Ancient homeobox gene loss and the evolution of chordate brain and pharynx development: deductions from amphioxus gene expression

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Homeobox genes encode a large superclass of transcription factors with widespread roles in animal development. Within chordates there are over 100 homeobox genes in the invertebrate cephalochordate amphioxus and over 200 in humans. Set against this general trend of increasing gene number in vertebrate evolution, some ancient homeobox genes that were present in the last common ancestor of chordates have been lost from vertebrates. Here, we describe the embryonic expression of four amphioxus descendants of these genes—AmphiNexa, AmphiNexb, AmphiMsxlx and AmphiNKx7. All four genes are expressed with a striking asymmetry about the left–right axis in the pharyngeal region of neurula embryos, mirroring the pronounced asymmetry of amphioxus embryogenesis. AmphiMsxlx and AmphiNKx7 are also transiently expressed in an anterior neural tube region destined to become the cerebral vesicle. These findings suggest significant rewiring of developmental gene regulatory networks occurred during chordate evolution, coincident with homeobox gene loss. We propose that loss of otherwise widely conserved genes is possible when these genes function in a confined role in development that is subsequently lost or significantly modified during evolution. In the case of these homeobox genes, we propose that this has occurred in relation to the evolution of the chordate pharynx and brain.

Keywords: cephalochordate; homeobox; gene loss; pharynx evolution; brain evolution

1. INTRODUCTION

Homeobox genes encode a large group of transcription factors that expanded very early in metazoan evolution (Ryan et al. 2006), and the diversification of these genes has been implicated in the evolution of the diversity of animal body plans. Frequently, homeobox genes have major directive roles in developmental gene-regulatory networks and many, such as the Hox genes in the context of the anterior–posterior axis (Deschamps 2007; Duboule 2007) and Pax6 in the case of eye specification (Gehring 2002), function in aspects of development that are highly conserved across bilaterian taxa. Our understanding of the evolution of the homeobox genes, and of animal development more generally, has in recent years been greatly impacted by the progressive advance of genome-sequencing projects across the Metazoa. In this regard, the genome of the chordate amphioxus (Branchiostoma floridae; Putnam et al. 2008) has been particularly revealing.

The lancelets, or amphioxus, were long thought to be the closest invertebrate relatives of the vertebrates, but recent molecular evidence has led to their relocation to the most basal lineage within the chordates (Bourlat et al. 2006; Delsuc et al. 2006; Putnam et al. 2008). Notwithstanding this repositioning, amphioxus remains a most informative extant outgroup taxon for studying the early evolution of the vertebrates (Holland & Chen 2001). In large part, this importance is a result of the derived nature of development in the other invertebrate chordate lineage, the urochordates. In contrast, amphioxus in many ways resembles a typical vertebrate: gastrulation is coordinated by an organizer (Yu et al. 2007), and is followed by neurulation, producing an animal with a subepidermal dorsal hollow nerve cord (Holland & Chen 1999) that is enlarged, albeit not very much, at its anterior end, where the various sense organs are situated, and that is surrounded laterally and ventrally by mesoderm that develops from somites (Beaster-Jones et al. 2008). A notochord sits atop a through gut that at the anterior end includes a muscular pharynx. Following a larval stage, amphioxus undergoes a metamorphic transition that is homologous to that seen in amphibians (Paris et al. 2008a,b), and as an adult even resembles a small, poorly cephalized fish.

Classical descriptive morphological studies (Hatschek 1893; Conklin 1932) underlined the differences as well as the similarities between the lancelets and their vertebrate cousins, and the embryological and larval development of amphioxus differs from that of vertebrates in a number of important ways. Amphioxus possesses unpaired sense organs: a single photoreceptive eyespot (though this is not the only photoreceptive structure), and a single putative balance organ located in the cerebral vesicle (Lacalli & Kelly 2000) but no clear olfactory organ (Lacalli 2004). The notochord of amphioxus, unlike its
vertebrate counterparts, is muscular (Flood et al. 1969) and extends the full length of the body, acting as an antagonist to the lateral muscles during swimming or burrowing (Guthrie & Banks 1970). The formation of the mesodermal somites also differs between amphioxus and vertebrates, with the first eight somites in B. floridai (Holland et al. 1997) forming by enteroctodermally rather than schizocoelely, which instead occurs during the addition of more posterior somites in amphioxus and in vertebrate development. Interestingly, the somites are generally asymmetric with the right series offset by half a somite length to the posterior relative to the left series, a result of their alternating production from the posterior growth zone (Schubert et al. 2001). Perhaps the most striking difference is the asymmetry that accompanies the development of the pharyngeal region of the amphioxus neurula. The mouth opens on the left-hand side, just behind a pre-oral pit, a product of the fusion of the left anterior head cavity with the ectoderm. Both these structures have no equivalent on the right-hand side, where the enigmatic club-shaped gland develops just below the endostyle, the presumed thyroid homologue (Ogasawara 2000). Posterior to these, the first few gill slits form ventrally and migrate to the right side, where they break through the body wall (Whittaker 1997). Thus, despite the extensive similarity and homology, amphioxus embryogenesis and larval development differs from that of vertebrates in several important aspects, not least of which is profound asymmetry in early development.

In genomic terms, amphioxus has retained an unprecedented number of ancestral features, both in terms of gene organization at the microsyntenic (e.g. Ferrier et al. 2005; Mazet et al. 2006; Butts et al. 2008; Horton et al. 2008) and macrosyntenic (Castro & Holland 2003; Putnam et al. 2008) scales, and in terms of gene retention. Indeed, following the completion of the genome project, several genomic surveys of different gene families have underscored the conclusion that amphioxus generally has undergone comparatively little gene loss in its evolutionary history (D’Aniello et al. 2008; Holland et al. 2008; Paris et al. 2008b; Schubert et al. 2008; Shimeld 2008; Vu et al. 2008; Dai et al. 2009).

Interestingly, in regard to the homeobox gene complement, amphioxus has retained members of seven gene families (Abox, Bari, Msxl, Nedx, NK7, Repo and Rough) that date back to at least the last common ancestor of the chordates: 37 families (Abox, Bari, Msxl, Nedx, NK7, Repo and Rough) that date back to at least the last common ancestor of the chordates: 37 families characterized by whole-genome duplications (Dehal & Boore 2005; Putnam et al. 2008), and the recruitment of duplicated genes and gene-regulatory networks to developmental innovations is one of the key themes of vertebrate developmental evolution. Given that homeobox genes frequently function in developmental cascades in determinative roles, specifying embryonic territories and fates, the loss of ancient homeobox genes presents a particularly interesting contrast to the genetic expansion widely described for early vertebrate evolution. In order to shed light on this transition, we have examined the developmental expression of the amphioxus orthologues of the pan-bilaterian homeobox gene families lost from vertebrates. We find that four genes are detectably expressed during amphioxus embryogenesis, and that intriguingly, all these four are expressed in territories that, in vertebrates, have undergone significant modification since the last common ancestor of the chordates: the anterior central nervous system and the pharynx.

2. MATERIAL AND METHODS

(a) Animal collection

Adult amphioxus, Branchiostoma floridai, were collected in July and August 2006 from Old Tampa Bay, FL, USA and fertilized in vitro as described by Holland & Holland (1993). Developing embryos were fixed for in situ hybridization at regular intervals by incubation for 60 min at room temperature or overnight at 4°C in 4 per cent PFA in MOPS buffer (0.1 M MOPS, 0.5 M NaCl, pH 7.0). After fixation, embryos were washed twice in 70 per cent ethanol and stored at −20°C in 70 per cent ethanol.

(b) Probes

All aqueous solutions for subsequent stages were treated for 2 h with 0.5 per cent diethyl pyrocarbonate (DEPC) and autoclaved as a precaution against RNAse contamination. Probes were obtained by polymerase chain reaction on amphioxus genomic DNA obtained by phenol–chloroform extraction from fixed adult specimens. Primers to exonic sequences were designed using the v. 1.0 genome assembly of Branchiostoma floridai (http://genomeportal.jgi-psf.org/Braf/Braf1.home.html). Primers used to clone exons of AmphiNedxa, AmphiNedxb, AmphiMsxl and AmphiNKx7 were as follows: BfNedxaex1f, 5’-ATGCGCCGCTGAC-3’; BfNedxaex1r, 5’-TCTCCGTCGCCAGCT-3’; BfNedbxex1f, 5’-ACGTGCCAGAGGAGAGGAGAG-3’; BfNedbxex1r, 5’-TGGCTTCTCATCTCTTGCTG-3’; BfMsxllex1f, 5’-GCCAATCTGATCGCAGTC-3’; BfMsxllex2f, 5’-GAGTTTGGCCGCTTTGATCC-3’; BfMsxllex3f, 5’-GATTGTTGCGAACTTTTGGAGAG-3’; BfMsxllex3r, 5’-CGAGCCGAGAGAGACGG-3’; BfMsxllex3f, 5’-GTTCTCTGTCCAGAAGATA-3’; BfMsxllex3r, 5’-AGGCGGAGAGAGAGAAG-3’; BfMsxllex3f, 5’-GATTTTCGGACAGGTTG-3’; BfMsxllex3r, 5’-GATTTTCGGACAGGTTG-3’; BfMsxllex3r, 5’TCAATAGTGGACGACGACGAGGAGG-3’; BfNkex1f, 5’-GGCAGATGACGAGGAGAGT3’; BfNkex1r, 5’-CTCGGATGACTGTCTCTGCG-3’; BfNkex3f, 5’-TCTCGGTTCGAACTTGCAGG-3’; BfNkex3r, 5’-TAGTCCGTCGTTGGCGACGTTTG-3’.

For the probe AmphiMxl2 consisted of 373 bp in exon 1, for AmphiMxl3 an equimolar mixture of probes of length 514, 161 and 210 bp from exons 1, 2 and 3, respectively, and for AmphiNKx7 an equimolar mixture of probes of length 298 and 353 bp from exons 1 and 3, respectively.

Gene fragments were cloned into pGEMT-easy (Promega). Plasmid DNA was isolated using QIAprep Spin Mini Kit (Qiagen) according to the manufacturer’s instructions. Fragments were re-amplified from minipreps using M13 primers. This re-amplification product was run on a gel, excised and purified using the GFX gel extraction kit (Amersham).

Antisense and sense (control) probes were transcribed in vitro using DIG-RNA labelling (Roche) according to the manufacturer’s instructions. The reaction was incubated at 37°C for at least 2 h. One microlitre of probe was run on an agarose gel to check for complete transcription and the probes were subsequently cleaned using Mini Quick Spin RNA columns (Roche) and then precipitated with 70 per
cent ethanol and 100 mM LiCl. The probes were then washed with 70 per cent ethanol, dried, resuspended in DEPC-treated H₂O and stored at −20°C.

(c) In situ hybridization

In situ hybridizations were performed essentially as described elsewhere (Holland et al. 1996; Osborne et al. 2009) with the following modifications. After rehydration, embryos were digested with 7.5 μg ml⁻¹ Proteinase K for 5–30 min depending on the size and stage of development. Mid-neurula stages (9/10 somites; 15 h development) were digested for 7 min. Late neurula stages (12 somites; 18 h development) were digested for 10 min. Prehybridization was conducted at 50–65°C with gentle shaking for at least 3 h. Hybridization was undertaken overnight at the same temperature with 50–200 ng of labelled probe. The embryos were blocked in 10 per cent sheep serum (in phosphate-buffered saline–Tween buffer) for at least 3 h overnight. Following staining, embryos mounted in 80 or 100 per cent glycerol were visualized and photographed under a Zeiss Axioskop 2 microscope. All digital images were processed with Adobe Photoshop, with adjustments to brightness, colour balance and contrast being made uniformly across the entirety of each image.

3. RESULTS

The ancient homeobox genes that are the subject of this study have been identified previously and classified based upon phylogenetic reconstruction (Holland et al. 2008; Takatori et al. 2008). As with most ancient homeobox gene families, phylogenetic classification at the family level is robust and reflects the conservation of diagnostic residue combinations within the homeodomain (figure 1). The four genes studied here belong to three families: Nedx, Msx1 and NK7, and are all expressed in specific spatio-temporal patterns during amphioxus embryogenesis, only within the first day of amphioxus development.

(a) AmphiNedx expression

The amphioxus genome possesses two lineage-specific duplicates of Nedx: AmphiNedxa and AmphiNedxb (Takatori et al. 2008). We find that both are expressed in an overlapping pattern solely in the neurula stage of amphioxus embryogenesis, although AmphiNedxb is expressed in a broader territory and at higher levels than AmphiNedxa. Expression is observed in the 9-somite neurula in a region of ventrolateral epidermis on the left-hand side of the anterior portion of the embryo (figure 2). At the 12-somite stage, approximately 3 h later, expression is no longer detectable (data not shown). At this stage in embryogenesis, many of the morphological features of the ventral pharyngeal region are yet to develop and the transient nature of the expression coupled with the lack of lineage tracing studies in amphioxus makes it hard to identify whether Nedx expression is confined to a particular organ-specific territory. In this regard, comparison with the previously reported expression patterns of AmphiPax3/7 (Holland et al. 1999), AmphiPax2/5/8 (Kozmik et al. 1999) and AmphiSix4/5 (Kozmik et al. 2007) is informative. AmphiPax2/5/8 is expressed in a number of tissues including asymmetrical structures. The strongest pharyngeal expression at neurula stages is endodermal and is observed in a region that at the early larval stage will mark the position of the mouth. In the early larva, as the mouth breaks through the body wall, the expression marks both the ectoderm and endoderm surrounding the mouth, which are in the process of fusing (Kozmik et al. 1999). In contrast, the expression of AmphiPax3/7 in the neurula stage is much broader throughout the mesendoderm in the anterior third of the embryo. In the mouth region of the early larva, it specifically marks the endoderm both dorsal and ventral of the mouth opening (Holland et al. 1999). AmphiSix4/5 is also widely expressed in the developing pharyngeal endoderm at neurula stages, but in and around the opening larval mouth its expression is confined to the endoderm located ventral to the mouth opening (Kozmik et al. 2007). The expression of the Nedx paralogues is comparable with the ventral half of the pharyngeal AmphiPax2/5/8 territory in the neurula stage, though it is epidermal. Potentially these ectodermal cells then proceed to locate to the region ventral of the mouth opening in a position adjacent to the AmphiSix4/5-expressing cells, though this is speculative as by the larval stages of development Nedx expression is not detectable by in situ hybridization.
(b) **AmphiMsxlx expression**
AmphioxusMsxlx is expressed in two territories during amphioxus embryogenesis: the anterior central nervous system and the left anterior gut diverticulum of the nine-somite neurula (figure 3). The neural expression is in the ventral half of the neural tube and is situated at the same anteroposterior level as the neuropore, within the territory that will develop into the cerebral vesicle. Expression does not extend to the anterior tip of the cerebral vesicle but is within the anterior vesicle, which is putatively homologous to the vertebrate diencephalon (Lacalli 2008). A second site of **AmphiMsxlx** expression is in the anterior endoderm. **AmphiMsxlx** is expressed here solely on the left-hand side in Hatschek’s left diverticulum, which will go on to fuse with the ectoderm at the left-hand body wall and form the larval pre-oral pit, the amphioxus homologue of the vertebrate adenohypophysis (Candiani et al. 2008).

(c) **AmphiNKx7 expression**
**AmphiNKx7**, the amphioxus orthologue of the NK7.1 gene of *Drosophila*, is also expressed in a left-sided territory in the anterior endoderm and an anterior neural territory in the region that will develop into the cerebral vesicle (figure 4) in the neurula stage. The expression of **AmphiNKx7** is slightly less transient than the other genes discussed here and its expression is detectable at very low levels in the neural ectoderm just after hatching (roughly 12 h post-fertilization) and persists in the 12-somite stage (figure 4), though is not detectable at the time of mouth opening at 24 h post-fertilization (data not shown). The endodermal expression of **AmphiNKx7** is qualitatively weaker than the neural expression and diffusely covers a relatively extended area, especially at the 12-somite stage (figure 4).

4. **DISCUSSION**
(a) **Gene loss and the rewiring of gene-regulatory networks**
The expression of **AmphiNedxa**, **AmphiNedxb**, **AmphiMsxlx** and **AmphiNKx7** has important implications for the evolution of the structures in which they are expressed: the anterior central nervous system and the pharynx. The expression of **AmphiMsxlx** in Hatschek’s left anterior diverticulum, and its inferred role in the early development of the pre-oral pit along with **POUIF1/Pir-1**, is an instance where expression resides within a structure that has vertebrate homologues, in this case the adenohypophysis (Candiani et al. 2008). A scenario can be envisaged whereby amphioxus represents the ancestral condition and the loss of **Msxlx** expression from this territory in vertebrates was associated with a change in the function of the ancestral adenohypophysis-like organ from external secretion to internal secretion of peptide hormones. An alternative possibility is that the function of **Msxlx** in the homologue of the adenohypophysis is a derived feature of amphioxus embryogenesis. Whether **Msxlx** exists and performs a similar function in hemichordates, perhaps in the protocoel and proboscis pore, will be of considerable importance in choosing between these hypotheses and in clarifying the evolution of the coelomopore complex and an adenohypophysis-like organ.

A whole suite of genes have been found to be expressed in the pre-oral pit including **AmphiEomes/Tbr1** (Satoh et al. 2002), **AmphihairA**, **AmphihairD** (Minguillón et al. 2002), **AmphiNKx7**, and **AmphiMsxlx**.
et al. 2003), Amphithię (Kozmik et al. 2001), Amphi-POU1F1/Pit-1 (Candiani et al. 2008), Amphihx3 (Wang et al. 2002) and AmphiPax-6 (Glardon et al. 1998). Importantly, the expression of AmphiPOU1F1/Pit-1, which is one of the earliest specific markers of the ver- tebrate adenohypophysis and amphioxus pre-oral pit (Candiani et al. 2008) is first detected at the same stage as the AmphiMsxlx expression reported here, implying that AmphiMsxlx is one of the earliest genes active in the initial phase of the development of this organ. Two of the genes mentioned above, namely Amphihx3 and AmphiPax-6 are expressed in the same territories as AmphiMsxlx in both the neuroectoderm and the endo- derm. Thus, AmphiMsxlx may act as part of a regulatory circuit of transcription factors in concert with these genes during the development of both the pre-oral pit and the brain of amphioxus.

In common with the AmphiMsxlx gene, AmphiNKx7 and the two Nedx paralogues—AmphiNedxa and Amphi- Nedxb—are expressed asymmetrically on the left-hand side during early pharyngeal development. However, the asymmetrical expression of the latter three genes is more caudal than that of AmphiMsxlx, in the regions where the mouth and first gill slit will later break through the body wall. The endodermal expression of AmphiNKx7 is at the same anteroposterior position within the embryo as the ectodermal Nedx expression and accords well with the pharyngeal expression of AmphiPax2/5/8 (Kozmik et al. 1999). In addition, the pattern is almost identical to that of AmphiNK2-2 in this region, though this gene is expressed in the mid- and hindgut also (Holland et al. 1998). It is tempting to speculate therefore that the function of AmphiNKx7, the AmphiNedx genes, AmphiPax2/5/8 and AmphiNK2-2 are intertwined in specifying aspects of the asymmetric early development of pharyngeal structures in the amphioxus embryo, and that NKx7 and Nedx have been lost in the vertebrate and ascidian lineages because of modulations in the developmental programme controlling pharyngeal development.

The expression of Nedx has also been examined in Drosophila (Nedx is CG13424 in D. melanogaster) where it is specific to developing larval muscle, initially in the thoracic segments but later in all segments (Tomancak et al. 2007). It has recently been found to be a direct target of Twist, the central transcription factor in the early development of Drosophila mesoderm (Sandmann et al. 2007). The amphioxus Twist gene is likewise expressed during early mesodermal differentiation (Yasui et al. 1998) and its expression does not overlap with Nedx, implying that Nedx possesses distinct, non- homologous developmental roles in Drosophila and amphioxus.

The implication of changes in transcription factor net- works is mirrored in the brain. The anterior end of the neural tube in amphioxus has been homologized with the vertebrate anterior central nervous system based upon both neuroanatomical and developmental genetic studies (Williams & Holland 1996; Lecallie 2004; Holland 2005). In both taxa, the anterior border of the Hox gene- expressing hindbrain is caudal to a domain expressing Otx family genes (Williams & Holland 1996). The expression of AmphiMsxlx and AmphiNKx7 within the AmphiOtx-positive region of the cerebral vesicle suggests that these genes act to regionalize this structure in a manner that is accomplished in a different way in its putative verte- brate homologue, the diencephalon. The expression of AmphiNKx7 is located posterior to that of AmphiMsxlx and possibly the two genes act to regionalize the AmphiOtx-expressing cerebral vesicle. This makes the loss of these genes in vertebrates particularly striking as it implies profound regulatory change during the evol- ution of a well-conserved part of the ancestral chordate brain, the diencephalon (Williams & Holland 1996; Holland & Holland 1999).

(b) The gain (or loss) of asymmetry
The profound asymmetry of the amphioxus embryo has to date been largely ignored in molecular terms owing to the understandable concentration upon those many features that are conserved between amphioxus and other bilaterians, particularly the vertebrates. Traditional embryological study has led to the hypothesis that the mouth and the club-shaped gland represent modified gill slits (Whittaker 1997), a theory that is supported by the presence of Hatschek’s nephron above the mouth, coupled with the actual gill slit pairs each being accompanied by a pair of nephrons, and the fact that the club-shaped gland has no known homologue in other chordate phyla. The corollary is that the original primordial mouth has been lost, with the implication that the asymmetric nature of amphioxus is secondarily derived.

Data from developmental genetic studies over the past decade have further refined the question of the origin of amphioxus pharyngeal asymmetry with many genes pos- sessing sites of expression on one side of the pharyngeal
endoderm in addition to sites in other developmental contexts that are conserved with vertebrates. Crucially though, the ectodermal invagination that will form the mouth, the stomodeum, has been homologized across chordates (Christiaen et al. 2007) using the expression of Pitx downstream of conserved ventralizing BMP signalling (Yu et al. 2007). This pattern of mouth development, and a loss of Brachyury and Goosecoid expression, is distinct from non-chordate deuterostomes and protostomes (Arendt et al. 2001; Christiaen et al. 2007) and has led to the suggestion that a ‘new mouth’ is a chordate innovation (Christiaen et al. 2007; Hejnol & Martindale 2008).

In addition to its role in chordate oral ectoderm specification, Pitx involvement in left–right asymmetry downstream of Nodal signalling is conserved across deuterostomes (Duboc & Lepage 2008) and probably Bilateria (Grande & Patel 2009). It has thus been hypothesized that the evolution of the ‘new’ chordate mouth was the result of the evolution of a stomodeal Pitx expression domain that was under the control of BMP signalling and distinct from the asymmetric domain of Pitx expression that was downstream of Nodal (Christiaen et al. 2007). Evidence for the distinct regulation of these domains of expression has been uncovered in both mouse (Goodyer et al. 2003; Shiratori et al. 2006) and Ciona (Christiaen et al. 2005).

Against this background, the correspondence between the expression of AmphimMsxl, AmphimNedxa, AmphimNedxb and AmphimNkx7 and the subsequent asymmetric development of the pharyngeal apparatus in amphioxus is striking, and the expression is likely, from its spatial and temporal profile, to be downstream of the conserved Nodal–Pitx symmetry-breaking pathway (Yasui et al. 2000; Boorman & Shimeld 2002; Yu et al. 2002). We propose that the morphogenetic asymmetry of the amphioxus embryo/larva is dependent upon these ancient homeobox genes that are integrated downstream of the ancestral symmetry-breaking pathway. Under this hypothesis, amphioxus would represent an ancestral developmental gene-regulatory programme, where Pitx is expressed asymmetrically and is responsible for directing subsequent asymmetric pharyngeal (including oral and pre-oral) development, with Msxl, Nedx and NKx7 being part of the ancestral, asymmetric pharyngeal patterning system.

In Olfactores (vertebrates + urochordates), the stomodeal function of Pitx (which is under the control of BMP signalling) has dissociated from the asymmetric function (under the control of Nodal signalling), and this correlates with the loss of asymmetry in pharyngeal development (figure 5). The loss of the genes examined here from vertebrates, would thus be correlated with a loss of ancestral pharyngeal developmental programmes that paved the way for the evolution of the neural crest-dependent pharyngeal development characteristic of vertebrates (Gans & Northcutt 1983; Graham 2001; Ota et al. 2007). In light of this suggestion, the expression of the Oikopleura dioica Nedx paralogues, which represent the only one of the ANTP-class homeobox families discussed here that is retained in a member of Olfactores, will be of considerable interest. A direct prediction of the model presented here would be that the Oikopleura...

Figure 5. A hypothesis for the evolution of pharynx development. The evolution of chordates was marked by the relocation of the mouth to a Pitx-expressing territory, the expression of which may have been controlled by BMP signalling (Olfactores) or Nodal signalling (amphioxus). In the Olfactores lineage, the pharynx has undergone significant modification with the loss of ancestral pharyngeal developmental programmes that paved the way for the evolution of the neural crest-dependent pharyngeal development characteristic of vertebrates (Gans & Northcutt 1983; Graham 2001; Ota et al. 2007). In light of this suggestion, the expression of the Oikopleura dioica Nedx paralogues, which represent the only one of the ANTP-class homeobox families discussed here that is retained in a member of Olfactores, will be of considerable interest. A direct prediction of the model presented here would be that the Oikopleura...
genes are expressed in a novel and species-specific fashion that is distinct from BMP-dependent oral Pitx expression.

Of course, it remains possible that amphioxus pharyngeal development is derived relative to the chordate ancestor, with the asymmetric role of the genes examined here representing a co-option of ancient genes for a novel function. To resolve this uncertainty, comparative developmental data from non-chordate phyla will be necessary, especially from enteropneusts, which pattern their anteroposterior and dorsoventral axes in a comparable way to chordates (Lowe et al. 2003, 2006) and represent a crucial reference for reconstructing the ancestral deuterostome (Gerhart 2006), assuming that the genes discussed here are present in enteropneust genomes. Interestingly, an expression domain of Pitx in the model hemichordate Saccoglossus kowalevskii (Lowe et al. 2006) is reminiscent of the position of the chordate mouth in the conserved ectodermal expression map, and corresponds to the site where the proboscis pore later forms, the structure that indicates left–right asymmetry in this animal (Lowe et al. 2003).

5. CONCLUSION

We have described the expression of four genes from three ancient ANTP-class homeobox families in the basal chordate lineage of amphioxus. These three ancient gene families were all lost during vertebrate evolution. It is striking that the expression of these ‘lost’ genes is both transient and spatially restricted, and the structures that the genes are expressed in amphioxus have been extensively modified during chordate evolution, namely the pharynx and the anterior CNS. This raises the distinct possibility that these genes have been ‘sidetracked’ into restricted developmental roles that were dispensed with during vertebrate evolution. The role of gene loss in the evolution of development has not been widely considered (reviewed in De Robertis 2008), but loss of ancient genes is clearly widespread (e.g. Kortschak et al. 2003; Wyder et al. 2007; Kuraku et al. 2008; Takahashi et al. 2009). A more complete appreciation of the role of gene loss in evolutionary developmental biology will come from extending the type of comparative genomic and embryological work highlighted here.

Irrespective of the ancestral role of Msx1, Neds and NKx7, it is clear that the evolution of the vertebrates was accompanied not just by the addition of developmental networks and structures (like the telencephalon and neural crest) onto a pre-existing amphioxus-like body plan, but that some ancestral gene-regulatory networks were dismantled leading ultimately to the loss of some of the constituent genes.

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REFERENCES

Arendt, D., Technau, U. & Wittbrodt, J. 2001 Evolution of the bilaterian foregut. Nature 409, 81–85. (doi:10.1038/3501075)

Beaster-Jones, L., Kaltenbach, S. L., Koop, D., Yuan, S., Chastain, R. & Holland, L. Z. 2008 Expression of somite segmentation genes in amphioxus: a clock without a wavefront? Dev. Genes Evol. 218, 599–611. (doi:10.1007/s00427-008-0257-5)

Boorman, C. J. & Shimeld, S. M. 2002 Pitx homeobox genes in S. purpuratus and amphioxus. How left–right asymmetry is conserved. Curr. Opin. Genet. Dev. 17, 422–427. (doi:10.1016/j.gde.2007.07.008)

Duboc, V. & Lepage, T. 2008 A conserved role for the nodal signalling pathway in the establishment of dorso-ventral and left-right axes in deuterostomes. J. Exp. Zool. B (Mol. Dev. Evol.) 310, 41–53.

Dufour, B. 2007 The rise and fall of Hox gene clusters. Development 134, 2549–2560. (doi:10.1242/dev.001065)

Dubin, D. E. K., Devar, K., Cook, A., Chang, J. L., Hill-Force, A. & Amemiya, C. 2005 The chordate Parahox cluster. Curr. Biol. 15, R820–R822. (doi:10.1016/j.cub.2005.10.014)
insights into chordate myogenesis, neurogenesis, and the possible evolutionary precursor of definitive vertebrate neural crest. *Evol. Dev.* 1, 153–165. (doi:10.1046/j.1525-142x.1999.99019.x)

Holland, L. Z. et al. 2008 The amphioxus genome illuminates vertebrate origins and cephalochordate biology. *Genome Res.* 18, 1100–1111. (doi:10.1101/gr.073676.107)

Holland, N. D. & Chen, J. 2001 Origin and early evolution of the vertebrates: new insights from advances in molecular biology, anatomy, and palaeontology. *Bioessays* 23, 142–151. (doi:10.1022/1521-1878(200102)23:2<142::AID-BIES1021>3.0.CO;2-5)

Horton, A. C. et al. 2008 Conservation of linkage and evolution of developmental function within the Tbx23/4/5 subfamily of T-box genes: implications for the origin of vertebrate limbs. *Dev. Genes Evol.* 218, 613–628. (doi:10.1007/s00427-008-0249-5)

Kortschak, R. D., Samuel, G., Saint, R. & Miller, D. J. 2003 EST analysis of the cnidarian *Acropora millepora* reveals extensive gene loss and rapid sequence divergence in the model invertebrates. *Curr. Biol.* 13, 2190–2195. (doi:10.1016/j.cub.2003.11.030)

Kozmik, Z., Holland, N. D., Kalousova, A., Paces, J., Schubert, M. & Holland, L. Z. 1999 Characterisation of an amphioxus paired box gene, *AmphiPax2/5/8*: developmental expression patterns in optic support cells, nephridium, thyroid-like structures and pharyngeal gill slits, but not in the midbrain-hindbrain boundary region. *Development* 126, 1295–1304.

Kozmik, Z., Holland, L. Z., Schubert, M., Lacalli, T. C., Kreslova, J., Vícek, C. & Holland, N. D. 2001 Characterisation of amphioxus *AmphiKout*, an evolutionarily conserved marker for chordate ventral mesoderm. *Genesis* 29, 172–179. (doi:10.1002/gene.1021)

Kozmik, Z. et al. 2007 *Pax–Six–Eya–Dach* network during amphioxus development: conservation *in vivo* but context specificity in *vivo*. *Dev. Biol.* 306, 143–159. (doi:10.1016/j.ydbio.2007.03.009)

Kuraku, S., Takio, Y., Tamura, K., Aono, H., Meyer, A. & Kuratani, S. 2008 Noncanonical role of Hox14 revealed by its expression patterns in lamprey and shark. *Proc. Natl Acad. Sci. USA* 105, 6679–6683. (doi:10.1073/pnas.0710947105)

Lacalli, T. C. 2004 Sensory systems in amphioxus: a window on the ancestral chordate condition. *Brain Behav. Evol.* 64, 148–162. (doi:10.1159/000079744)

Lacalli, T. C. 2008 Basic features of the ancestral chordate brain: a protochordate perspective. *Brain Res. Bull.* 75, 319–323. (doi:10.1016/j.brainresbull.2007.10.038)

Lacalli, T. C. & Kelly, S. J. 2000 The infundibular balance organ in amphioxus larvae and related aspects of cerebral vesicle organisation. *Acta Zool.* 81, 37–47. (doi:10.1046/j.1463-6395.2000.0036.x)

Lowe, C. J., Wu, M., Salic, A., Evans, L., Lander, E., Stange-Thomann, N., Gruber, C. E., Gerhart, J. & Kirschner, M. 2003 Anteroposterior patterning in hemichordates and the origins of the chordate nervous system. *Cell* 113, 853–865. (doi:10.1016/S0092-8674(03)00469-0)

Lowe, C. J. et al. 2006 Dorsoventral patterning in hemichordates: insights into early chordate evolution. *PloS Biol.* 4, e291. (doi:10.1371/journal.pbio.0040291)

Mazet, F., Amemiya, C. T. & Shimeld, S. M. 2006 An ancient Fox gene cluster in bilaterian animals. *Curr. Biol.* 16, R314–R316. (doi:10.1016/j.cub.2006.03.088)

Mingüllón, C., Jiménez-Delgado, S., Panopoulou, G. & García-Fernández, J. 2003 The amphioxus Hairy family: differential fate after duplication. *Development* 130, 5903–5914. (doi:10.1242/dev.00811)
Ogasawara, M. 2000 Overlapping expression of amphioxus homologues of the thyroid transcription factor-1 gene and thyroid peroxidase gene in the endostyle: insight into the evolution of the thyroid gland. *Dev. Genes Evol.* **210**, 231–242. (doi:10.1007/s00427-005-0309)

Osborne, P. W., Benoit, G., Laudet, V., Schubert, M. & Ferrier, D. E. K. 2009 Differential regulation of ParaHox genes by retinoic acid in the invertebrate chordate amphioxus (*Branchiostoma floridae*). *Dev. Biol.* **327**, 252–262.

Ota, K. G., Kuraku, S. & Kuratani, S. 2007 Hagfish embryology with reference to the evolution of the neural crest. *Nature* **446**, 672–675. (doi:10.1038/nature05633)

Paris, M. et al. 2008a Amphioxus postembryonic development reveals homology of chordate metamorphosis. *Curr. Biol.* **18**, 825–830. (doi:10.1016/j.cub.2008.04.078)

Paris, M., Brunet, F., Markov, G. V., Schubert, M. & Lau, V. 2008b The evolution of the thyroid hormone signalling pathway. *Dev. Genes Evol.* **218**, 667–680. (doi:10.1002/dge.2008-0025-5-7)

Putnam, N. H. et al. 2008 The amphioxus genome and the evolution of the chordate karyotype. *Nature* **453**, 1064–1071. (doi:10.1038/nature06967)

Ryan, J. F., Burton, P. M., Maza, M. E., Kwon, G. K., Mullikin, J. C. & Finnerty, J. R. 2006 The cnidarian-bilaterian ancestor possessed at least 56 homeoboxes: evidence from the starlet sea anemone, *Nematostella vectensis*. *Genome Biol.* **7**, R64. (doi:10.1186/gb-2006-7-7-r64)

Sandmann, T., Girardot, C., Brehme, M., Stole, V. & Furlong, E. E. M. 2007 A core transcriptional network for early mesoderm development in *Drosophila melanogaster*. *Genes Dev.* **21**, 436–449. (doi:10.1101/gad.1509007)

Satoh, G., Takeuchi, J. K., Yasui, K., Tagawa, K., Saiga, H., Zhang, S. & Satoh, N. 2002 *Amphi-Boneless/Tbr1*: an amphioxus cognate of vertebrate *Homeobox* and *T-Brain1* genes whose expression reveals an evolutionarily distinct domain in amphioxus development. *J. Exp. Zool. B* (Mol. Dev. Evol.) **294**, 136–145. (doi:10.1002/jez.10149)

Schubert, M., Holland, L. Z., Stokes, M. D. & Holland, N. D. 2001 Three amphioxus Wnt genes (*AmphiWnt3*, *AmphiWnt5*, and *AmphiWnt6*) associated with the tail bud: the evolution of somitogenesis in chordates. *Dev. Biol.* **240**, 262–273. (doi:10.1006/dbio.2001.0460)

Schubert, M., Brunet, F., Paris, M., Bertrand, S., Benoit, G. & Lau, V. 2008 Nuclear hormone receptor signalling in amphioxus. *Dev. Genes Evol.* **218**, 651–665. (doi:10.1007/s00427-008-0251-y)

Shimeld, S. M. 2008 C2H2 zinc finger genes of the *Gli, Zic, KLF, SP, Wilms’ tumour, Hoxcbein, Snai1, Ovo, Spald, Odd, Blimp1, Foz* and related gene families from *Branchiostoma floridae*. *Dev. Genes Evol.* **218**, 639–649. (doi:10.1007/s00427-008-0248-6)

Shiratori, H., Yashiro, K., Shen, M. M. & Hamada, H. 2006 Conserved regulation and role of *Ptx2* in *in situ*-specific morphogenesis of visceral organs. *Development* **133**, 3015–3025. (doi:10.1242/dev.02470)

Takahashi, T., McDougall, C., Troscianko, J., Chen, W.-C., Jayaraman-Nagarajan, A., Shimeld, S. M. & Ferrer, D. E. K. 2009 An EST screen from the annelid *Pomatoceros lamarckii* reveals patterns of gene loss and gain in animals. *BMC Evol. Biol.* **9**, 240. (doi:10.1186/1471-2148-9-240)

Takatori, N., Butts, T., Candiani, S., Pestarino, M., Ferrier, D. E. K., Saiga, H. & Holland, P. W. H. 2008 Comprehensive survey and classification of homebox genes in the genome of amphioxus, *Branchiostoma floridae*. *Dev. Genes Evol.* **218**, 579–590. (doi:10.1002/dge.2008-00245-9)

Tomancak, P., Berman, B. P., Beaton, A., Kwan, R. W. E., Hartenstein, V., Celniker, S. E. & Rubin, G. M. 2007 Global analysis of gene expression during *Drosophila* embryogenesis. *Genome Biol.* **8**, R145. (doi:10.1186/gb-2007-8-7-r145)

Wang, Y., Zhang, P.-J., Yasui, K. & Saiga, H. 2002 Expression of *Bblhx3*, a LIM homeobox gene, in the development of amphioxus *Branchiostoma belcheri tingtawense*. *Mech. Dev.* **117**, 315–319. (doi:10.1016/S0925-4773(02)00197-1)

Whittaker, J. R. 1997 Cephalochordates, the lancelets. In *Embryology: constructing the organism* (eds S. F. Gilbert & A. M. Rainio), pp. 365–381. Sunderland, MA: Sinauer Associates Inc.

Williams, N. A. & Holland, P. W. H. 1996 Old head on young shoulders. *Nature* **383**, 490. (doi:10.1038/38349oa0)

Wyder, S. et al. 2007 Quantification of ortholog losses in insects and vertebrates. *Genome Biol.* **8**, R242. (doi:10.1186/gb-2007-8-11-r242)

Yasui, Zhang, S.-C., Uemura, M., Aizawa, S. & Ucki, T. 1998 Expression of a twist-related gene, *BhTwist*, during the development of a lancelet species and its relation to cephalochordate anterior structures. *Dev. Biol.* **195**, 49–59. (doi:10.1006/dbio.1997.8834)

Yasui, K., Zhang, S., Uemura, M. & Saiga, H. 2000 Left-right asymmetric expression of *BhPtx*, a *Ptx*-related gene, in a lancelet species and the developmental left-sidedness in deuterostomes. *Development* **127**, 187–195.

Yu, J. K., Holland, L. Z. & Holland, N. D. 2002 An amphioxus nodal gene (*AmphiNodal*) with early symmetrical expression in the organiser and mesoderm and later asymmetrical expression associated with left-right axis formation. *Dev. Biol.* **240**, 418–425. (doi:10.1006/dbio.2002.02030.x)

Yu, J. K., Satou, Y., Holland, N. D., Shin-I, T., Kohara, Y., Satoh, N., Bronner-Fraser, M. & Holland, L. Z. 2007 Axial patterning in cephalochordates and the evolution of the organizer. *Nature* **445**, 613–617. (doi:10.1038/nature05472)

Yu, J. K., Mazet, F., Chen, Y. T., Huang, S. W., Jung, K. C. & Shimeld, S. M. 2008 The Fox genes of *Branchiostoma floridae*. *Dev. Genes Evol.* **218**, 629–638. (doi:10.1007/s00427-008-0229-9)