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Review

The chordate amphioxus: an emerging model organism for developmental biology

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Abstract. The cephalochordate amphioxus is the closest living invertebrate relative of the vertebrates. It is vertebrate-like in having a dorsal, hollow nerve cord, notochord, segmental muscles, pharyngeal gill slits and a post-anal tail that develops from a tail bud. However, amphioxus is less complex than vertebrates, lacking neural crest and having little or no mesenchyme. The genetic programs patterning the amphioxus embryo are also similar to those patterning vertebrate embryos, although the amphioxus genome lacks the extensive gene duplications characteristic of vertebrates. This relative structural and genomic simplicity in a vertebrate-like organism makes amphioxus ideal as a model organism for understanding mechanisms of vertebrate development.

Key words. Cephalochordate; retinoic acid; neural crest; genome evolution; pharyngeal patterning; tail bud.

Introduction

To gain insights into human development, developmental biologists have traditionally relied on vertebrate models such as the mouse, chick, frog and zebrafish. However, the discovery that developmental genes are conserved across wide phylogenetic distances has given additional impetus to the use of such invertebrates as the fruit fly *Drosophila* and the nematode *Caenorhabditis*. These invertebrate models have been very useful for elucidating core signaling cascades because, compared with vertebrates, they have small genomes with little gene duplication. For example, the *Drosophila* genome is only 5% the size of the human with only one *Hox* cluster (split into two groups) compared to four in mammals [1–3]. Comparative studies have shown that many core signaling cascades (e.g. intercellular signaling by Notch and Delta, the Pax/Six/Eya network in eye development, short-range signaling by Wnt proteins) are similar in *Drosophila* and vertebrates [4–6]. However, due to independent gain and loss of genes and to the considerable difference in the body plans of flies and humans, there are clear limitations in extrapolating entire developmental gene networks from one organism to the other [7, 12, 14]. Even though the core signaling pathways are evolutionarily conserved, the downstream gene targets of these pathways must necessarily differ considerably between such phylogenetically distant organisms.

The ideal organism to bridge the gap between the *Drosophila* and vertebrate models would be a vertebrate with a relatively unduplicated genome, like that of invertebrates. Unfortunately, even the agnathan fishes, which are basal within the vertebrates, have sizeable genomes (50–70% of that of the human genome) with considerable gene duplication (e.g. three or four *Hox* clusters) [8–11; see also http://www.genomesize.com]. The organisms that come closest to bridging the gap are the cephalochordates and tunicates, which together comprise

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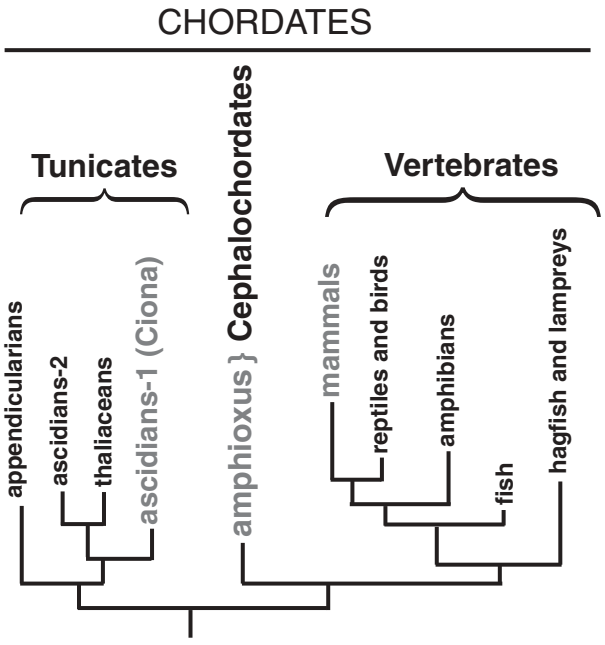

Figure 1. Phylogenetic tree of the chordates. After [206].

the invertebrate chordates (fig. 1) [12]. Both have small, relatively unduplicated genomes and share a number of characteristics with vertebrates (e.g. dorsal hollow nerve cord, perforate pharynx and notochord). However, cephalochordates, also called lancelets and amphioxus, share additional features with vertebrates (e.g. kidneys and segmental paraxial muscles) that are not present in tunicates (figs 2, 3). Moreover, the larger genome of amphioxus (17% that of the human genome) is less divergent from that of the human than the smaller one of tunicates (5% that of the human genome) [13–15]. For example, amphioxus has a single cluster of 14 *Hox* genes (*Hox14* is an independent duplication in amphioxus), whereas tunicates have lost four *Hox* genes (*Hox 7–9* and *11*), and the cluster is split onto five different scaffolds [13, 14]. Consequently, amphioxus is the best available model to use as a proxy for the ancestral vertebrate.

The use of amphioxus as a model for the ancestral vertebrate gains additional support from the fossil record. Although amphioxus and vertebrates split about 520 million years ago, modern amphioxus resembles early vertebrate fossils from the Cambrian such as *Haikouella* [16, 17] (figs 1, 2), indicating that the body plan of amphioxus has not evolved particularly far from that of the common ancestor it shares with vertebrates. However, although amphioxus superficially resembles the larva of a modern lamprey (fig. 3), it lacks some key vertebrate innovations such as neural crest, ectodermal placodes and a cartilaginous or bony endoskeleton. Nonetheless, the absence of these structures can be an advantage in understanding the gene networks essential for initiating neural crest migration, skeletal formation, and the separate contributions of neural crest derivatives and other tissues in the development of vertebrate organs.

The present review discusses the utility of amphioxus as a model organism for understanding the developmental mechanisms that pattern the vertebrate embryo. First, we present examples to demonstrate (i) how under-

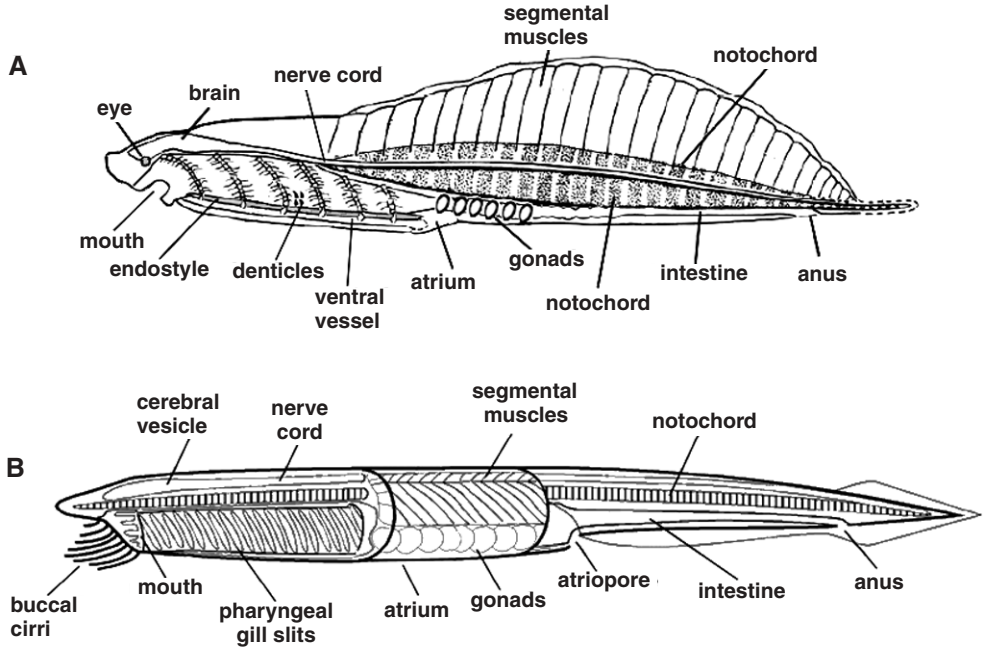

Figure 2. Diagram of the Cambrian chordate fossil *Haikouella* (*A*) and amphioxus (*B*). Although *Haikouella* resembles fossil modern cephalochordates, it has vertebrate characteristics such as paired eyes and pharyngeal denticles. After [14, 86].

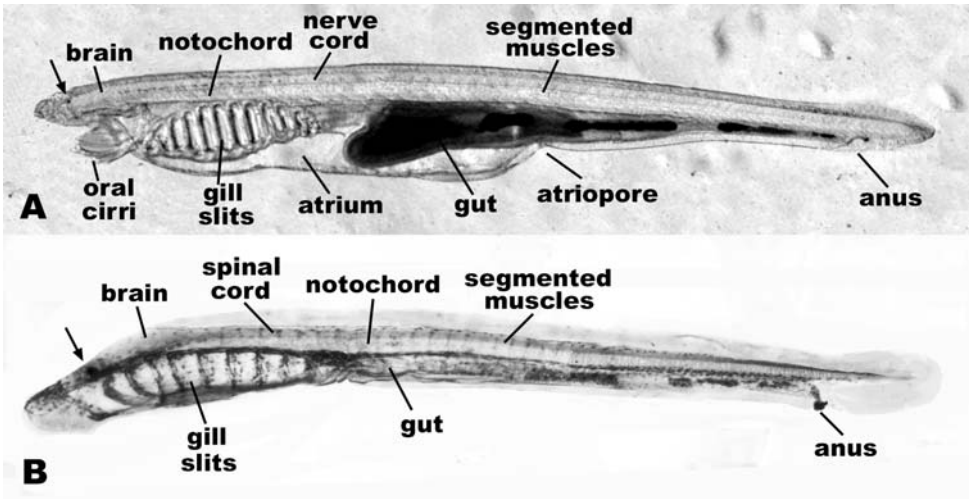

Figure 3. Photomicrographs of (*A*) a living amphioxus juvenile (*Branchiostoma floridae)* and (*B*) a fixed ammocoete larva of *Lampetra japonica*. The juvenile amphioxus is not sexually mature and lacks visible gonads. The arrow in *A* indicates the medial frontal photoreceptor, sometimes called the frontal eye, and in *B* one of the paired eyes of the ammocoete. Both individuals are about 5 mm long.

standing the development of structures in amphioxus can give insights into development of their more complex vertebrate homologs, (ii) how amphioxus can be used to differentiate the role of neural crest from that of other tissues in the development of a particular structure and (iii) how amphioxus can be used to understand the critical steps in development of vertebrate-specific structures such as neural crest. Finally, we discuss the utility of the amphioxus genome in understanding how changes in gene regulation and protein function that occurred subsequent to gene duplication relate to the separate roles of the individual duplicates in vertebrates.

The amphioxus tail bud: insights into the fundamental structure of the vertebrate tail bud

During amphioxus and vertebrate development, the posterior portions of the notochord, somites and neural tube are generated by the tail bud, located at the extreme posterior tip of the embryo [18–23]. Although the larvae of ascidian tunicates also have a tail and the early larval stage is referred to as the tailbud stage, tunicates do not, in fact, have a tail bud. Instead, the tail elongates by rearrangement of preexisting cells [24].

It has been controversial whether development of the vertebrate tail from the tail bud is an entirely separate process from formation of the trunk or is a continuation of gastrulation (reviewed in [25]). In the former view, based chiefly on studies in chick and mouse, the tail bud is a blastema consisting of pluripotent mesenchymal cells [26, 27]. These cells are presumed to be actively proliferating and to contribute to a wide spectrum of tissues. In the contrasting view, based largely on studies of frog development, the tail bud, derived from the blastoporal lip, is not a blastema, but is already partitioned into germ layers, and elongation of the tail is really a continuation of gastrulation [28–30]. Some recent work in the mouse and chick has shown, in contrast with the blastema view, that the precursors of the myotomes and spinal cord, which are a self-renewing population, are regionalized within the tail bud – cells in each location giving rise to specific parts of the myotome and nerve cord [31–34]. Conversely, studies in the frog have shown that small groups of adjacent cells in the tail bud can contribute to somitic muscle, as well as to the notochord and neural tube, suggesting that some tail bud cells may be pluripotent [35]. Clearly, while the majority view appears to be that the vertebrate tail bud contains at least some cell populations that are restricted in their potency, the debate about the organization of vertebrate tail buds is still not settled.

Understanding the organization of the vertebrate tail bud has been complicated because gastrulation differs considerably among the vertebrate classes, at least in part, due to the large differences in the amount of yolk and/or extra-embryonic membranes. In the chick and mouse, but not in the frog, gastrulation continues in the posterior region of the embryo long after the anterior end of the embryo has formed the neural tube. Consequently, there may be real differences among vertebrates in the structure of the tail bud.

Vertebrates are generally thought to have evolved from an amphioxus-like ancestor with relatively small, non-yolky eggs. Thus, the amphioxus gastrula probably approximates that of the ancestral vertebrate, and the amphioxus tail bud probably reflects the fundamental structure of

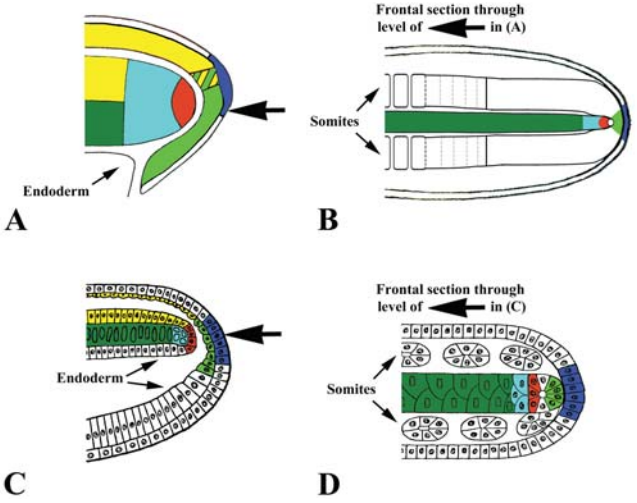

Figure 4. Comparison of *Xenopus* (*A*, *B*) and amphioxus (*C*, *D*) tail buds in medial longitudinal section (*A*, *C*) and in frontal section through the notochord (*B*, *D*) of neurula-stage embryos. Large arrows in *A* and *C* show the level of sections in *B* and *D*. The diagrams are based on both morphology and patterns of gene expression. Yellow, neural tube region; light blue, chordoneural hinge; red, posterior tip of the chordoneural hinge; light green, the posterior wall of the neurenteric canal; dark blue, the posterior tip of the posterior wall; dark green, notochord. Cross-hatching in *A*, overlap of domains. *A* modified from [67]. *C*, *D* modified from [23].

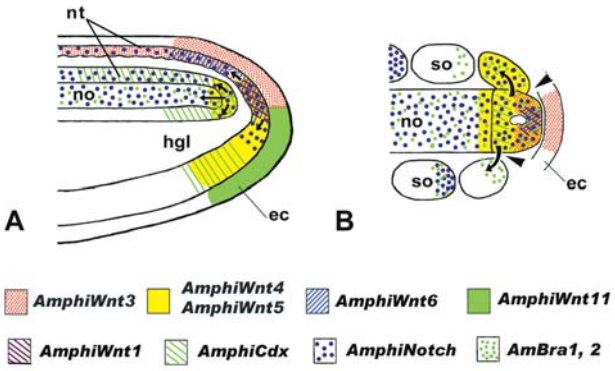

Figure 5. Overlapping domains of gene expression domains define regions within the tailbud of the amphioxus neurula. (*A*) Medial longitudinal section. (*B*) Frontal section through notochord. The posterior ectoderm (ec) expresses *Wnt3* dorsally and *Wnt11* ventrally. The posterior wall of the neurenteric canal (nec) expresses *Wnt1, Wnt4, Wnt5* and *notch*. The chordoneural hinge expresses *caudal*, *Wnt4, Wnt5* and *notch.* The notochord (no) and the posterior portion of nascent somites (no) express *notch* and the two *brachyury* genes (*AmBra 1 and Ambra 2*). Notch, however, is not expressed in the most newly formed somite, while the two *brachyury* genes turn off in the anterior somites. The neural tube (nt) expresses *notch*, and *Wnt3 dorsally* and *caudal* (*cdx*) ventrally. *Caudal* is also expressed in the posterior part of the gut. Arrows show somites budding off from the chordoneural hinge. Hgl, hindgut lumen. After [23, 36, 37, 46, 56–58].

the vertebrate tail bud upon which variations were built. Amphioxus gastrulates by simple invagination. Since there is little involution over the lips of the blastopore, the tissues around the blastopore are posterior during all of gastrulation and are incorporated into the tailbud [36, 38]. At the onset of neurulation, the non-neural ectoderm migrates over the blastopore, and the neuroectoderm becomes continuous posteriorly with the endoderm (figs 4, 5, 8). The blastopore persists as part of the neurenteric canal, and the dorsal lip of the blastopore is incorporated into the chordoneural hinge. As figures 4 and 5 show, the amphioxus tail bud initially consists of discrete epithelia, each with a single layer of cells. At the neurula stage there is no detectable mesenchyme [39]. As in vertebrates, as the amphioxus embryo elongates, the tail bud gives rise to the axial and paraxial mesoderm, posterior endoderm and the posterior part of the neural tube [23]. One major difference between amphioxus and vertebrates is that amphioxus somites bud off directly from the wall of the neurenteric canal rather than, as in vertebrates, deriving from segmentation of bands of presomitic mesoderm that in turn derive from the tail bud (figs 4, 5).

Comparisons of gene expression in the amphioxus and vertebrate tail buds support the idea that the fundamental plan of the vertebrate tail bud is similar to that in amphioxus, and that mesenchymal components of vertebrate tail buds arose in connection with modified modes of gastrulation due to the increase in egg size. A suite of genes, including the T-box gene *brachyury*, as well as *caudal*, *Notch*, *BMPs* and several *Wnt* genes, is expressed around the blastopore and in the tail buds of both amphioxus and vertebrates (table 1) [23, 37, 40–58]. There are numerous *Wnt* genes in both vertebrates (19 in the mouse) and amphioxus (8 described to date in amphioxus), several of which signal through each of three pathways (the canonical Wnt/β-catenin, Wnt/Ca^{++} and planar cell polarity pathways) [59–61]. *Wnt* genes expressed around the blastopore and/or in the tail bud of vertebrates include *Wnt3*, *Wnt6* and *Wnt8*, which signal via the canonical pathway, and *Wnt5* and *Wnt11*, which appear to signal preferentially via the Ca^{++} pathway and the planar cell polarity pathway, respectively [62–66]. In contrast, in amphioxus, but not in vertebrates, *Wnt1*, *Wnt4* and *Wnt7* are also expressed around the blastopore and/or in the tail bud [23, 37, 55–57]. Because different *Wnt* genes signaling via the same pathway can apparently compensate for one another (e.g. *Wnt1*, *Wnt8*, *Wnt3a*) [61; see also www.stanford.edu/~musse/wntwindow.html for a complete list of reviews on Wnt signaling], there may be fewer constraints on maintaining the expression of a particular *Wnt* gene. *Wnt1*, for example, is not only expressed around the blastopore in amphioxus, but also in a wide variety of other invertebrates [37, 38]. The loss of *Wnt1* expression around the vertebrate blastopore and a gain of expression in the nerve cord appear to be examples of loss of function combined with neo-functionalization (see below).

Table 1. Expression domains of selected genes within sub-regions of the tail bud in amphioxus (a) and the frog *Xenopus* (f).

Genes	Wnt1		Wnt3		Wnt4		Wnt5		Wnt6		Wnt11		cdx		notch		bra	
Organism	a	f	a	f	a	f	a	f	a	f	a	f	a	f	a	f	a	f
Posterior wall NEC	+	–	+	+	+	+/?	+	+	+	?	–	?	+	+	+	+	+	+
Posterior tip of epidermis	–	–	+	–	–	–	–	–	–	?	+	?	–	–	–	–	–	–
Posterior neural tube	–	–	+	+	–	–	–	–	+	?	–	?	+	+	+	–	–	–
Posterior notochord	–	–	–	–	+	?	+	+	–	?	–	?	–	–	+	–	+	+
Nascent somites	–	–	–		+	+	+	?	–	?	–	?	–	–	+	–	+	–
CNH	–	–	–	+	+	+	+	+	–	?	–	?	+	–	+	–	+	+

The regions of the tail bud correspond to those in figures 4 and 5. For some *Xenopus* genes, detailed expression in the tail bud has not been determined. However, *Xenopus Wnt6* and *Wnt11* are probably expressed in the tail bud, since they are expressed in the tail bud of other vertebrates [24, 23, 37, 40–58, 211]. NEC, neurenteric canal; CNH, chordoneural hinge.

In amphioxus, and in at least *Xenopus* among the vertebrates, the genes discussed above are expressed in discrete, but partially overlapping, regions of the tail bud [23, 28, 67–69]. Temporally, the expression of these genes also overlaps. For example, in amphioxus, *Wnt8* is expressed first, followed in order by *Wnt11* and *Wnt1*, and then, by the mid-gastrula, *Wnt3*, *Wnt4*, *Wnt5*, *Wnt6* and *Wnt7b* [38]. By the early neurula, *Wnt8* and *Wnt7b* turn off around the blastopore, while expression of the others persists into the tail bud as the tail elongates (fig. 4, 5). This suggests that combinatorial codes of developmental genes subdivide the tail bud into a mosaic of regions, each contributing to a specific differentiated tissue.

In vertebrates, there is considerable experimental evidence for the roles of these genes in formation of the tail and/or tail elongation. Null mutations or knock-downs of *cdx, Notch* or *bmp* typically result in failure of the tail to elongate [45, 48, 49, 51, 53, 70–81]. Moreover, several of these genes have been shown to interact directly with one another in controlling tail formation. For example, *caudal* is directly regulated by fgfs [49, 82] and by Wnt/βcatenin via tcf [83, 84], while brachyury and Wnt5 synergistically regulate expression of *BMP4* [70]. Moreover, at least in the presomitic mesoderm, Wnt3a appears to regulate *Notch* indirectly [85].

The functions of amphioxus homologs of vertebrate genes involved in formation of the tail bud and elongation of the tail have yet to be studied. Currently, genetic experiments are not possible in amphioxus as there are no continuous breeding cultures in the laboratory. Nevertheless, knock-down of gene function with antisense morpholino oligonucleotides is possible [86], and future experiments should demonstrate the role of specific genes in the amphioxus tail bud.

Even in the absence of experimental data in amphioxus, the strikingly similar patterns of gene expression in the vertebrate and amphioxus tail buds suggest that the vertebrate tail bud is not a blastema of pluripotent cells. Rather, the contributions of pluripotent mesenchymal cells to the tail bud are superimposed on a fundamentally epithelial organization of tissues derived from the blastoporal lip, which are subdivided into a mosaic of specific cellular populations.

Pharyngeal patterning in amphioxus: key to the separate roles of the endoderm and neural crest in development of the vertebrate pharynx

During development of aquatic vertebrates (agnathans, fish, amphibians) and invertebrate chordates (amphioxus and tunicates), the pharynx forms a series of gill slits (figs 2, 3) through localized fusions of the pharyngeal endoderm and ectoderm. The mammalian homologs of the gill slit primordia are the pharyngeal pouches, a segmental series of diverticula in the pharyngeal endoderm (figs 2, 3).

There are several pharyngeal derivatives in both vertebrates and amphioxus. In vertebrates, the thyroid gland forms from the pharyngeal endoderm near the 2nd pharyngeal pouch, while in amphioxus, its homolog, the endostyle, forms anterior to the first gill slit. However, amphioxus has no known counterparts of the vertebrate thymus and parathyroids, which begin, at least in the mouse, as evaginations of the 3rd pharyngeal pouches, or the ultimobranchial bodies, which develop as evaginations of the 4th pharyngeal pouches [87–91]. This is probably because inductive interactions between mesenchyme derived from neural crest migrating into the pharyngeal arches and the pharyngeal endoderm are essential for the development of these organs [92–99], and while amphioxus has pharyngeal endoderm, it has no neural crest (figs 6, 8). Neural crest also gives rise to various components of the thyroid, parathyroid and ultimobranchial bodies, such as the connective tissue, as well as to the calcitonin-producing cells of the thyroid. The absence of neural crest and neural crest derivatives in amphioxus is advantageous for embryological studies, in that it allows the role of the endoderm in pharyngeal patterning to be differentiated from that of neural crest.

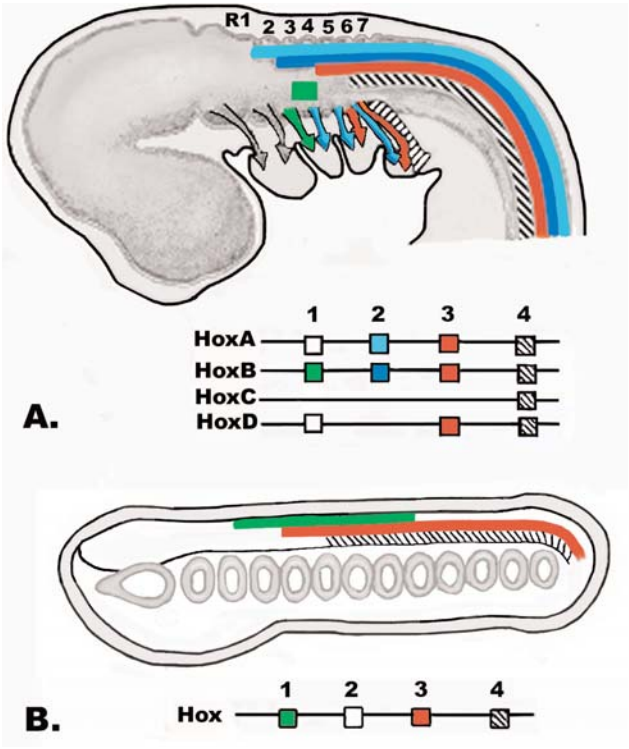

Figure 6. Expression of *Hox1–4* homologs in the nerve cord and neural crest of (*A*) the mouse and (*B*) amphioxus. *Hox1*, *3* and *4* are similarly expressed in the nerve cord of both organisms. In the mouse, hindbrain neural crest carries the Hox code into the pharyngeal arches. Arrows in *A* show neural crest expressing specific *Hox* genes into the pharyngeal arches. Amphioxus (*B*) lacks neural crest and expression of *Hox2* in the nerve cord.

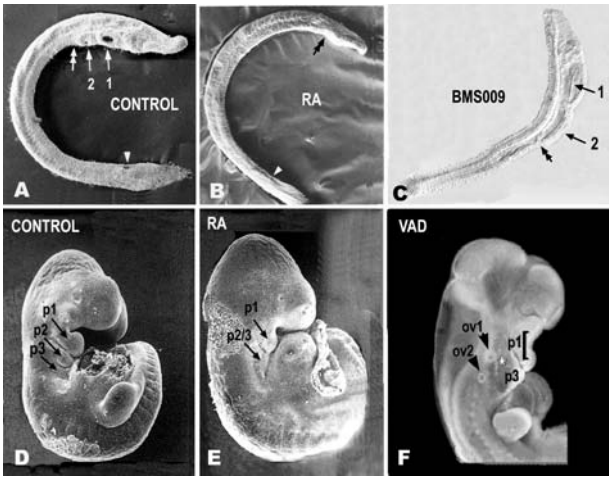

Figure 7. Altered levels of retinoic acid (RA) signaling affect the development of the pharynx in both amphioxus and vertebrates. (*A–C*) 2.5-day amphioxus larvae. (*D–F*) Rat and mouse embryos stage 9.5. (*A*, *B*, *D*, *E*) Scanning electronmicrographs. (*C*, *F*) Light micrographs. Anterior to right in all panels. (*A*) control amphioxus larvae. Arrows, forming first and 2nd gill slits. Double arrow, posterior limit of the pharynx. Arrowhead, anus. (*B*) amphioxus larva treated with 1×10^{-6} M all-*trans* retinoic acid during the gastrula stage. Gill slits are absent and the posterior limit of the pharynx has shifted anteriorly. (*C*) Amphioxus larva treated with the RA-antagonist BMS009 (1×10^{-6} M) during the gastrula stage. The first and second gill slits (arrows) are elongated but have not penetrated. The posterior limit of the pharynx (double arrow) is shifted posteriorly. The anus has formed, but is not visible in this photograph. (*D*) Control rat embryo. The first three pharyngeal pouches (p1–p3) are indicated by arrows. (*E*) Rat embryo from a mother treated with 1×10^{-6} all-*trans* retinoic acid. The first three pharyngeal pouches are fused. (*F*) Mouse embryo from a vitamin-A deficient mother treated in culture with a low concentration of RA. The first and second pharyngeal pouches are fused, and the otic vesicle has shifted posteriorly and duplicated. *A*, *B*, after [104]. *D*, *E* from [207] with permission from Blackwell publishers. *F* from [208] with permission from John Wiley and Sons publishers.

For vertebrates, the prevailing model for some time was that most of the patterning information for the pharynx resided in the neural crest-derived mesenchyme. This idea came chiefly from experiments with retinoic acid (RA), a vitamin A derivative resulting in craniofacial defects when taken as the acne drug Accutane during early pregnancy. When applied to vertebrate embryos at the gastrula stage, RA posteriorizes the hindbrain and shifts the expression patterns of *Hox* genes anteriorly [100]. Consequently, neural crest migrating from the hindbrain carries an altered *Hox* code into the branchial arches (fig. 7). Lending support to the idea that patterning the pharynx is due entirely to influences from neural crest is the observation that the phenotype of vertebrate embryos treated with RA, in which the branchial arches are fused and have abnormal cartilages (fig. 7) [101], is similar to that of embryos in which *Hox1* and *Hox2* genes are misexpressed in neural crest [102, 103].

However, studies of pharyngeal development in amphioxus focused attention on intrinsic signals from the pharyngeal endoderm [104, 105]. Even though amphioxus lacks neural crest, concentrations of RA that cause pharyngeal defects in vertebrates result in a similar phenotype in amphioxus (fig. 7). In the mouse, RA blocks formation of the pharyngeal clefts, resulting in fusion of the arches. Similarly, in amphioxus, the gill slits and the mouth, which is thought to be a modified gill slit, do not form (fig. 7). Correspondingly, lack of RA has similar effects in both amphioxus and vertebrates. In amphioxus embryos treated with the RA antagonist BMS009, the mouth is enlarged, the pharynx is expanded posteriorly and the gill slits do not form. This phenotype is similar to that of vitamin A-deficient chick embryos or of mouse embryos treated with a similar RA antagonist, BMS493. In the vitamin A-deficient quail, the pharynx extends further caudally than normal. The first pharyngeal pouch forms normally, the second pouch is abnormal, and the third and fourth pouches are absent [106]. Similarly, in BMS493-treated mouse embryos, the second pharyngeal pouch is enlarged, and the 3rd and 4th pouches are absent [107]. The comparisons between amphioxus and vertebrates show that in both, RA acting directly on the endoderm establishes the posterior limit of

the pharynx. Excess RA shifts the posterior limit of the pharynx anteriorly, while RA antagonists shift it posteriorly.

In the pharyngeal endoderm of vertebrates and amphioxus, the intrinsic signals downstream of RA are similar. These include both the group I and group II *Pax* genes. The amphioxus genome, which has undergone very little gene duplication, has only one gene in each group (*Pax1/9* and *Pax2/5/8,* respectively), compared to two group I genes (*Pax1* and *Pax9*) and three group II genes (*Pax2*, *Pax5* and *Pax8*) in the mouse [109–113]. These genes code for transcription factors with a paired DNA binding domain. The group II genes also have a partial homeodomain, which is lacking in the group I genes [108]. In both amphioxus and vertebrates, the group II genes have several alternatively spliced isoforms. Alternatively spliced forms of Pax9 have also been described in vertebrates. In vitro assays show that the single amphioxus Pax2/5/8 protein has features in common with several of the vertebrate homologs. For example, the DNA binding properties of one AmphiPax2/5/8 isoform are identical to those of mammalian Pax2 and Pax5 [110]. In addition, the two isoforms of AmphiPax2/5/8 function similarly to human Pax8 in transactivation assays and in the ability to bind to the *Groucho* family of transcriptional corepressors [110].

Pax1/9 genes are widely expressed in the pharyngeal endoderm in both amphioxus and vertebrates. Studies in both amphioxus and mammals suggest a role for *Pax1* and *Pax9* genes in promoting cell proliferation and preventing fusion of adjacent tissues in the pharynx [112, 114]. In the amphioxus neurula, *Pax1/9* is downregulated in the pharyngeal endoderm where the endoderm and ectoderm will fuse to form gill slits [109]. In vertebrates, expression of these genes correlates with focal increases in cell proliferation in the pharyngeal endoderm that are responsible for the growth of the pharyngeal pouches; there is no expression in regions of the developing foregut where fusions are occurring [112, 113, 116]. Moreover, both *Pax1* and *Pax9* single-mutant mice have defects in pharyngeal organogenesis, including reduced or absent formation of organ primordia [115, 116]. Correspondingly, in the mouse *Hoxa3* mutant, in which *Pax1* expression in the endoderm of the 3rd pharyngeal pouch is reduced, there are fusions between the endoderm and ectoderm in the pouch, supporting a role of *Pax1* in preventing fusion of tissue layers.

Group II *Pax* genes appear to act in opposition to the group I genes. In amphioxus, *Pax2/5/8* is expressed in the ectoderm and endoderm of the gill slit primordia as well as in the endostyle. It has been proposed that *AmphiPax2/5/8* functions to promote fusion of the ectoderm and endoderm to form gill slits [111]. Vertebrate *Pax2/5/8* genes are expressed in the homolog of the endostyle, the thyroid. In addition, in *Xenopus*, *Pax2* is expressed in the ectoderm of the three visceral furrows, comparable to the pharyngeal clefts of the mouse, where it may function to regulate fusion of cell layers during gill slit formation [113,117], while in the zebrafish, *Pax8* is expressed in the caudal pharyngeal pouches [118]. *Pax2/5/8* genes are not expressed in the imperforate pharyngeal pouches in mammals and birds, reinforcing the idea that expression of *Pax2/5/8* genes promotes epithelial fusions during gill slit formation.

Group I and group II Pax genes appear to be downstream targets of RA signaling. In both amphioxus and vertebrates, RA treatment shifts the expression of *Pax1/9* anteriorly and prevents downregulation of *Pax1/9* in the mouth and gill slit primordia. Conversely, the RA antagonist BMS009 expands expression of *Pax1/9* expression posteriorly, but also prevents its downregulation in gill slit primordia, although not in that of the mouth [105]. Similarly, in vitamin A- deficient quail, the *Pax1* expression domain is expanded posteriorly [119], while in mouse embryos treated with BMS493, *Pax9* is expressed where the 4th pouch would normally have formed, although the 4th pouch in controls does not express *Pax1*. This expression is in agreement with an anteriorization of the pharynx, with the endoderm posterior to the 2nd pharyngeal pouch adopting a 2nd pouch fate [107]. RA and BMS009 also affect expression of *AmphiPax2/5/8* – in the absence of gill slit formation, it does not turn on in the gill slit primordia [our unpublished results]. The effects of altered RA signaling on expression of Pax2/5/8 genes in the pharynx of aquatic vertebrates have not been determined.

Several other genes are expressed in similar patterns in the pharyngeal endoderm in both amphioxus and vertebrates, including *Otx, Ptx, Shh* and *HNF3-β* [42, 120–126]. This list is likely to grow as the expression of more genes is determined in amphioxus.

Since the first paper on the effects of RA in pharyngeal patterning in amphioxus, work in vertebrates has underscored the fundamental role of the endoderm in pharyngeal patterning and shown that neural crest does not establish initial patterning of the pharyngeal pouches and arches. When neural crest is ablated or prevented from forming, development of the pharyngeal arches is initially normal [90, 127]. Conversely, in a mutant zebrafish lacking pharyngeal arch segmentation, gill slits do not form, although early migration of neural crest cells is normal [125, 128]. Moreover, patterning of the endoderm into organ-specific domains may also be endoderm intrinsic: heterotopic transplants of prospective 3rd pouch endoderm generate both thymus and parathyroid glands in the absence of contact with neural crest cells [129]. Furthermore, data from both frogs and chickens suggest that pharyngeal endoderm may be a source for patterning and/or differentiation signals for the skeletal elements derived from neural crest mesenchyme [130].

In sum, primary patterning of the pharynx in both amphioxus and vertebrates is mediated by RA signaling and a suite of downstream genes, including group I and II *Pax* genes acting in the pharyngeal endoderm. Neural crest adds additional complexity in vertebrates, allowing for the differentiation of the thymus, parathyroids and ultimobranchial bodies and for the formation of the branchial cartilages.

Expression of genes at the edges of the neural plate in amphioxus: insights into the mechanism of neural crest formation

Although amphioxus lacks neural crest, neurulation has much in common between neurulation amphioxus and vertebrates (fig. 8). In amphioxus, as in frogs, birds and mammals, a neural plate forms in the dorsal ectoderm and rounds up to form the neural tube. One difference between neurulation in amphioxus and vertebrates is that in the former, the non-neural ectoderm detaches from the edges of the neural plate and migrates over it to fuse in the dorsal midline. Only then does the neural plate round up. In contrast, in frogs, birds and mammals, the neural tube rounds up before detaching from the non-neural ectoderm [131]. Nevertheless, most of the genes expressed in premigratory and migratory neural crest in vertebrates are expressed in the edges of the neural plate and/or the adjacent non-neural ectoderm in amphioxus (fig. 8). These include *Sox1/2/3, Snail* and *Pax3/7*, among others [132–134; reviewed in 93] (fig. 8). Interestingly, the edges of the migrating ectodermal sheets in amphioxus express *Dll,* homologs of which are characteristically expressed at the neuroectoderm/non-neuroectoderm boundary and in migrating neural crest in vertebrates. However, the *Dll*-expressing cells in amphioxus remain epidermal and do not differentiate into the wide variety of tissues that neural crest gives rise to (e.g. pigment cells, cartilage, connective tissue, adrenal chromaffin cells, parts of numerous ganglia) [131]. In some cases, genes expressed at the edges of the neural plate in amphioxus are expressed in the adjacent non-neural ectoderm in vertebrates or vice versa. For example, *Wnt6* is expressed in the edges of the neural plate in amphioxus but in non-neural ectoderm adjacent the neural plate in vertebrates [23, 135].

The similarity in gene expression at the edges of the neural plate in amphioxus and vertebrates raises the question of what genes are responsible for the generation of migratory neural crest. Not surprisingly, some genes that are expressed in vertebrate neural crest only after migration begins (e.g. *AP2*) are not expressed in either the edges of the neural plate or non-neural ectoderm in amphioxus [136]. Moreover, some amphioxus homologs of premigratory neural crest markers are not expressed at the edges of the neural plate during neurulation. For example, *Zic* is expressed in the edges of the neural plate in the very early neurula of amphioxus, but turns off there while the neural plate rounds up. After neurulation is complete, it turns back on in the dorsal part of the neural tube [137]. Similarly, the single amphioxus homolog of the four vertebrate Id genes, some of which mark premigratory and migratory neural crest, is expressed throughout the anterior neural plate at the early gastrula stage, but turns off altogether in the neural plate as neurulation begins [138].

A potentially key gene in the evolution of neural crest is *FoxD3*, the only one of the five vertebrate *FoxD* genes that is expressed in neural crest. The single amphioxus *FoxD* gene (table 1) is not expressed in the edges of the neural plate or in the adjacent non-neural ectoderm [139], suggesting that *FoxD3* acquired a role in neural crest subsequent to duplication of the *FoxD* genes. Overexpression of *FoxD3* in vertebrate embryos suggests that it has an important role in neural crest. Under some conditions, overexpression of *FoxD3* keeps neural crest progenitor cells in a proliferating, relatively undifferentiated state

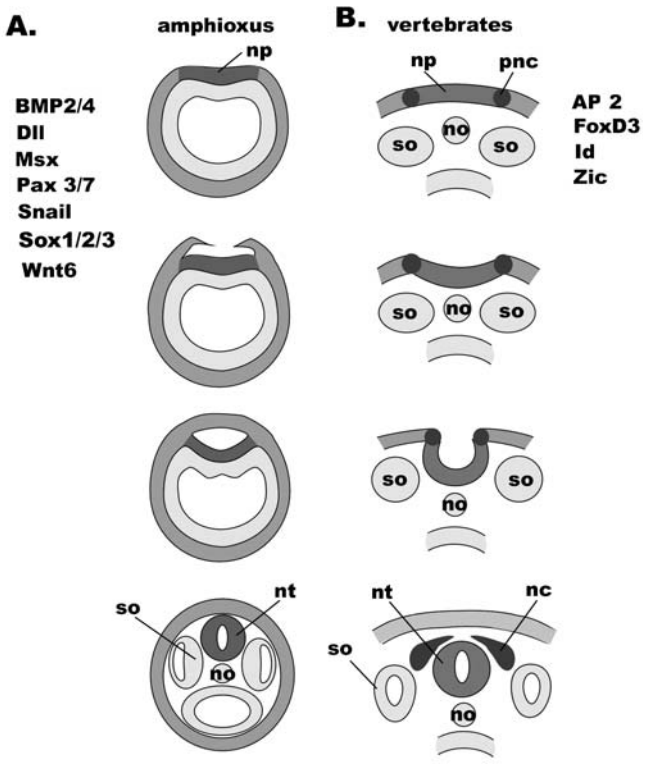

Figure 8. Schematic diagrams comparing neurulation (*A*) in amphioxus and (*B*) in vertebrates. Genes listed in *A* are expressed at the boundary of neuroectoderm and non-neuroectoderm in both amphioxus and vertebrates. Genes listed in *B* are expressed during neurulation at the boundary of neuroectoderm and non-neuroectoderm in vertebrates, but not in amphioxus, and therefore may be essential for the ability of neural cells to migrate and/or differentiate into many cell types. After [23, 131, 132, 134, 136–138, 159, 209].

[140], although under other conditions, it promotes neural crest cell determination and induces a migratory morphology and behavior [141–144]. These experiments suggest that *FoxD3* expression at the edges of the neural plate may have recruited the genetic program necessary for determination and migration of neural crest cells.

To date, the comparison of neural crest markers in vertebrates and amphioxus has included relatively few genes (fig. 8). It is unlikely that changes in gene function/regulation in *FoxD* genes subsequent to gene duplication are solely responsible for the migratory behavior of cells at the edges of the vertebrate neural plate. Additional work may reveal a spectrum of differences in genes expressed at the edges of the neural plate in amphioxus and vertebrates.

Amphioxus as a genomic model system: chordate genome evolution and the fate of duplicated genes

Amphioxus is key to understanding the pattern of gene duplications in vertebrates

Since the pioneering work of Ohno [145], it has been widely accepted that vertebrates have more genes than invertebrates due to large-scale gene duplications during vertebrate evolution. The majority view is that one round of gene duplications occurred after the divergence of amphioxus and the vertebrates, and a second round after the divergence of agnathans and gnathostomes (reviewed in [146]). Thus, similar to the *Id* and *FoxD* genes mentioned above, amphioxus has one complete *Hox* cluster, whereas mammals have four [147–151]. Attempts to reconstruct the history of gene duplications are clouded by loss of some duplicate genes and independent duplications of others. For example, it is uncertain whether lampreys have three or four *Hox* clusters since there are no more than three copies of any one of the *Hox* genes. Recent analyses suggest that there was an independent duplication of the two ancestral vertebrate *Hox* clusters within the lamprey lineage (producing four copies), followed by loss of several genes from each of the clusters [7–9]. Additional studies of genes from cartilaginous fish showed that within the gnathosomes, most gene duplications occurred before the divergence of cartilaginous fish [152]. Whether such large-scale duplications involved entire genomes or only parts of genomes remains controversial [10, 152–157].

For understanding the evolutionary history of gene duplications and loss in vertebrates, amphioxus is particularly important as an outgroup. Genomes of tunicate and protostome models such as *Drosophila* and *Caenorhabditis* are not as useful because they have undergone considerable gene loss and because the protostomes diverged from deuterostomes early in evolution [11, 92]. Importantly, in phylogenetic analyses, amphioxus genes typically mark the base of vertebrate diversification [158–161]. Thus, given its phylogenetic position and its unduplicated genome, amphioxus promises to be especially helpful in unraveling the evolutionary history of the structure and organization of vertebrate genomes.

To understand the evolution of gene duplication and loss in vertebrates, a ‘phylogenomic’ approach has recently been developed that compares paralogous regions of linked genes in the genomes of amphioxus and vertebrates [11, 160, 161]. For such analyses, mammalian genomes are more useful than those of vertebrates such as *Xenopus laevis* (the African clawed frog), which is a rediploidized tetraploid, or teleost fish (such as the zebrafish), which are characterized by large-scale, teleost-specific gene duplications [165–168].

Since the amphioxus genome has not yet been completely sequenced [the sequence of *Branchiostoma floridae* should be completed by the Joint Genome Institute of the Department of Energy (USA) early in 2005], only a few genomic regions have to date been analyzed by the phylogenomic approach. These include the *Hox*, *ParaHox* and *NKL* clusters as well as the major histocompatibility complex (*MHC*) region [42, 149, 162–164]. Examples include analyses of the *Hox*, *ParaHox*, *MHC* and *NKL* gene loci. For each of these, the organization in amphioxus appears to represent the ancestral chordate condition. As noted above, mammals have four clusters of *Hox* genes (39 genes in all), while amphioxus has a single cluster of 14 *Hox* genes [149, 169]. The ancestral number of *Hox* genes appears to be 13, with *Hox14* in amphioxus possibly representing an independent duplication [169]. The single amphioxus *ParaHox* gene cluster (there are four such clusters in mammals) consists of three *Hox*-like genes (*AmphiGsx*, *AmphiXlox* and *AmphiCdx*) [42, 162]. The *Hox* and *ParaHox* clusters probably arose by duplication of an ancestral *ProtoHox* gene cluster consisting of four linked genes [42, 170].

Similarly, there is a single *MHC* region in amphioxus (which lacks key genes for adaptive immunity, but includes the anchor genes as in vertebrates) and four in humans, each on a different chromosome (fig. 9), providing additional support for large-scale gene duplications during vertebrate evolution. This analysis has highlighted the possible fates of large genome regions after duplication [163]. Whereas one of the human *MHC* regions (9q32-q34.3) appears to have largely retained an ancestral state comparable to that in amphioxus (fig. 9), the other three have faster substitution rates and massive gene losses [163]. However, this is not always the case. For example, substitution rates are similar in three paralogous regions on human chromosomes 4, 5 and 10 (8p11.21–8p21.3) [171, 172]. For the *NKL* cluster, comparisons suggest that a single *NKL* gene cluster of five genes in the bilaterian ancestor became broken into three clusters, two with two genes each and one with a single gene. These three clus-

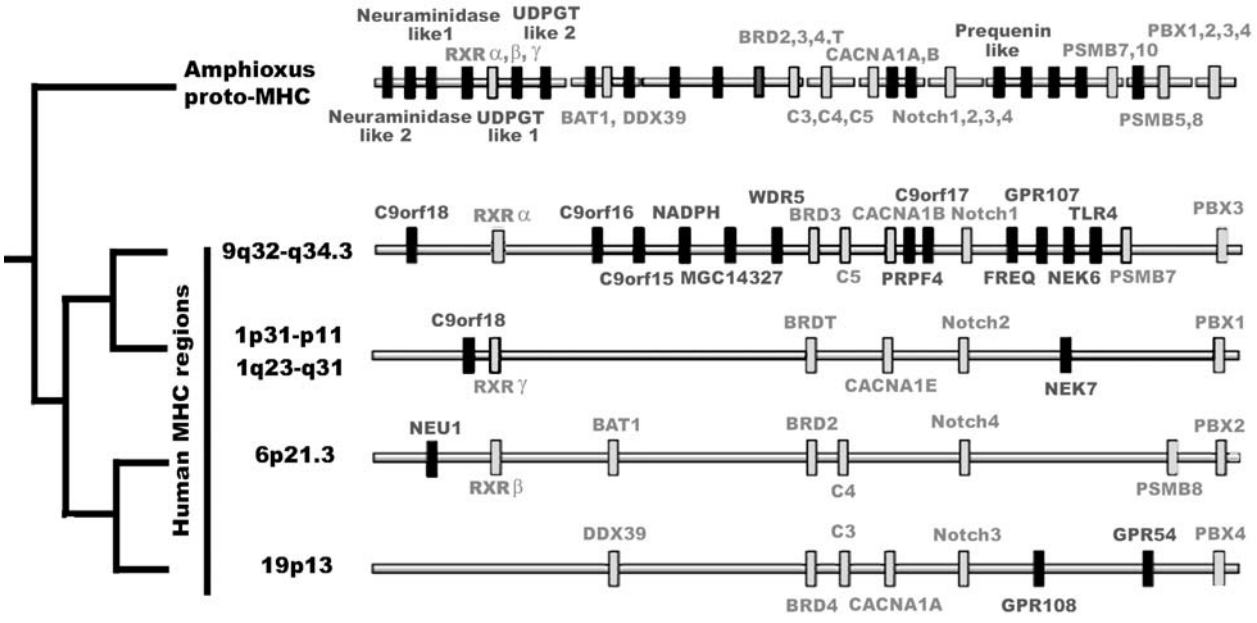

Figure 9. Scheme for evolution of the major histocompability (MHC) genomic region. Cosmids corresponding to the amphioxus MHC region and the four genomic human MHC regions are shown. Cosmids encompassing the MHC gene have recently been localized to a single amphioxus chromosome [13]. For the amphioxus and vertebrate MHC regions, the anchor genes are indicated in grey, whereas genes surrounding the anchor genes are shown in black. After [163].

ters dispersed within a single chromosome in the deuterostome lineage before the split of amphioxus and vertebrates. This organization has been maintained in the vertebrate lineage after two rounds of duplication and subsequent loss and additional duplication of individual genes [164]. Thus, vertebrates have 20 NKL genes in four clusters, while amphioxus has only a single cluster with 6 genes [164].

The fate of duplicated genes: sub-functionalization and neo-functionalization of regulatory regions

The amphioxus genome is also important for analyzing the fate of individual genes after duplication. Classically, two fates have been predicted for genes that have been duplicated in their entirety (i.e. a duplication that includes the regulatory regions of the gene) [145, 173–175]: (i) The most likely fate is that one duplicate either degenerates to become a pseudogene or is completely lost from the genome due to chromosomal remodeling or deletion (non-functionalization). (ii) Alternatively, since duplicated genes are initially identical and functionally redundant, only one of the two copies needs to be maintained for the ancestral function, whereas the second is free to acquire a new one (neo-functionalization). This requires the fixation of rare beneficial mutations in one of the duplicates and would suggest that the retention of both is extremely rare. However, since duplicate genes appear to be retained more often than predicted by this model [176–180], the duplication-degeneration-complementation (DDC) model has been developed, which predicts preservation of duplicated genes by partitioning the several expression domains of an ancestral gene among the duplicates [181–182]. The duplicates lose congruent expression domains due to loss/change in regulatory elements, leading to retention of both duplicates, allowing each to evolve independently.

Sub-functionalization and neo-functionalization of regulatory DNA after gene duplication

Within the vertebrate lineage, most duplicated gene families include examples of neo-functionalization [183]. In addition, there is strong evidence for sub-functionalization of recently duplicated genes in both amphioxus and vertebrates [181, 182; reviewed in 175]. In sub-functionalization, the multiple roles of a pleiotropic ancestral gene are divided amongst the duplicates, thus keeping the duplicates from becoming pseudogenes. For example, amphioxus has eight *Hairy* genes resulting from independent, amphioxus-specific gene duplications. The combined expression domains of four of these resemble those of the vertebrate *Hairy* genes, suggesting that after duplication of an ancestral gene, the amphioxus *Hairy* genes underwent sub-functionalization in which regulatory elements directing expression to particular tissues were lost from some of the duplicates [184]. Since sub-functionalization is more common than neo-functionalization [181,

182], preservation by sub-functionalization may be an important first step for a duplicated gene before it undergoes neo-functionalization [175, 185]. This may explain why sub-functionalization seems to be more common amongst recently duplicated genes, whereas neo-functionalization is more evident in genes resulting from an ancient gene duplication event.

Comparisons of developmental genes between amphioxus and vertebrates reveal numerous examples of neo- and/or sub-functionalization after gene duplication in vertebrates (e.g. in *FoxD, RAR, Wnt1, Wnt8, Sox1/2/3, Adh*, *AP-2*, *Distalless*, *Engrailed*, *Eomes*/*Tbr1*/*Tbx21*, *Fringe*, *Hedgehog*, *Hox*, *Islet*, *Mox*, *Otx*, *Pax1/9*, *Pax3/7*, *Snail*, *Id*, *Pax2/5/8* and *Pitx* gene families), [109, 111, 120, 121, 126, 136, 138, 139, 183, 186–191]. The *FoxD* family exemplifies both sub-functionalization and neo-functionalization subsequent to gene duplication (table 1). Amphioxus has a single *FoxD* gene that is homologous to four mammalian genes. A fifth *FoxD* gene is present in *Xenopus* and zebrafish. In addition, this gene and the human *FoxD4* gene have been independently duplicated in their respective lineages. The single amphioxus *FoxD* is expressed in four domains: the somites, the dorsal part of the notochord, the anterior part of the nerve cord and the posterior part of the hindgut [139] (table 2). In vertebrates, expression in the forebrain has been retained by three of the four duplicates (*FoxD1*, *D2* and *D4*), while expression in the notochord has been retained only by *FoxD4*. In contrast, two of the duplicates (*Fox D2* and *D3*) retain expression in the somites. In addition, the vertebrate *FoxD* genes have acquired new expression domains in new tissues. Vertebrate *Fox D3* is expressed in neural crest and spinal cord, *D4* in the mesencephalon, *D1* and *D2* in mesenchyme and mesenchymal derivatives, *D2* in the hindgut endoderm, *D4* in the foregut endoderm and *D2* in the abdominal muscle. A second example of sub-functionalization is RAR. In amphioxus the single RAR gene is expressed in all three tissue layers at low levels in the anterior third, highest in the middle third and at intermediate levels in the posterior third of the embryo [105]. In contrast, the three vertebrate RAR genes have subdivided this expression domain along the anterior/posterior embryonic axis: *RARα* is expressed in the head, *RARβ* in the trunk and mesonephros, and *RARɣ* most strongly in the tail [101, 192].

Sub-functionalization and/or neo-functionalization can also occur together with non-functionalization. Interesting examples are the *Wnt1* and *Wnt8* genes (table 3). The single amphioxus Wnt1 gene retains the ancestral bilaterian expression around the blastopore. This expression persists into the tail bud. The gene is never expressed anywhere else. Vertebrates also have a single *Wnt1* gene. However, it is expressed at the midbrain/hindbrain boundary, not around the blastopore. This suggests two possible scenarios. In one, the single *Wnt1* gene added a second expression domain and then lost the original one. In the second, the gene duplicated in the vertebrate lineage, with one duplicate retaining the ancestral function and the second acquiring a new one. Subsequently, the copy with the original function was lost. The single amphioxus *Wnt8* gene is expressed around the blastopore during the early gastrula and later in the presomitic and somitic mesoderm and still later in the forebrain. One of the two *Xenopus* genes, *Wnt8b*, has apparently retained the forebrain domain, while the other, *Wnt8a*, has retained expression in the early presomitic mesoderm [58]. However, *Wnt8a* has added new domains in the *Xenopus* midbrain, hindbrain and the posterior part of the nerve cord, and both have added new domains in the lens. The vertebrate genes have no counterpart of the expression domain in the definitive amphioxus somites. This raises the question of whether vertebrates have lost this expres-

Table 2. Sub-functionalization, neo-functionalization and non-functionalization of FoxD genes after duplication in the vertebrate lineage.

	Amphioxus	Mouse			
	AmphiFoxD	D1	D2	D3	D4
Forebrain	+	+	+	–	+
Edge of np	–	–	–	+	–
Spinal cord	–	–	–	+	–
Midbrain		–	–	+	–
Notochord	+	–	–	–	+
Somites	+	–	+	+	–
Mesenchyme		+	+	–	–
Hindgut endo	+	–	+	–	–
Foregut endo	–	–	–	–	+
abd muscle		–	+	–	–

Expression domains of the single amphioxus genes and the comparable domains of their vertebrate homologs are indicated. Endo, endoderm; abd, abdominal; nc, nerve cord; np, neural plate, pre som mes, pre somitic mesoderm.

Table 3. Sub-functionalization, neo-functionalization and non-functionalization of Wnt8 duplicates in the vertebrate lineage.

	Amphioxus	Mouse	
	AmphiWnt8	Wnt8a	Wnt8b
Forebrain	+	–	+
Midbrain		+	–
Hindbrain	–	+	–
Posterior nc	–	+	–
Pre-som mes	+	+	–
Somites	+	–	–
Ventral mes	–	+	–
Lens		+	+

Expression domains of the single amphioxus genes and the comparable domains of their vertebrate homologs are indicated. Ventral mes, ventral mesoderm; nc, nerve cord; pre som mes, pre somitic mesoderm.

sion domain or whether amphioxus has added it. The coming complete genome sequence of amphioxus will help answer such questions. What is still needed is more information of genomes and gene expression of agnathans. No *Wnts* have been cloned from lampreys, and as yet, neither the lamprey nor hagfish genome appears to be scheduled for sequencing.

Sub-functionalization can be either spatial or temporal or both. The examples above concern spatial sub-functionalization. An example of the temporal sub-functionalization is the estrogen-related receptor (ERR) [193; our unpublished data]. In both amphioxus and zebrafish, *ERR* genes are expressed in specific subsets of cells of the hindbrain. However, in the zebrafish, the three *ERR* genes turn on sequentially in overlapping regions of the hindbrain.

Taken together, these examples of non-, neo- and sub-functionalization of duplicated genes in vertebrates highlight the potential of amphioxus as a comparative model when addressing questions about the fate of duplicated genes in vertebrates. To reiterate, we conclude that amphioxus, due to its phylogenetic position and its relatively unduplicated genome, is critical (i) for understanding the evolution of vertebrate genomes and (ii) for studying the fate of individual genes after duplication in vertebrates. In addition, it will be particularly valuable to have genome sequences of lampreys and hagfish. Lampreys are amenable to developmental studies (in contrast to hagfish, for which embryos are close to impossible to obtain), and lamprey BAC libraries are under construction.

The evolution of receptor-ligand interactions in nuclear hormone receptors: insights from amphioxus

In the examples above, sub-functionalization and neo-functionalization subsequent to gene duplication involve the regulatory regions of genes. However, they can also include the coding region. There are relatively few examples of evolutionary changes in protein function subsequent to gene duplication. Perhaps the best are the retinoic acid receptor (RAR) proteins within the nuclear hormone receptor superfamily. This superfamily includes ligand-activated transcription factors that mediate physiological processes ranging from development to homeostasis, such as receptors for retinoic acid (RAR), thyroid hormone, vitamin D3 and cholesterol-derived steroid hormones, including glucocorticoids, progesterone, androgens and estrogens. There are also numerous so-called orphan receptors, for which no ligands are known [193]. During vertebrate evolution, the nuclear hormone receptors apparently underwent many gene duplications followed by some gene loss (fig. 10). For example, vertebrates have 49 nuclear hormone receptors including three each of the *RAR*, *RXR*, *ERR* and *PPAR* genes, and two each of the *SF1/LRH1*, *LXR*, *COUP-TF*, *TR2/4*, *TR*, *ER* and *Rev-erb* genes, compared to just one of each of these in amphioxus [104, 191–196]. It is likely that, as for most other gene families, amphioxus has one representative of each nuclear receptor subfamily, a total of 21 genes (fig. 10). In contrast, in the other chordate group, the tunicates, *Ciona intestinalis* has only 19 nuclear hormone receptors, having apparently lost four genes, including the estrogen receptor, which is present in both mollusks and amphioxus [12, 197; our unpublished data]. Such a gene loss appears to be relatively common in the tunicate genome. *C. intestinalis* has also lost several *Hox* genes as well as those for the circadian rhythm protein clock, the *Lim* gene *Lhx6/7* and others [12]. Thus, amphioxus is the best invertebrate chordate for studying the evolutionary diversification of the nuclear hormone receptor superfamily in vertebrates. These amphioxus genes can be used to understand both the original interactions between receptors and their ligands and the ancestral function of a given receptor before gene duplication.

One example of neo-functionalization of the coding region is the RAR:RXR heterodimer [101, 105, 198–203]. In vertebrates, the three RARs and three RXRs (RXRα, RXRβ and RXRγ) combine to make nine heterodimers (fig. 10). In contrast, amphioxus has just one RAR:RXR heterodimer. RARs have at least two natural ligands: all-*trans* retinoic acid and its isomeric form, 9-*cis* retinoic acid. Since altered RA signaling appears to be involved in several diseases (e.g. cancer, diabetes and skin disorders), numerous retinoid analogs with varying affinities to the different vertebrate RAR isoforms have been designed to change levels of RA signaling [193, 204]. To understand the evolution of protein-ligand interactions, these synthetic retinoids can be used in transactivation assays with the three vertebrate and the single amphioxus RARs. Together with site-directed mutagenesis and three-dimensional modeling experiments, these assays can reveal which amino acids within the ligand binding domain of the RAR proteins are crucial for recognizing different retinoid ligands [105]. In addition, comparisons of the amino acid sites in the vertebrate receptors that mediate isoform-specific binding with the corresponding sites in the amphioxus ligand binding domain can give insights into the ligand binding potential of the RAR protein of the last invertebrate ancestor of vertebrates.

As with RARs, there are three peroxisome proliferator activated receptors (*PPARs*) in vertebrates (*PPAR*α, *PPAR*β and *PPAR*γ), and only one in amphioxus [193, our unpublished data] (fig. 10). However, although the RARs all bind the same retinoids in vivo, each of the vertebrate PPARs binds a different ligand. For example, PPARα binds leukotriene B4, an important molecule in inflammatory processes, while PPARγ, an important mediator of adipogenesis, binds prostaglandins, such as PGJ2, and PPARβ probably binds a fatty acid or its derivative [193].

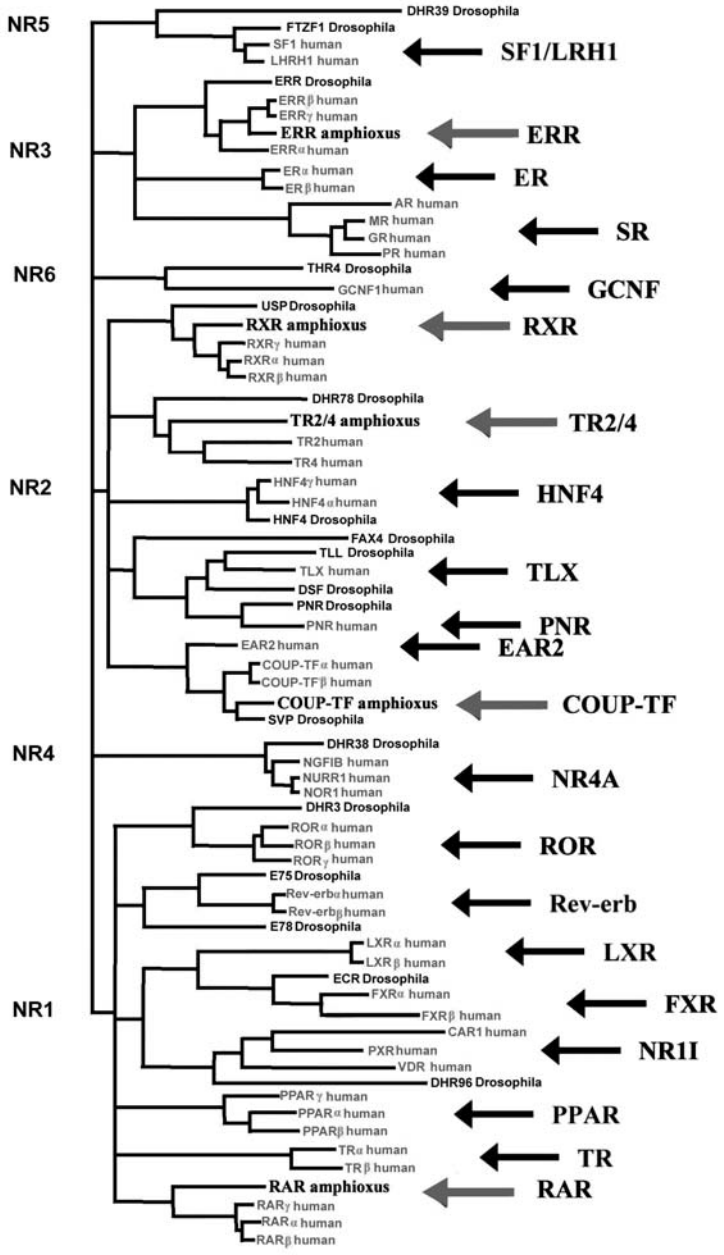

Figure 10. Phylogeny of the nuclear hormone receptors. Sequences from *Drosophila* are shown in black. The six subfamilies within the nuclear hormone receptor superfamily are numbered NR1–6. For the nuclear hormone receptor groups indicated with the grey arrow, a single amphioxus homolog has been isolated. Black arrows indicate groups for which a single amphioxus homolog is hypothesized, but has not yet been identified. After [152].

PPARα will also bind synthetic compounds called fibrates, which are used in medicine to decrease the level of triglycerides in the blood, while PPARγ binds synthetic thiazolodinediones, used in the treatment of diabetes. Although PPAR has been cloned from amphioxus, it is not known what its natural ligand(s) are. Such an understanding would be very useful for elucidating the evolution of ligand-binding specificity of the three vertebrate PPAR proteins. For example, if amphioxus PPAR binds only one of the three ligands of the vertebrate PPAR genes, it would suggest that two PPAR receptors changed their ligand specificity after duplication of the PPAR genes in the vertebrate lineage.

In addition, a study of the amphioxus steroid receptor, which has been started in the Laudet laboratory, should provide important insights into the evolution of steroid receptor specificity and may illuminate the origin of endocrine signaling in vertebrates. Vertebrates have four paralogous steroid receptors in vertebrates. AR and PR both bind sex steroids (androgens and progesterone, re-

spectively), while GR and MR both bind corticosteroids [193] (fig. 10). An analysis of steroid receptors within the vertebrates has shown they are paralogs that derived from an ancestral gene by duplication early during vertebrate diversification [197], suggesting that amphioxus has a single such receptor. However, existing biochemical models and molecular phylogenies do not reveal which of the main functions of steroid receptors evolved first, the role in sexual differentiation, the regulation of metabolism and homeostasis or the control of inflammation and stress responses [193].

Conclusions

It has been proposed that the complexity of the vertebrate body plan, with such new structures as jaws, image-forming eyes and ears, was made possible by large-scale gene duplications, allowing an extensive neo-functionalization and sub-functionalization of genes [150]. It is not known how these large-scale gene duplications came about. There could have been an increased rate of gene duplication, or single isolated events in which either the whole vertebrate genome or a substantial part of it was duplicated or because of a decreased loss of duplicate genes. Answers may be forthcoming with the current emphasis on comparative genomics. At present, genomic resources are being developed for a wide range of organisms, including agnathans and invertebrates. Genome sequencing projects are currently scheduled for several of these organisms, including amphioxus and the annelid worm *Platynereis.* The genome sequence of amphioxus will be particularly valuable for understanding the evolution of new genes (e. g. those involved in adaptive immunity) and new structures (e. g. neural crest). Moreover, comparative studies between amphioxus and vertebrates are helping to reveal the regulatory elements that are responsible for new roles of genes in new structures as well as the protein motifs that correlate with new protein functions. In sum, amphioxus, with its vertebrate-like but relatively simple body plan and relatively unduplicated genome that does not appear to have undergone substantial gene loss, is an excellent organism to use in comparative studies for understanding the mechanisms of vertebrate development.

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