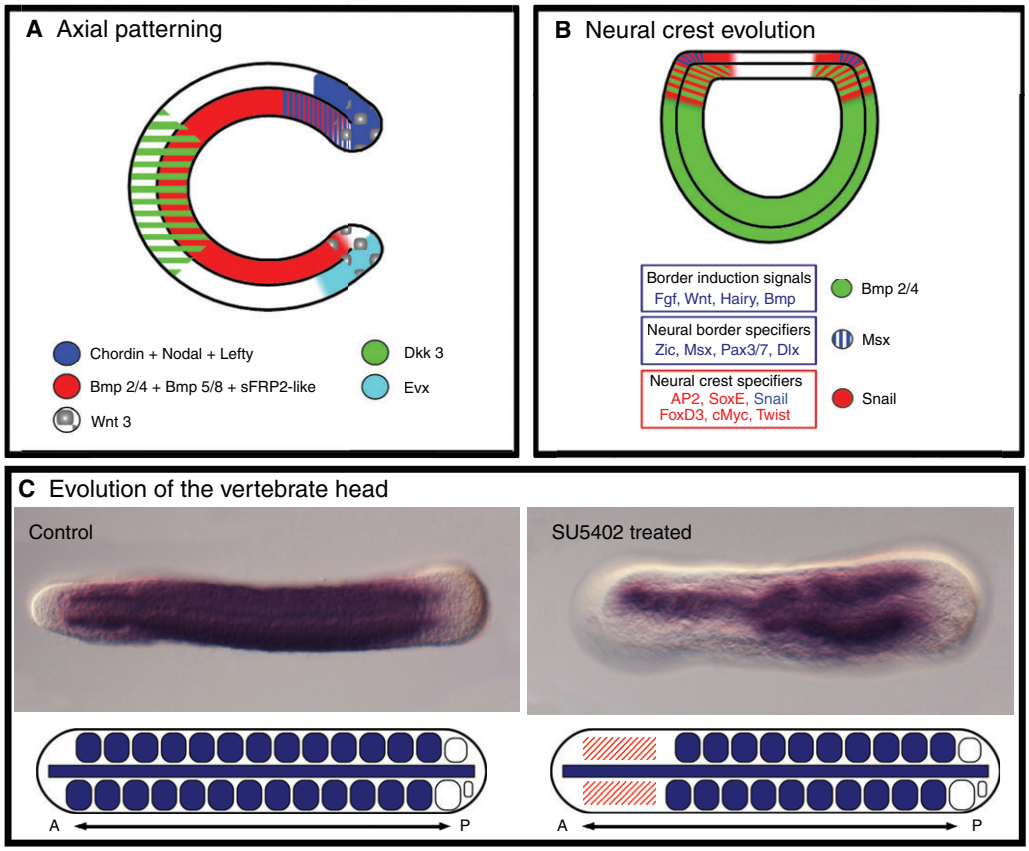

Fig. 5. Key findings obtained using the amphioxus model. (**A**) Axial patterning. Recent studies suggest a functional conservation between amphioxus and vertebrate genes implicated in axial patterning in the organizer; some examples of gene expression in the amphioxus gastrula are shown (e.g. *Chordin*, *Nodal*, *Lefty* and *Wnt3* are expressed in the dorsal blastoporal lip; *Bmp2/4*, *Bmp5/8* and *sFRP2-like* in the mesendoderm; *Evx* in the ventral ectoderm; and *Dkk3* in the anterior ectoderm and mesendoderm). (**B**) Neural crest gene regulatory network (GRN) and evolution. The neural crest cell GRN (NC-GRN) is partially conserved between amphioxus and vertebrates. The vertebrate NC-GRN includes 'border induction signals', 'neural border specifiers' and 'neural crest specifiers', and the expression of some of these (*Bmp2/4*, *Msx*, *Snail*) within the amphioxus neural plate border is depicted. Although the signals for border induction and neural border specifiers are conserved between amphioxus and vertebrates, only one gene (*Snail*) from the neural crest specifiers group shows a conserved expression pattern between amphioxus and vertebrates. Genes showing a conserved expression pattern with vertebrates are written in blue and genes showing divergent expression patterns are in red. (**C**) Evolution of the vertebrate head. Control amphioxus larva show expression of *myosin light chain* (*MLC*) in all somites, as detected by in situ hybridization (top panel) and as depicted in the schematic (bottom panel). The inhibition of fibroblast growth factor signalling (with SU5402) disrupts *MLC* expression and prevents the formation of the three anterior-most somites (shown in top panel and indicated by red lines in the schematic bottom panel), suggesting that changes in FGF signalling were instrumental for the evolution of the vertebrate head. AP2, activating protein 2; Bmp, bone morphogenetic protein; Dkk, dickkopf; Dlx, distal-less homeobox; Evx, even-skipped; FoxD3, forkhead box D3; Msx, Msh-like; Pax, paired-box; Sox, SRY box containing; sFRP2, secreted frizzled-related protein 2; Zic, zinc-finger protein of the cerebellum.

been shown that the interaction between Wnt and RA signals, as well as the opposition between FGF and RA signals for the posterior elongation of the body, were probably also acquired specifically in vertebrates (Bertrand et al., 2011b; Onai et al., 2009; Petersen and Reddien, 2009). Thus, the evolution of the AP axis in vertebrates combined the evolution of different signalling cascades with the apparition of migratory NCCs.

Evolution of vertebrate structures

Although amphioxus share typical chordate morphological traits with vertebrates (Fig. 2), they lack some vertebrate characteristics, such as migratory neural crest cells and their derivatives, placodes, a mineralized skeleton, and paired appendages. Thus, one of the major issues in this area of research concerns how these vertebrate morphological innovations appeared during evolution from an amphioxus-like chordate ancestor.

The head

One of the most important structures that appeared specifically in vertebrates is the head. The vertebrate head contains many vertebrate-specific tissues or organs, including NCC derivatives (head cartilage, bones, muscles and nerves), placodes and paired sensory organs. Studies in the early 1980s proposed that at least part of the vertebrate head is a neomorphic unit that probably appeared through the evolution of migratory NCCs and placodes (Gans and Northcutt, 1983; Northcutt and Gans, 1983). Although ectoderm derivatives (i.e. placodes and NCCs) probably played a crucial role in the evolution of the vertebrate head, recent studies using amphioxus have shed light on the notable importance of the mesoderm. It is widely accepted that the paraxial mesoderm in the chordate ancestor was segmented from its most anterior to its most posterior part, as is observed in extant amphioxus (reviewed by Sambasivan et al., 2011) in which epithelial somites form during

development (Holland et al., 2008b). As the most anterior paraxial mesoderm of vertebrates is not segmented, and as it does not form somites, it has been hypothesized that vertebrates lost the anterior segmentation that was present in their ancestors (Holland et al., 2008b). This morphological loss was probably crucial for the appearance of the vertebrate head, as it may have relaxed the developmental constraints usually imposed by the somites, thus allowing NCCs and placodes to evolve new specific structures. A recent study of FGF signalling in amphioxus has shown that inhibition of this pathway before gastrulation causes specific loss of the most anterior somites (Bertrand et al., 2011b) (Fig. 5C,D). This induced somite-loss phenotype produces an amphioxus larva with an anterior non-segmented 'vertebrate-like' region. These data strongly suggest that a slight modification of FGF signalling during the early embryonic development of the vertebrate ancestor could have been responsible for the loss of the anterior segments in the vertebrate lineage, which may have been instrumental for the appearance of the vertebrate 'new head'.

Migratory neural crest cells

Migratory cells generated in the neural tube have been found outside the vertebrate lineage (e.g. in urochordates). These cells migrate from the neural plate border and differentiate into pigment cells, but their homology to vertebrate NCCs is still debated (Baker, 2008; Jeffery, 2006; Jeffery et al., 2008; Jeffery et al., 2004). It is clear, however, that amphioxus do not possess such cells. Recent efforts have aimed to determine whether the gene regulatory network (GRN) implicated in neural crest formation in vertebrates (NC-GRN) is conserved in amphioxus. In vertebrates, a three-level GRN has been proposed for neural crest formation. The first level includes 'border induction' signals, the second includes 'neural border specifiers' and the third level includes 'neural crest specifiers' (reviewed by Sauka-Spengler and Bronner-Fraser, 2008). A recent study has shown that many genes orthologous to those expressed in the neural plate border of vertebrates, including both border inducers and specifiers, are also expressed in the homologous region of the amphioxus embryo (Fig. 5B) (Yu et al., 2008). However, among the neural crest specifiers (i.e. the third level of signals), only *Snail* is transiently expressed in the neural plate border of amphioxus, whereas orthologues of other vertebrate neural crest specifier genes are not expressed in this region (Yu et al., 2008). Thus, of the vertebrate NC-GRN, only the first two levels seem to be conserved in amphioxus. It was thus proposed that existing genes were specifically recruited (i.e. co-opted) in vertebrates to be under the control of the first two ancestral levels of the NC-GRN, thus providing the cells present at the neural plate border with the ability to migrate. One of these neural crest specifier genes in vertebrates is forkhead box D3 (*Foxd3*). In amphioxus, only one *FoxD* gene is present in the genome (the orthologue of vertebrate *Foxd1*, *Foxd2*, *Foxd3*, *Foxd4* and *Foxd5*). The expression pattern of *FoxD* in the amphioxus embryo recapitulates that of the vertebrate paralogues *Foxd1*, *Foxd2*, *Foxd4* and *Foxd5*, but, in contrast to vertebrate *Foxd3*, amphioxus *FoxD* is not expressed in the amphioxus neural plate border (Yu et al., 2004; Yu et al., 2008). It was suggested, therefore, that, following the genome duplications at the base of the vertebrate lineage that gave rise to the five FoxD paralogues, *Foxd3* acquired a new regulatory element in its promoter region, allowing its expression in NCCs. This hypothesis is further supported by the fact that regulatory elements in the promoter region of amphioxus *FoxD* are not able to drive expression in the neural plate border or NCC in chick (Yu et al., 2008).

Placodes

The placodes are thickenings of the embryonic non-neural ectoderm that develop into several sensory and non-sensory tissues or organs (Schlosser, 2005). The non-neurogenic placodes of vertebrates give rise to hair, lens, teeth or the adenohypophysis, whereas the neurogenic placodes give rise to several sensory structures, such as the lateral line, the olfactory epithelium, some ganglia and the inner ear. Many attempts have been made to elucidate the evolution of such structures in the chordate phylum through studies of both urochordates and cephalochordates. Comparative morphological studies have, until now, failed to show whether distinct invertebrate-chordate structures are real homologues of the vertebrate placodes. However, gene expression data show that genes of the paired-box (*Pax*), sine oculis homeobox (Six) and eyes absent (Eya) families, which are known to be major players in the formation of vertebrate placodes, are also co-expressed in some non-neural ectodermal regions of urochordates and cephalochordates that usually develop into sensory cells (Schlosser, 2005). In amphioxus, functional protein-protein interactions and protein/DNA interactions were also tested for members of the Pax-Six-Eya-Dach (dachsund) network and have been shown to be relatively similar to those described in vertebrates (Kozmik et al., 2007). Moreover, other orthologues of genes expressed in the vertebrate placodes, such as paired-like homeodomain transcription factor (*Pitx*), sex-determining region Y-box B1c (*SoxB1c*), *Col/Olf-1/EBF* (*Coe*) or brain 3 (*Brn3*; *Pou4f1* – Mouse Genome Informatics), are also expressed in amphioxus epidermal sensory cells (Meulemans and Bronner-Fraser, 2007a). Again, these data suggest that a pre-existing network, which is implicated in sensory epidermal cell formation in the ancestor of chordates, was recruited for placode formation during evolution.

Bones

Bones, which are made of cartilage and mineralized skeleton, are also a vertebrate innovation. Adult amphioxus possess some acellular cartilage that contains fibrillar collagens, as well as elastin-like fibres in the gill bars (see Glossary, Box 1) and in the skeletal rods of the cirri (see Glossary, Box 1) (Wright et al., 2001). The expression pattern of genes known to be implicated in the control of vertebrate cartilage formation has been studied during amphioxus embryonic development (Meulemans and Bronner-Fraser, 2007b). Co-expression of these genes, which would have been the sign of regulatory interactions among them, was not found. This led the authors to propose that there was no pre-existing regulatory network co-opted in vertebrates for cartilage formation (Meulemans and Bronner-Fraser, 2007b). However, the acellular cartilages of amphioxus form late in development, and are absent from the larva. This could explain why no embryonic structure co-expresses cartilage genes in amphioxus. Recently, a study using regeneration of the oral cirri after amputation as a model for cartilage genesis in amphioxus, has shown that several orthologous genes functioning in cartilage and mineralized bone formation [i.e. secreted protein, acidic, rich in cysteines (SPARC/osteonectin), SoxE, Runt-related transcription factor (Runx) and genes coding for fibrillar collagens] are co-expressed during the regeneration of skeleton rods (Kaneto and Wada, 2011). This suggests that a gene regulatory unit implicated in acellular cartilage formation might indeed have existed in the chordate ancestor and was thereafter recruited in the vertebrate lineage for both cartilage and bone formation. This recruitment was probably associated with some specific genetic events: the Scpp (secretory calcium-binding phosphoprotein) genes, which are crucial for

mineralization, appeared by tandem duplications of the SPARC-like 1 (*Sparcl1*) gene in vertebrates (Kawasaki et al., 2004; Kawasaki and Weiss, 2006), whereas the gene coding for aggrecan, a major component of the cartilage matrix in vertebrates, appeared by domain shuffling in the ancestor of vertebrates (Kawashima et al., 2009).

Paired appendages

In gnathostomes, the T-box genes, *T-box4* (*Tbx4*) and *T-box5* (*Tbx5*), are required to initiate limb outgrowth (Logan, 2003). In amphioxus, a unique *Tbx4/5* gene is present, and it is expressed in the ventral mesoderm of larva (Minguillon et al., 2009). Interestingly, transgenesis experiments showed that the amphioxus *Tbx4/5* gene is able to induce limb outgrowth in mice when expressed at the right time and place (Minguillon et al., 2009). These results suggest that the evolution of limb formation was not due to a modification of the biochemical function of Tbx proteins. The authors also tested the ability of the genomic region surrounding amphioxus *Tbx4/5* to drive gene expression in the lateral plate mesoderm of the mouse embryo, which is the region in which *Tbx4* and *Tbx5* are naturally expressed. But the amphioxus *Tbx4/5* gene regulatory elements were not able to induce the correct spatiotemporal expression pattern of *Tbx4/5*, suggesting that the evolution of *Tbx4* and *Tbx5* function in vertebrate limb outgrowth was associated with the acquisition of new regulatory elements and not new properties of the protein (Minguillon et al., 2009).

Evolution of the immune system

Understanding the evolution from innate to vertebrate-specific adaptive immunity is a major issue in immunology. Over the past few years, several studies using amphioxus have shed light on this complex evolutionary history.

A genomic survey of the immune gene repertory in amphioxus has shown that it shares an overall conserved framework for the innate immune system with vertebrates (Huang et al., 2008). However, some gene families [e.g. Toll-like receptor, nucleotide oligomerization domain/NALP (NACHT-LRR-PYD protein)-like receptor and scavenger receptor] have been expanded in amphioxus and in the sea urchin, and some families show further enlargement in amphioxus [e.g. C-type lectin-containing proteins; leucine-rich repeat (LRR)- and IGcam-containing proteins; LRR-only, C1q-like, ficolin-like and complement-related genes] (Huang et al., 2008; Zhang et al., 2008). These gene expansions suggest that, instead of adaptive immunity, invertebrate deuterostomes were adapted to use innate diversity at the population level. Moreover, the presence in amphioxus of a new family of genes containing immunoglobulin variable regions [variable region containing chitin-binding proteins (VCBPs)] has been described (Cannon et al., 2002). VCBPs show structural characteristics of innate immune receptors and a high degree of polymorphism in the germline (Cannon et al., 2004), suggesting that increased innate diversity was the prevalent immunological strategy in the chordate ancestors. Altogether, these results suggest that innate diversity was reduced in vertebrates concurrently with the rise of somatic diversity of the adaptive immune system. But how did this adaptive immune system arise? Lymphocyte-like cells have recently been reported to be present in amphioxus (Huang et al., 2007). In response to microbial challenge, these cells change their morphology and overexpress some orthologues of vertebrate genes implicated in lymphocyte function. The putative presence of lymphocyte-like cells and of a complex set of immune genes in amphioxus suggests that the evolution of the vertebrate adaptive immune system was due to the appearance of only a few genes, and particularly to the emergence of recombination-activating gene (RAG)-mediated immunity.

Evolution of metamorphosis

All metazoan phyla contain members that follow a late developmental process called metamorphosis, in which morphological, physiological and ecological changes occur between the larval and juvenile stages. The wide diversity of this process in different lineages has led to the proposal that metamorphosis evolved several times independently. The demonstration that thyroid hormone (TH) controls this process in amphioxus, as it does in vertebrates, supports the hypothesis that metamorphosis controlled by such a hormone is a synapomorphy of chordates (Paris et al., 2008; Paris et al., 2010). Besides this, it has been shown that the active TH in amphioxus is slightly different from that found in vertebrates. Indeed, although the active vertebrate hormone is T3 (3,3′,5-triiodo-L-thyronine), the active hormone in amphioxus is TRIAC (a T3 deaminated derivative, 3,3′,5-triiodothyroacetic acid) (Paris et al., 2008; Paris et al., 2010). This discovery provides evidence for a new in vivo ligand of the thyroid hormone receptor, but also raises new questions concerning the evolution of thyroid hormone signalling, which is probably linked to the evolution of thyroid hormone-producing glands (the endostyle in amphioxus and the thyroid gland in vertebrates).

Limitations and future directions

The main drawbacks for the use of amphioxus as a model system are the limited technical approaches that it is amenable to. Although a number of techniques in molecular and developmental biology, including methods for genetic manipulation, have been developed in recent years, these approaches are still underexploited. Thus, the development of improved aquaculture techniques that allow work with amphioxus in both marine stations and inland laboratories is crucial (Theodosiou et al., 2011). The development of methods for keeping multiple species of amphioxus in captivity for a whole life cycle (Zhang et al., 2007) will also be extremely important. The current in vivo approaches rely on embryo availability (i.e. the spawning season); thus, the elaboration of methods to obtain ripe animals in captivity in periods outside the spawning season, in order to obtain embryos all year round, will be essential.

The small number of amphioxus species, most of which stem from the *Branchiostoma* genus, and the high degree of morphological and genetic conservation among them (Somorjai et al., 2008a), is an important factor for comparative studies, as the experimental results obtained in a model species can easily be extrapolated to the others. However, this is also a limiting factor, as diversity is very important for establishing conserved versus divergent issues. Thus, the development of breeding techniques and of studies for the closest genus *Asymmetron* will be of great importance in the immediate future.

Publication of the first complete amphioxus genome sequence (Putnam et al., 2008) has been of great importance for comparative genomic studies. However, the low coverage and defective annotation of this genome hampers its use for the study of the evolution of genome structure in chordates. New amphioxus genome sequences will certainly be of great importance for comparative genomics at the inter- and intra-species levels, but also for evolutionary developmental biology studies. Genome sequencing projects for both *B. lanceolatum* and *B. belcheri* have begun, and are likely to provide valuable resources for the community.

In summary, given the key phylogenetic position of amphioxus, an exponential increase in scientific publications using this model has been observed since 2008. The future development of technical approaches, of new model species and of access to new complete genome sequences will be instrumental in understanding how the evolution of developmental mechanisms produced the vertebrate body plan.

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References

Amemiya, C. T., Prohaska, S. J., Hill-Force, A., Cook, A., Wasserscheid, J., Ferrier, D. E., Pascual-Anaya, J., Garcia-Fernandez, J., Dewar, K. and Stadler, P. F. (2008). The amphioxus Hox cluster: characterization, comparative genomics, and evolution. *J. Exp. Zool. B Mol. Dev. Evol.* **310**, 465-477.
Baker, C. V. (2008). The evolution and elaboration of vertebrate neural crest cells. *Curr. Opin. Genet. Dev.* **18**, 536-543.
Bertrand, S., Belgacem, M. R. and Escriva, H. (2011a). Nuclear hormone receptors in chordates. *Mol. Cell Endocrinol.* **334**, 67-75.
Bertrand, S., Camasses, A., Somorjai, I., Belgacem, M. R., Chabrol, O., Escande, M. L., Pontarotti, P. and Escriva, H. (2011b). Amphioxus FGF signaling predicts the acquisition of vertebrate morphological traits. *Proc. Natl. Acad. Sci. USA* **108**, 9160-9165.
Bourlat, S. J., Juliusdottir, T., Lowe, C. J., Freeman, R., Aronowicz, J., Kirschner, M., Lander, E. S., Thorndyke, M., Nakano, H., Kohn, A. B. et al. (2006). Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida. *Nature* **444**, 85-88.
Cannon, J. P., Haire, R. N. and Litman, G. W. (2002). Identification of diversified genes that contain immunoglobulin-like variable regions in a protochordate. *Nat. Immunol.* **3**, 1200-1207.
Cannon, J. P., Haire, R. N., Schnitker, N., Mueller, M. G. and Litman, G. W. (2004). Individual protochordates have unique immune-type receptor repertoires. *Curr. Biol.* **14**, R465-R466.
Chen, Y., Shin, P. K. S. and Cheung, S. G. (2008). Growth, secondary production and gonad development of two co-existing amphioxus species (Branchiostoma belcheri and B. malayanum) in subtropical Hong Kong. *J. Exp. Marine Biol. Ecol.* **357**, 64-74.
Chin, T. G. (1941). Studies on the biology of the Amoy amphioxus Branchiostoma belcheri Gray. *Philipp. J. Sci.* **75**, 369-421.
Costa, O. G. (1834). Cenni zoologici, ossia descrizione somaria delle specie nuove di animali discoperti in diverse contrade del regno nell'anno 1834. In *Annuario Zoologico*, Vol. 12, pp. 49-50. Napoli, Italy: Tipografia di Azzolino e Comp.
Courtney, W. A. M. (1975). The temperature relationships and age-structure of North sea and Mediterranean populations of Branchiostoma lanceolatum. *Symp. Zool. Soc. Lond.* **36**, 213-233.
D'Aniello, S., Irimia, M., Maeso, I., Pascual-Anaya, J., Jimenez-Delgado, S., Bertrand, S. and Garcia-Fernandez, J. (2008). Gene expansion and retention leads to a diverse tyrosine kinase superfamily in Amphioxus. *Mol. Biol. Evol.* **25**, 1841-1854.
da Silva, L. F. B., Tavares, M. and Soares-Gomes, A. (2008). Population structure of the lancelet Branchiostoma caribaeum (Cephalochordata: Branchiostomidae) in the Baía de Guanabara, Rio de Janeiro, southeastern Brazil. *Rev. Bras. Zool.* **25**, 617-623.
De Robertis, E. M., Larrain, J., Oelgeschlager, M. and Wessely, O. (2000). The establishment of Spemann's organizer and patterning of the vertebrate embryo. *Nat. Rev. Genet.* **1**, 171-181.
Dehal, P. and Boore, J. L. (2005). Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biol.* **3**, e314.
Delsuc, F., Brinkmann, H., Chourrout, D. and Philippe, H. (2006). Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* **439**, 965-968.
Desdevises, Y., Maillet, V., Fuentes, M. and Escriva, H. (2011). A snapshot of the population structure of Branchiostoma lanceolatum in the Racou Beach, France, during its spawning season. *PLoS ONE* **6**, e18520.
Escriva, H., Holland, N. D., Gronemeyer, H., Laudet, V. and Holland, L. Z. (2002a). The retinoic acid signaling pathway regulates anterior/posterior patterning in the nerve cord and pharynx of amphioxus, a chordate lacking neural crest. *Development* **129**, 2905-2916.
Escriva, H., Manzon, L., Youson, J. and Laudet, V. (2002b). Analysis of lamprey and hagfish genes reveals a complex history of gene duplications during early vertebrate evolution. *Mol. Biol. Evol.* **19**, 1440-1450.
Friedman, R. and Hughes, A. L. (2001). Pattern and timing of gene duplication in animal genomes. *Genome Res.* **11**, 1842-1847.
Fuentes, M., Schubert, M., Dalfo, D., Candiani, S., Benito, E., Gardenyes, J., Godoy, L., Moret, F., Illas, M., Patten, I. et al. (2004). Preliminary observations on the spawning conditions of the European amphioxus (Branchiostoma lanceolatum) in captivity. *J. Exp. Zool. B Mol. Dev. Evol.* **302**, 384-391.
Fuentes, M., Benito, E., Bertrand, S., Paris, M., Mignardot, A., Godoy, L., Jimenez-Delgado, S., Oliveri, D., Candiani, S., Hirsinger, E. et al. (2007). Insights into spawning behavior and development of the european amphioxus (Branchiostoma lanceolatum). *J. Exp. Zool. B Mol. Dev. Evol.* **308**, 484-493.
Futch, C. R. and Dwinell, S. E. (1977). Nearshore marine ecology at Hutchinson Island, Florida: 1971-1974: IV. lancelets and fishes. *Fla. Mer. Res. Publ.* **24**, 1-23.
Gans, C. and Northcutt, R. G. (1983). Neural crest and the origin of vertebrates: a new head. *Science* **220**, 268-274.
Garcia-Fernandez, J. and Holland, P. W. H. (1994). Archetypal organization of the amphioxus Hox gene cluster. *Nature* **370**, 563-566.
Gerhart, J. (2001). Evolution of the organizer and the chordate body plan. *Int. J. Dev. Biol.* **45**, 133-153.
Gosselck, F. (1975). The distribution of Branchiostoma senegalense (Acrania, Branchiostomidae) in the off-shore shelf region off North West Africa. *Int. Revue Ges. Hydrobiol.* **60**, 199-207.
Gosselck, F. and Spittler, P. (1979). Age structure, growth and weight of Branchiostoma senegalense (Acrania, Branchiostomidae) off North-West Africa. *Int. Revue Ges. Hydrobiol.* **64**, 541-550.
Gray, J. E. (1847). Description of a new species of Amphioxus from Borneo. *Proc. Zool. Soc. Lond.* **15**, 35-36.
Hirakow, R. and Kajita, N. (1990). An electron microscopic study of the development of amphioxus, Branchiostoma belcheri tsingtauense: Cleavage. *J. Morphol.* **203**, 331-344.
Holland, L. Z. and Holland, N. D. (1996). Expression of AmphiHox-1 and AmphiPax-1 in amphioxus embryos treated with retinoic acid: insights into evolution and patterning of the chordate nerve cord and pharynx. *Development* **122**, 1829-1838.
Holland, L. Z. and Yu, J. K. (2004). Cephalochordate (amphioxus) embryos: procurement, culture, and basic methods. *Methods Cell Biol.* **74**, 195-215.
Holland, L. Z., Panfilio, K. A., Chastain, R., Schubert, M. and Holland, N. D. (2005). Nuclear beta-catenin promotes non-neural ectoderm and posterior cell fates in amphioxus embryos. *Dev. Dyn.* **233**, 1430-1443.
Holland, L. Z., Albalat, R., Azumi, K., Benito-Gutierrez, E., Blow, M. J., Bronner-Fraser, M., Brunet, F., Butts, T., Candiani, S., Dishaw, L. J. et al. (2008a). The amphioxus genome illuminates vertebrate origins and cephalochordate biology. *Genome Res.* **18**, 1100-1111.
Holland, L. Z., Holland, N. D. and Gilland, E. (2008b). Amphioxus and the evolution of head segmentation. *Int. Comp. Biol.* **48**, 630-646.
Holland, N. D. (2011). Spawning periodicity of the lancelet, Asymmetron lucayanum (Cephalochordata), in Bimini, Bahamas. *Ital. J. Zool.* (in press).
Holland, N. D. and Holland, L. Z. (1989). Fine structural study of the cortical reaction and formation of the egg coats in a lancelet (=Amphioxus), Branchiostoma floridae (Phylum Chordata: Subphylum Cephalochordata=Acrania). *Biol. Bull.* **176**, 111-122.
Holland, N. D. and Chen, J. (2001). Origin and early evolution of the vertebrates: new insights from advances in molecular biology, anatomy, and palaeontology. *BioEssays* **23**, 142-151.
Holland, N. D. and Holland, L. Z. (2010). Laboratory spawning and development of the Bahama lancelet, Asymmetron lucayanum (cephalochordata): fertilization through feeding larvae. *Biol. Bull.* **219**, 132-141.
Holland, P. W., Holland, L. Z., Williams, N. A. and Holland, N. D. (1992). An amphioxus homeobox gene: sequence conservation, spatial expression during development and insights into vertebrate evolution. *Development* **116**, 653-661.
Huang, G., Xie, X., Han, Y., Fan, L., Chen, J., Mou, C., Guo, L., Liu, H., Zhang, Q., Chen, S. et al. (2007). The identification of lymphocyte-like cells and lymphoid-related genes in amphioxus indicates the twilight for the emergence of adaptive immune system. *PLoS ONE* **2**, e206.
Huang, S., Yuan, S., Guo, L., Yu, Y., Li, J., Wu, T., Liu, T., Yang, M., Wu, K., Liu, H. et al. (2008). Genomic analysis of the immune gene repertoire of amphioxus reveals extraordinary innate complexity and diversity. *Genome Res.* **18**, 1112-1126.
Hufton, A. L., Mathia, S., Braun, H., Georgi, U., Lehrach, H., Vingron, M., Poustka, A. J. and Panopoulou, G. (2009). Deeply conserved chordate

noncoding sequences preserve genome synteny but do not drive gene duplicate retention. *Genome Res.* **19**, 2036-2051.
Jeffery, W. R. (2006). Ascidian neural crest-like cells: phylogenetic distribution, relationship to larval complexity, and pigment cell fate. *J. Exp. Zool. B Mol. Dev. Evol.* **306**, 470-480.
Jeffery, W. R., Strickler, A. G. and Yamamoto, Y. (2004). Migratory neural crest-like cells form body pigmentation in a urochordate embryo. *Nature* **431**, 696-699.
Jeffery, W. R., Chiba, T., Krajka, F. R., Deyts, C., Satoh, N. and Joly, J. S. (2008). Trunk lateral cells are neural crest-like cells in the ascidian Ciona intestinalis: insights into the ancestry and evolution of the neural crest. *Dev. Biol.* **324**, 152-160.
Kaneto, S. and Wada, H. (2011). Regeneration of amphioxus oral cirri and its skeletal rods: implications for the origin of the vertebrate skeleton. *J. Exp. Zool. B Mol. Dev. Evol.* **316**, 409-417.
Kawasaki, K. and Weiss, K. M. (2006). Evolutionary genetics of vertebrate tissue mineralization: the origin and evolution of the secretory calcium-binding phosphoprotein family. *J. Exp. Zool. B Mol. Dev. Evol.* **306**, 295-316.
Kawasaki, K., Suzuki, T. and Weiss, K. M. (2004). Genetic basis for the evolution of vertebrate mineralized tissue. *Proc. Natl. Acad. Sci. USA* **101**, 11356-11361.
Kawashima, T., Kawashima, S., Tanaka, C., Murai, M., Yoneda, M., Putnam, N. H., Rokhsar, D. S., Kanehisa, M., Satoh, N. and Wada, H. (2009). Domain shuffling and the evolution of vertebrates. *Genome Res.* **19**, 1393-1403.
Kmita, M. and Duboule, D. (2003). Organizing axes in time and space; 25 years of colinear tinkering. *Science* **301**, 331-333.
Kon, T., Nohara, M., Yamanoue, Y., Fujiwara, Y., Nishida, M. and Nishikawa, T. (2007). Phylogenetic position of a whale-fall lancelet (Cephalochordata) inferred from whole mitochondrial genome sequences. *BMC Evol. Biol.* **7**, 127.
Kowalevsky, A. (1867). Entwickelungsgeschichte des Amphioxus lanceolatus. *Mem. Acad. Imp. Sci. St-Petersb. (Ser. VII)* **11**, 1-17.
Kozmik, Z., Holland, N. D., Kreslova, J., Oliveri, D., Schubert, M., Jonasova, K., Holland, L. Z., Pestarino, M., Benes, V. and Candiani, S. (2007). Pax-Six-Eya-Dach network during amphioxus development: conservation in vitro but context specificity in vivo. *Dev. Biol.* **306**, 143-159.
Kozmikova, I., Smolikova, J., Vlcek, C. and Kozmik, Z. (2011). Conservation and diversification of an ancestral chordate gene regulatory network for dorsoventral patterning. *PLoS ONE* **6**, e14650.
Kuraku, S., Meyer, A. and Kuratani, S. (2009). Timing of genome duplications relative to the origin of the vertebrates: did cyclostomes diverge before or after? *Mol. Biol. Evol.* **26**, 47-59.
Logan, M. (2003). Finger or toe: the molecular basis of limb identity. *Development* **130**, 6401-6410.
Manzanares, M., Wada, H., Itasaki, N., Trainor, P. A., Krumlauf, R. and Holland, P. W. (2000). Conservation and elaboration of Hox gene regulation during evolution of the vertebrate head. *Nature* **408**, 854-857.
Meulemans, D. and Bronner-Fraser, M. (2007a). The amphioxus SoxB family: implications for the evolution of vertebrate placodes. *Int. J. Biol. Sci.* **3**, 356-364.
Meulemans, D. and Bronner-Fraser, M. (2007b). Insights from amphioxus into the evolution of vertebrate cartilage. *PLoS ONE* **2**, e787.
Minguillon, C., Gibson-Brown, J. J. and Logan, M. P. (2009). Tbx4/5 gene duplication and the origin of vertebrate paired appendages. *Proc. Natl. Acad. Sci. USA* **106**, 21726-21730.
Northcutt, R. G. and Gans, C. (1983). The genesis of neural crest and epidermal placodes: a reinterpretation of vertebrate origins. *Q. Rev. Biol.* **58**, 1-28.
Ohno, S. (1970). *Evolution by Gene Duplication*. Heidelberg, Germany: Springer-Verlag.
Onai, T., Lin, H. C., Schubert, M., Koop, D., Osborne, P. W., Alvarez, S., Alvarez, R., Holland, N. D. and Holland, L. Z. (2009). Retinoic acid and Wnt/beta-catenin have complementary roles in anterior/posterior patterning embryos of the basal chordate amphioxus. *Dev. Biol.* **332**, 223-233.
Onai, T., Yu, J. K., Blitz, I. L., Cho, K. W. and Holland, L. Z. (2010). Opposing Nodal/Vg1 and BMP signals mediate axial patterning in embryos of the basal chordate amphioxus. *Dev. Biol.* **344**, 377-389.
Pallas, P. S. (1774). Limax lanceolatus. p. 19 + Plate I. *In* Spicilegia zoologica, Vol. I (Quadrupedium, avium, amphibiorum, piscium, insectorum, molluscorum, aliorumque marinorum). Fascicle 10 (quibus novae imprimus et obscurae animalium species iconibus, descriptionibus atque comentariis illustrantur cura P. S. Pallas). Berlin, Germany: G. A. Lange.
Paris, M., Escriva, H., Schubert, M., Brunet, F., Brtko, J., Ciesielski, F., Roecklin, D., Vivat-Hannah, V., Jamin, E. L., Cravedi, J. P. et al. (2008). Amphioxus postembryonic development reveals the homology of chordate metamorphosis. *Curr. Biol.* **18**, 825-830.
Paris, M., Hillenweck, A., Bertrand, S., Delous, G., Escriva, H., Zalko, D., Cravedi, J. P. and Laudet, V. (2010). Active metabolism of thyroid hormone during metamorphosis of amphioxus. *Int. Comp. Biol.* **50**, 63-74.
Petersen, C. P. and Reddien, P. W. (2009). Wnt signaling and the polarity of the primary body axis. *Cell* **139**, 1056-1068.
Poss, S. G. and Boschung, H. T. (1996). Lancelets (Cephalochordata: Branchiostomatidae): how many species are valid? *Israel J. Zool.* **42 Suppl.**, 13-66.
Putnam, N. H., Butts, T., Ferrier, D. E., Furlong, R. F., Hellsten, U., Kawashima, T., Robinson-Rechavi, M., Shoguchi, E., Terry, A., Yu, J. K. et al. (2008). The amphioxus genome and the evolution of the chordate karyotype. *Nature* **453**, 1064-1071.
Sambasivan, R., Kuratani, S. and Tajbakhsh, S. (2011). An eye on the head: the development and evolution of craniofacial muscles. *Development* **138**, 2401-2415.
Sauka-Spengler, T. and Bronner-Fraser, M. (2008). A gene regulatory network orchestrates neural crest formation. *Nat. Rev. Mol. Cell Biol.* **9**, 557-568.
Schaeffer, B. (1987). Deuterostome monophyly and phylogeny. *Evol. Biol.* **21**, 179-235.
Schlosser, G. (2005). Evolutionary origins of vertebrate placodes: insights from developmental studies and from comparisons with other deuterostomes. *J. Exp. Zool. B Mol. Dev. Evol.* **304B**, 347-399.
Schubert, M., Holland, N. D., Escriva, H., Holland, L. Z. and Laudet, V. (2004). Retinoic acid influences anteroposterior positioning of epidermal sensory neurons and their gene expression in a developing chordate (amphioxus). *Proc. Natl. Acad. Sci. USA* **101**, 10320-10325.
Schubert, M., Yu, J. K., Holland, N. D., Escriva, H., Laudet, V. and Holland, L. Z. (2005). Retinoic acid signaling acts via Hox1 to establish the posterior limit of the pharynx in the chordate amphioxus. *Development* **1**, 61-73.
Schubert, M., Escriva, H., Xavier-Neto, J. and Laudet, V. (2006). Amphioxus and tunicates as evolutionary model systems. *Trends Ecol. Evol.* **21**, 269-277.
Skrabanek, L. and Wolfe, K. H. (1998). Eukaryote genome duplication-where's the evidence? *Curr. Opin. Genet. Dev.* **8**, 694-700.
Somorjai, I., Bertrand, S., Camasses, A., Haguenauer, A. and Escriva, H. (2008a). Evidence for stasis and not genetic piracy in developmental expression patterns of Branchiostoma lanceolatum and Branchiostoma floridae, two amphioxus species that have evolved independently over the course of 200 Myr. *Dev. Genes Evol.* **218**, 703-713.
Somorjai, I. M. L., Camasses, A., Rivière, B. and Escrivà, H. (2008b). Development of a semi-closed aquaculture system for monitoring of individual amphioxus (Branchiostoma lanceolatum), with high survivorship. *Aquaculture* **281**, 145-150.
Stokes, M. D. (1996). Larval settlement, post-settlement growth and secondary production of the Florida lancelet (=amphioxus) Branchiostoma floridae. *Mar. Ecol. Prog. Ser.* **130**, 71-84.
Stokes, M. D. and Holland, N. D. (1996). Reproduction of the Florida lancelet (Branchiostoma floridae): spawning patterns and fluctuations in gonad indexes and nutritional reserves. *Invert. Biol.* **115**, 349-359.
Takatori, N., Butts, T., Candiani, S., Pestarino, M., Ferrier, D. E., Saiga, H. and Holland, P. W. (2008). Comprehensive survey and classification of homeobox genes in the genome of amphioxus, Branchiostoma floridae. *Dev. Genes Evol.* **218**, 579-590.
Terazawa, K. and Satoh, N. (1997). Formation of the chordamesoderm in the amphioxus embryo: Analysis with Brachyury and fork head/HNF-3 genes. *Dev. Genes Evol.* **207**, 1-11.
Theodosiou, M., Colin, A., Schulz, J., Laudet, V., Peyrieras, N., Nicolas, J. F., Schubert, M. and Hirsinger, E. (2011). Amphioxus spawning behavior in an artificial seawater facility. *J. Exp. Zool. B Mol. Dev. Evol.* **316**, 263-275.
Tung, T. C., Wu, S. C. and Tung, Y. F. (1958). The development of isolated blastomeres of Amphioxus. *Sci. Sin.* **7**, 1280-1320.
Tung, T. C., Wu, S. C. and Tung, Y. Y. F. (1962a). Experimental studies on the neural induction in amphioxus. *Sci. Sin.* **11**, 805-820.
Tung, T. C., Wu, S. C. and Tung, Y. Y. F. (1962b). The presumptive areas of the egg of amphioxus. *Sci. Sin.* **11**, 629-644.
Wada, H., Garcia-Fernandez, J. and Holland, P. W. (1999). Colinear and segmental expression of amphioxus Hox genes. *Dev. Biol.* **213**, 131-141.
Webb, J. E. (1956a). A note on the lancelets of Singapore, with a description of a new species of Branchiostoma. *Proc. Zool. Soc. Lond.* **127**, 119-123.
Webb, J. E. (1956b). On the populations of Branchiostoma lanceolatum and their relations with the West African lancelets. *Proc. Zool. Soc. Lond.* **127**, 125-140.
Webb, J. E. (1958). The ecology of Lagos lagoon III. The life history of Branchiostoma nigeriense Webb. *Philos. Trans. Royal Soc. Lond. Ser. B Biol. Sci.* **241**, 335-353.
Webb, J. E. (1971). Seasonal changes in the distribution of Branchiostoma lanceolatum (Pallas) at Helgoland. *Vie et Milieu* **22**, 827-839.
Webb, J. E. and Hill, M. B. (1958a). The ecology of Lagos lagoon. IV. On the reactions of Branchiostoma nigeriense Webb to its environment. *Philos. Trans. Royal Soc. Lond. Ser. B Biol. Sci.* **241**, 355-391.
Webb, J. E. and Hill, M. B. (1958b). The ecology of Lagos Lagoon. V. Some physical properties of lagoon deposits. *Philos. Trans. Royal Soc. Lond. Ser. B* **241**, 393-419.
Winchell, C. J., Sullivan, J., Cameron, C. B., Swalla, B. J. and Mallatt, J. (2002). Evaluating hypotheses of deuterostome phylogeny and chordate evolution with new LSU and SSU ribosomal DNA data. *Mol. Biol. Evol.* **19**, 762-776.
Woolfe, A., Goodson, M., Goode, D. K., Snell, P., McEwen, G. K., Vavouri, T., Smith, S. F., North, P., Callaway, H., Kelly, K. et al. (2005). Highly conserved

non-coding sequences are associated with vertebrate development. *PLoS Biol.* **3**, 116-130.

Wright, G. M., Keeley, F. W. and Robson, P. (2001). The unusual cartilaginous tissues of jawless craniates, cephalochordates and invertebrates. *Cell Tissue Res.* **304**, 165-174.

Wu, X. H., Zhang, S. C., Wang, Y. Y., Zhang, B. L., Qu, Y. M. and Jiang, X. J. (1994). Laboratory observation on spawning, fecundity and larval development of amphioxus (Branchiostoma belcheri tsingtauense). *Chin. J. Oceanol. Limnol.* **12**, 289-294.

Xu, Q. S., Ma, F. and Wang, Y. Q. (2005). Morphological and 12S rRNA gene comparison of two Branchiostoma species in Xiamen waters. *J. Exp. Zool. B Mol. Dev. Evol.* **304**, 259-267.

Yan, S. (1999). Contribution of late professor T. C. Tung to the experimental embryology of Amphioxus. In memory of the 20th anniversary of Professor T. C. Tung's death. *Dev. Growth Differ.* **41**, 503-522.

Yarrell, W. (1836). In *A History of British Fishes*, Vol. II, 1st edn, pp. i-iv + 1-472. London, UK: J. V. Voorst.

Yasui, K., Zhang, S. C., Uemura, M., Aizawa, S. and Ueki, T. (1998). Expression of a twist-related gene, Bbtwist, during the development of a lancelet species and its relation to cephalochordate anterior structures. *Dev. Biol.* **195**, 49-59.

Yasui, K., Urata, M., Yamaguchi, N., Ueda, H. and Henmi, Y. (2007). Laboratory culture of the Oriental lancelet Branchiostoma belcheri. *Zool. Sci.* **24**, 514-520.

Yu, J. K., Holland, N. D. and Holland, L. Z. (2004). Tissue-specific expression of FoxD reporter constructs in amphioxus embryos. *Dev. Biol.* **274**, 452-461.

Yu, J. K., Satou, Y., Holland, N. D., Shin, I. T., Kohara, Y., Satoh, N., Bronner-Fraser, M. and Holland, L. Z. (2007). Axial patterning in cephalochordates and the evolution of the organizer. *Nature* **445**, 613-617.

Yu, J. K., Meulemans, D., McKeown, S. J. and Bronner-Fraser, M. (2008). Insights from the amphioxus genome on the origin of vertebrate neural crest. *Genome Res.* **18**, 1127-1132.

Zhang, Q., Zmasek, C. M., Dishaw, L. J., Mueller, M. G., Ye, Y., Litman, G. W. and Godzik, A. (2008). Novel genes dramatically alter regulatory network topology in amphioxus. *Genome Biol.* **9**, R123.

Zhang, Q. J., Zhong, J., Fang, S. H. and Wang, Y. Q. (2006). Branchiostoma japonicum and B. belcheri are distinct lancelets (Cephalochordata) in Xiamen waters in China. *Zool. Sci.* **23**, 573-579.

Zhang, Q. J., Sun, Y., Zhong, J., Li, G., Lu, X. M. and Wang, Y. Q. (2007). Continuous culture of two lancelets and production of the second filial generations in the laboratory. *J. Exp. Zool. B Mol. Dev. Evol.* **308**, 464-472.