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Research Paper

The Amphioxus *SoxB* Family: Implications for the Evolution of Vertebrate Placodes

Daniel Meulemans and Marianne Bronner-Fraser

Division of Biology, Beckman Institute MC 139-74, California Institute of Technology, Pasadena, CA 91125, USA

Correspondence to: Dr. Daniel Meulemans, ph. 626 395 3361, fax. 626 449 8599, dm@caltech.edu

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Cranial placodes are regions of thickened ectoderm that give rise to sense organs and ganglia in the vertebrate head. Homologous structures are proposed to exist in urochordates, but have not been found in cephalochordates, suggesting the first chordates lacked placodes. *SoxB* genes are expressed in discrete subsets of vertebrate placodes. To investigate how placodes arose and diversified in the vertebrate lineage we isolated the complete set of *SoxB* genes from amphioxus and analyzed their expression in embryos and larvae. We find that while amphioxus possesses a single *SoxB2* gene, it has three *SoxB1* paralogs. Like vertebrate *SoxB1* genes, one of these paralogs is expressed in non-neural ectoderm destined to give rise to sensory cells. When considered in the context of other amphioxus placode marker orthologs, amphioxus SoxB1 expression suggests a diversity of sensory cell types utilizing distinct placode-type gene programs was present in the first chordates. Our data supports a model for placode evolution and diversification whereby the full complement of vertebrate placodes evolved by serial recruitment of distinct sensory cell specification programs to anterior pre-placodal ectoderm.

Key words: Evolution, development, chordates, vertebrates, placodes, amphioxus

1. INTRODUCTION

The evolution of active predation in the vertebrate lineage was facilitated by the appearance of anterior paired sense organs and ganglia. Developmentally, these structures are derived from tissues considered unique to vertebrate embryos, the neural crest and cranial placodes. However, recent evidence of putative placode homologs in urochordates[1, 2], and new molecular phylogenies positioning this subphylum as the vertebrate sister group[3, 4], have cast doubt on the status of placodes as vertebrate synapomorphies. Gene expression in the tunicate *Ciona* and the larvacean *Oikopleura* suggest that the ancestor of vertebrates and urochordates had one or two composite placodes homologous to the adenohypophyseal, olfactory, and otic. The full set of vertebrate placodes is thought to have arisen by partitioning of these ancestral placodes. The possible existence of proto-placodes in the prevertebrate chordate effectively pushes back the origins of placodes to before the urochordate/vertebrate split. In this context, the most relevant outgroup for understanding placode origins becomes the cephalochordata, the third chordate subphylum.

Regardless of phylogenetic position, the cephalochordates are widely accepted as possessing the most prototypically chordate body plan of the three subphyla[5-7]. The likely basal position of cephalochordates reinforces this view and the utility of cephalochordates as proxies for the ancestor of both urochordates and vertebrates. To date, there is no strong evidence for the existence of placodes in amphioxus except for the possible homology of the preoral pit to the vertebrate adenohypophyseal placode[8-11]. Unlike urochordates and vertebrates, no region of amphioxus ectoderm appears to combine generic placodal properties such as localized thickening, invagination, and delamination with sense organ formation. However, amphioxus, like other invertebrate deuterostomes, does possess an epidermal nerve plexus including individual sensory neurons. The relationship of this plexus to vertebrate placodes is unclear. Gans and Northcutt originally proposed that placodes and neural crest evolved by reorganization of such a primitive plexus [12, 13]. Northcutt later rejected this in favor of replacement of the plexus by a non-homologous system derived from neural crest and placodes[14], as suggested by Lacalli[15]. While likely non-homologous at the tissue level, Lacalli left open the possibility of cell-level homology between components of the amphioxus and vertebrate peripheral nervous systems[15].

To further investigate possible cell or gene network-level homologies between placodes and components of the amphioxus epidermal nerve plexus we examined the expression of *SoxB* genes in amphioxus. The *SoxB* family consists of *SoxB1* genes (vertebrate *Sox1,2,3*) and SoxB2 genes (*Sox14,21*)[16]. Both *SoxB1* and *SoxB2* genes are expressed broadly in the central nervous system (CNS) where they interact to drive neural induction and differentiation[17-19]. In the nascent peripheral nervous system (PNS), *SoxB1* genes mark a subset of cranial placodes [20-23] and

ectopic *Sox*3 is sufficient to induce supernumerary lens and otic vesicles [24]. *SoxB1* genes also control the final stages of sense organ differentiation, directly activating delta-crystallin expression in the embryonic lens [25].

Here we report the isolation and expression of the complete set of *SoxB* genes from amphioxus. We find that like vertebrates, amphioxus has three *SoxB1* genes, but like invertebrates, only possesses a single *SoxB2* gene. Phylogenetic and genomic analyses and gene expression suggest that amphioxus and vertebrate *SoxB1* paralogs arose via independent duplication events. In addition, we observe expression of an amphioxus *SoxB1* co-ortholog in two populations of putative epidermal sensory cells. In the context of placode marker homolog expression our data suggests that discrete subpopulations of sensory cells utilizing placode-type genetic programs predate the evolution of placodes. Taken together, these data support a model for placodal diversification via repeated recruitment of preexisting sensory cell programs to an anterior placodal primordium.

2. MATERIALS AND METHODS

Amphioxus Collection

Amphioxus adults (*Branchiostoma floridae*) were collected from Tampa Bay, Florida and electrostimulated to induce gamete release. Eggs were fertilized, and embryos were cultured and fixed per the methods of Holland et al. [26].

Isolation of amphioxus *SoxB* genes

The following completely degenerate primers were designed against the HMG box of vertebrate *Sox* E proteins: AAGCCBCAYGTIAARMGNCCIATGAA, and TAITCIGGGTRRTCYTTYTTRTGYTG. Using these primers, a 220 bp DNA fragment with high homology to vertebrate *SoxB2* genes was amplified by PCR from an amphioxus Lambda Zap II embryonic cDNA library (a gift from Jim Langeland). This fragment was then used to screen the plated library at low stringency (2XSSC/.1%SDS at 40˚C) for full-length cDNAs. Fourteen phagemid clones were isolated, excised, partially sequenced, and found to encode two different *SoxB* genes. The largest cDNAs of each were completely sequenced. Amphioxus *SoxB1* and *SoxB2* coding sequences were then used to search Amphioxus Genome Release v1.0 (Joint Genome Institute) for paralogs. An EST clone (clone ID bfne072e07) corresponding to amphioxus *SoxB1b* (gene model estExt_fgenesh2_pg.C_6920002) was obtained and sequenced (a gift from Jr Kai Yu).

Phylogenetic analysis

Full-length cDNA sequences were translated and their conceptual protein products were aligned to published sea urchin, ascidian, amphioxus, and vertebrate *Sox* group B sequences using ClustalX [27]. The *Sox* group E gene, chicken *Sox*8, was included as an outgroup. A bootstrapped Neighbor-Joining tree [28] and was then constructed using ClustalX, and drawn using NJplot [29]. Bootstrap values were calculated from 1000 resamplings of the alignment data. Maximum Likelihood trees and quartet-puzzling reliability scores were generated from the same alignment using TREE-PUZZLE [30]. Similar analyses were performed on truncated protein sequences encompassing only the conserved HMG domain.

In situ Hybridization

In situ hybridizations were as described previously [31]. Riboprobes were made against the entire transcript.

3. RESULTS AND DISCUSSION

The *SoxB* family in amphioxus

Using degenerate PCR and low-stringency library screening, we isolated two novel *SoxB* genes from amphioxus, one most similar to vertebrate *SoxB1* genes, the other to vertebrate *SoxB2* homologs. These genes were used to search Amphioxus Genome Release v1.0 for potential amphioxus paralogs. Two other putative *SoxB1* genes were identified. The first corresponded to the previously described *AmphiSox1/2/3[32]*. The second was a previously undescribed gene with high similarity to *AmphiSox1/2/3* located 9337bp downstream of *AmphiSox1/2/3* on the same genomic sequence scaffold. Due to sequence similarity and genomic context, this gene likely represents a recent amphioxus duplicate of *AmphiSox1/2/3*. Based on order of discovery, we propose renaming *AmphiSox1/2/3* as amphioxus *SoxB1a* and designating its most recent duplicate *SoxB1b*. The remaining amphioxus group B1 paralog is designated *SoxB1c*.

Bootstrapped Neighbor-Joining and Maximum Likelihood analyses group amphioxus *SoxB1c* and *SoxB2* with their respective vertebrate homologs at high confidence values (Figure 1A). In contrast, placement of amphioxus *SoxB1a* and *SoxB1b* within the *SoxB1* subgroup was only weakly supported by Neighbor-Joining and unsupported by Maximum Likelihood, despite high similarity to metazoan *SoxB1* genes when queried against GenBank. Similarly, a single *Ciona SoxB1* homolog failed to group with any other deuterostome *SoxB* sequences despite a clear affinity to *SoxB1* subgroup members. Phylogenies using only the conserved HMG domains gave similar results (data not shown), suggesting amphioxus *SoxB1a* and *SoxB1b* and *Ciona SoxB1* are fast-evolving members of the SoxB1 subgroup.

The failure of the three amphioxus *SoxB1* genes to group with any single vertebrate *SoxB1* paralog, and the presence of single *SoxB1* homologs in sea urchin and *Ciona*, suggests that amphioxus *SoxB1* genes arose by two lineage-specific duplication events. As indicated above, the similarity and genomic proximity of amphioxus *SoxB1a* and *SoxB1b* indicate they are the products of the most recent of these duplications, whereas *SoxB1c* arose during the first duplication. Several examples of similar amphioxus-specific gene duplications have been reported[33].

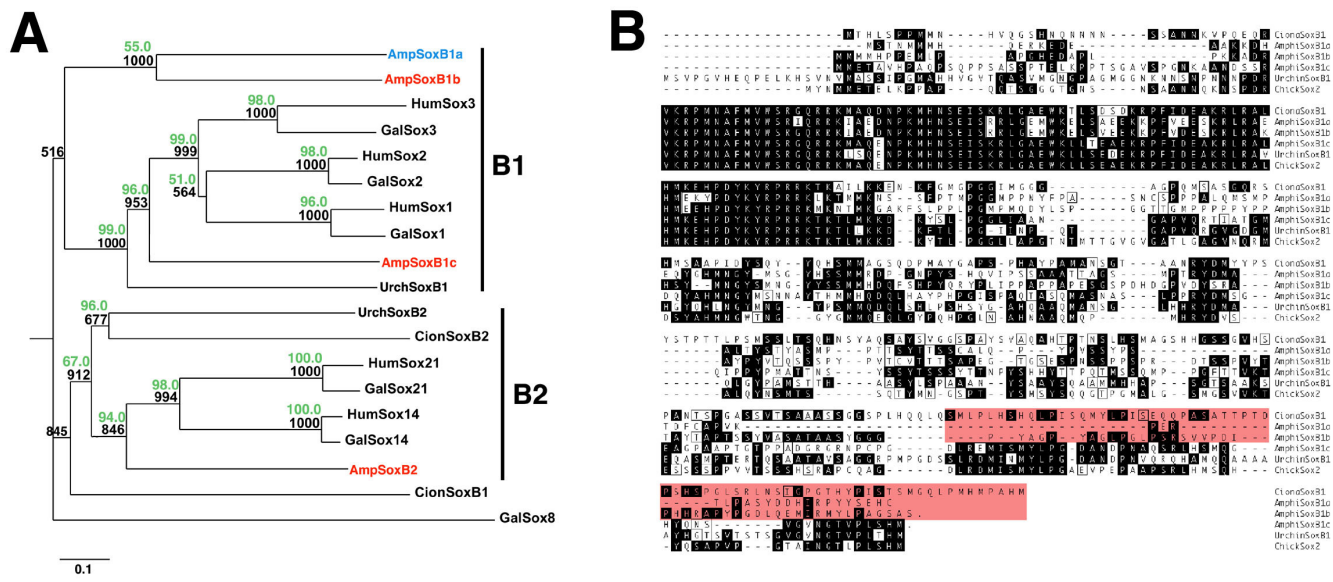

Figure 1. Phylogenetic tree and alignment of deuterostome SoxB proteins. (A) Phylogenetic tree created using the Neighbor-Joining method with chicken Sox8 as the outgroup. Black numbers at branch bases are confidence values derived from 1000 bootstrap resamplings of the alignment data. Sequence distance is indicated at the bottom left as substitutions per base. Numbers in green are quartet-puzzling reliability scores from Maximum Likelihood analysis of the same alignment. Amphioxus *SoxB1c* and amphioxus *SoxB2* group with their respective vertebrate homologs. Amphioxus *SoxB1a* and *SoxB1b* group together outside a clade including *SoxB1c* and vertebrate and sea urchin *SoxB1* genes. In both analyses, *Ciona SoxB1* failed to group with the other *SoxB1* genes. (B) Alignment of amphioxus, urchin, *Ciona* and chick *SoxB1* proteins. While all proteins are highly conserved in N-terminal HMG domain, the C-terminal transactivation domain is only conserved in chick *Sox2*, urchin *SoxB1* and amphioxus *SoxB1c*. Amphioxus *SoxB1a*, *SoxB1b* and *Ciona SoxB1* all have divergent transactivation domains, suggesting a similar loss of functionality.

Conserved roles for amphioxus *SoxB* genes in the CNS and gut

SoxB genes are required for the earliest steps of CNS formation in vertebrates [17] and *Drosophila* [34-36]. Consistent with an ancient role for *SoxB1* genes in neurectoderm specification, amphioxus *SoxB1* genes are all expressed in the nascent neural plate (Figure 2, Figure 3A-G) [32]. After neural induction and neurulation, vertebrate *SoxB1* genes also act to maintain progenitor cell identity in the developing CNS. Like its vertebrate cognates, amphioxus *SoxB1c* persists in the neural tube until larval stages (Figure 3H) suggestive of a conserved role in regulating neuronal differentiation. Like its vertebrate and *Drosophila* counterparts, amphioxus *SoxB2* is coexpressed with *SoxB1* paralogs in the developing CNS-- likely reflecting conserved interactions between these genes (Figure 4).

In vertebrates, *SoxB1* genes are expressed in the foregut and posterior-most hindgut [37-40]. Similar *SoxB1c* expression is seen in the foregut and hindgut of amphioxus, suggesting a highly conserved function in patterning the chordate endoderm (Figure 3, all panels). Low levels of amphioxus *SoxB2* transcripts are also detected in the endoderm at neurula stages (Figure 4, all panels). No similar expression is reported for vertebrate *SoxB2* genes, though the *Drosophila SoxB2* homolog, *dichaete,* is necessary for hindgut differentiation [41].

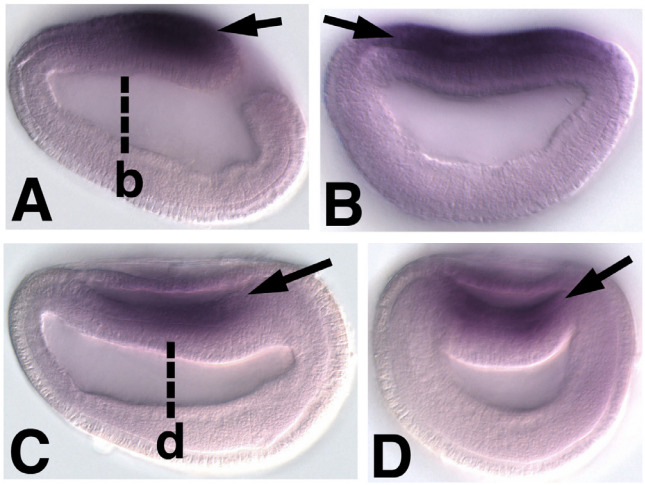

Figure 2. Embryonic expression of amphioxus *SoxB1b*. Anterior is to the left (A) Side view of 9-hour early neurula. *SoxB1b* expression is seen throughout the neural plate (arrow). (B) Optical cross section through 9-hour early neurula at approximately the level of b in A (arrow). (C) Side view of 12-hour neurula. *SoxB1b* is expressed in the neural plate as it rolls-up to form the neural tube (arrow). (D) Optical cross section through 12-hour neurula at the level of c in D. *SoxB1b* transcripts are detected in the neural plate (arrow) as it is overgrown by epidermal ectoderm.

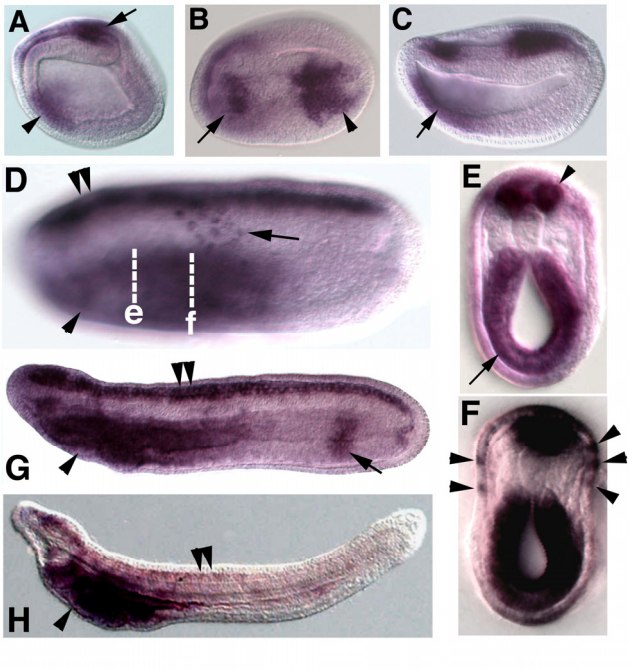

Figure 3. Embryonic expression of amphioxus *SoxB1c*. Anterior is to the left. (A) Side view of 9-hour early neurula. *SoxB1c* expression is seen in a patch of neurectoderm near the blastopore (arrow) and in anterior endoderm (arrowhead). (B) Dorsal view of 12-hour neurula, expression in restricted areas of the rostral (arrow) and caudal (arrowhead) neural plate. (C) Side view of 12-hour neurula. *SoxB1c* transcripts persist in the anterior gut (arrow). (D) Side view of 18-hour late neurula, focused in the plane of the epidermis. Scattered *SoxB1c* labelled cells are seen in the epidermal ectoderm (arrow). Out of focus, *SoxB1c* expression has expanded throughout the entire neural tube (double arrowheads) and marks the foregut (arrowhead). (E) Optical cross section through a bisected 18-hour neurula at the level of e in D. Strong expression is seen in the neural tube (arrowhead) and foregut (arrow). (F) Optical cross section through a bisected 18-hour neurula at the approximate the level of f in D. Arrowheads point to *SoxB1c*-positive cells in epidermis. (G) Side view of 24-hour late neurula. Expression in the neural tube (double arrowheads) and foregut (arrowhead) persists, and a band of expression also appears in the hindgut (arrow). (H) Side view of 36-hour late larva. Neural tube (double arrowheads) and hindgut expression have ceased while high levels of transcripts remain in the foregut (arrowhead).

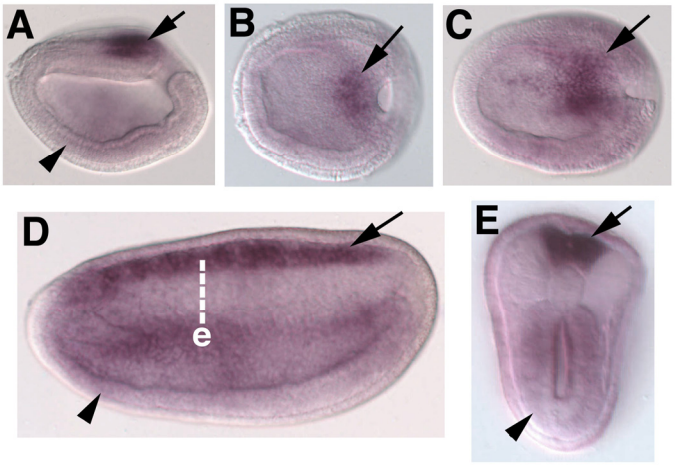

Figure 4. Embryonic expression of amphioxus *SoxB2*. Anterior is to the left. (A) Side view of 9-hour neurula. *SoxB2* transcripts are seen in the caudal-most neurectoderm (arrow) and weakly throughout the ventral mesendoderm (arrowhead). (B) Dorsal view of 9-hour early neurula. *SoxB2* transcripts are seen in a small region of neurectoderm bordering the blastopore (arrow). (C) Dorsal view of 12-hour neurula. *SoxB2* expression has begun to expand into the anterior neural plate (arrow). (D) Side view of 15-hour neurula. High levels of *SoxB2* transcripts are detected throughout the neural tube (arrow). Lower levels are seen in the gut (arrowhead). (E) Optical cross section through a bissected 15-hour neurula at the level of e in D. Strong *SoxB2* signal is observed in the neural tube (arrow), while lower levels persist in the gut (arrowhead). By 24 hours, detectable *SoxB2* expression has ceased (not shown).

Preservation of amphioxus *SoxB1* duplicates with and without obvious subfunctionalization

The duplication-degeneration-complementation (DDC) model predicts that partitioning of ancestral functions among paralogs after a duplication event will tend to preserve both duplicates, while redundancy will favor the maintenance of just one. In the case of amphioxus *SoxB1* paralogs we observe functional partitioning consistent with the DDC model, but also the apparent preservation of two redundant duplicates. Amphioxus *SoxB1a* is expressed throughout the nascent neural plate but is downregulated before the end of neurulation. It is also never expressed in the endoderm [32]. In contrast, early neurectodermal expression of amphioxus *SoxB1c* is restricted to rostral and caudal patches (Figure 3A-C) until the end of neurulation, when it expands throughout the CNS (Figure 3D). Furthermore, like vertebrate *Sox*2 and *Sox3*, amphioxus *SoxB1c* transcripts mark the foregut and hindgut (Figure 3F) and neurogenic ectoderm outside the CNS (discussed below). Thus, amphioxus *SoxB1a* appears to fulfill the early roles of *SoxB1* genes in neural induction, while amphioxus *SoxB1c* assumes later functions in the developing nervous system and gut. A similar partitioning of early and late functions between amphioxus-specific paralogs is seen in MRF genes [42].

The subfunctionalization of amphioxus *SoxB1* genes may explain the divergence of *SoxB1a* and *SoxB1b* proteins from other deuterostome *SoxB1* homologs, especially in the carboxy-terminal transactivation domain (Figure 1B)[43]. Motifs required for later neural and endodermal functions may have been lost in *SoxB1a*, resulting in a divergent sequence. Interestingly, this same domain is divergent in Ciona *SoxB1*, suggesting a similar loss of functionality possibly related to the abbreviated development of urochordates.

In addition to the clear subfunctionalization of

SoxB1c and *SoxB1a*, we also observed the redundant expression of *SoxB1a* and *SoxB1b* transcripts. Like amphioxus *SoxB1a*, amphioxus *SoxB1b* is expressed throughout the nascent neural plate (Figure 2) and is extinguished as *SoxB1c* is upregulated (Figure 3A-G). The sequence similarity and genomic proximity of *SoxB1a* and *SoxB1b* indicate they are the result of a relatively recent duplication event. One explanation for the coexpression of their transcripts is that one duplicate represents a transcribed pseudogene. Alternately, both paralogs may have been preserved to perform different late functions in adult tissues and their redundant early expression reflects responsiveness to the same embryonic enhancers.

Consistent with independent origins for vertebrate and amphioxus *SoxB1* paralogs, we noted differences in how the functions of the two sets of paralogs were partitioned during evolution. In vertebrates, all three *SoxB1* paralogs are expressed broadly in the early neural plate. Later, vertebrate *SoxB1* genes are expressed throughout nervous tissue but in progressively non-overlapping subsets of CNS and PNS neurons [37]. This contrasts with amphioxus in which partitioning of *SoxB1* paralogs was largely temporal, with *SoxB1a/b* being expressed from gastrula until early neurula stages, and *SoxB1c* being expressed from mid-neurula until larval stages. This difference in subfunctionalization strategy may reflect new roles for vertebrate *SoxB1* genes in contributing to the neural complexity of vertebrates.

Conservation of chordate sensory cell specification programs suggested by epidermal expression of amphioxus placode marker homologs

In the vertebrate PNS, *SoxB1* genes are expressed in a subset of neurogenic and non-neurogenic cranial placodes[44], suggestive of cell-type specific functions rather than a general neurogenic role. Anteriorly, vertebrate *SoxB1* genes mark the adenohypophysial, olfactory, and lens placodes. Posteriorly, they are expressed in the lateral line, otic and epibranchial placodes, but are excluded from the profundal or trigeminal placodes. Similarly, amphioxus *SoxB1c* expression is seen in specific subpopulations of general (non-CNS) ectoderm cells, likely presaging formation of two distinct groups of sensory cells. The first population consists of 10-12 cells confined along the anteroposterior axis to the middle third section of lateral epidermis at neurula and early larval stages (Figure 3D,F). This population appears to be a subset of the epidermal sensory cells marked by the more broadly expressed neural markers *Hu/Elav[45, 46]*, *ERR[47]*, *Trk[48]*, *b-tubulin[49]* and *Delta[50]* and the transcription factor *Brn3 (POU IV)[51]*. The second group of *SoxB1c* expressing cells surrounds the forming mouth in larvae (Figure 5A,B). These cells give rise to specialized mechanosensory cells (the oral spine cells) and associated peripheral neurons[15] (Figure 5C).

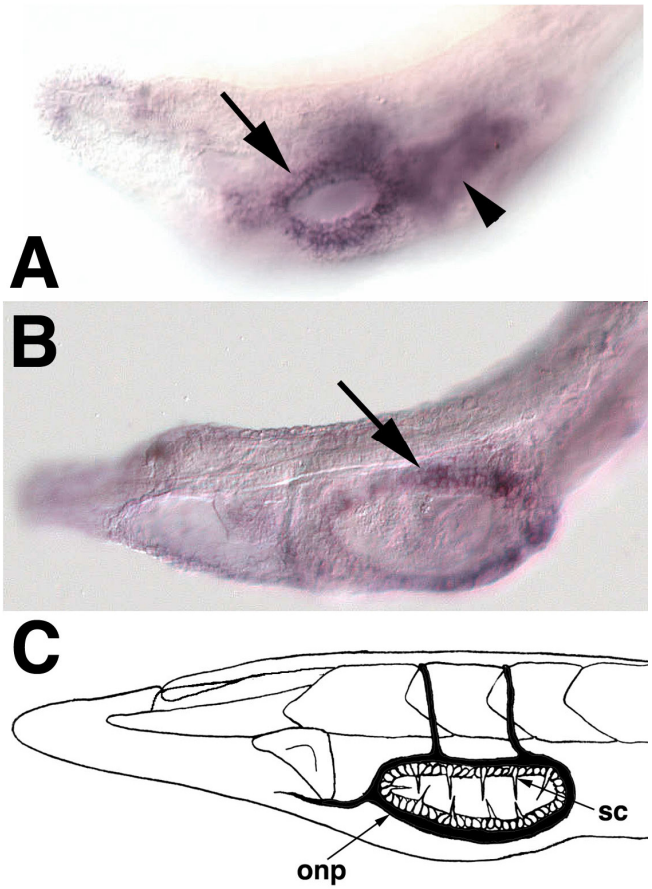

Figure 5. *SoxB1c* expression in oral ectoderm at early larval stages. Anterior is to the left (A) The head of a 2-day old larva focused in the plane of the epidermis. Expression is apparent in ectoderm surrounding the newly formed mouth (arrow). Out of focus is staining in the underlying pharyngeal endoderm (arrowhead).(B) In 3-day larvae, *SoxB1c* continues to mark ectoderm around the mouth corresponding to the future location of oral spine cells and neurons. (C) Schematic of oral spine cells, sc, and oral nerve plexus, onp, in 12-14 day larvae modified from Lacalli[63].

Other amphioxus homologs of vertebrate placode markers[44] are expressed in discrete ectodermal domains which generate particular sensory cell subtypes. Vertebrate *Pax6* genes are expressed in anterior placodes including the adenohypophyseal, olfactory, lens and trigeminal. In amphioxus, *Pax6* marks rostral ectoderm which gives rise to putative chemoreceptors expressing proteins similar in structure to vertebrate olfactory receptors[9, 15, 52]. Vertebrate *Msx* genes mark the trigeminal, profundal and otic placodes, as well as some lateral line placodes. Amphioxus *Msx* labels bilateral domains caudal to, and overlapping with, *Pax6*-positive epidermis which presages formation of putative pressure sensors [15, 53] (the corpuscles of de Quatrefages). Vertebrate *Pitx* genes collectively label the anteriormost cranial placodes including the adenohypophyseal, olfactory and lens placodes. In amphioxus, *Pitx* labels the preoral pit and, like *SoxB1c*, epidermis surrounding

the mouth which will generate oral spine cells and neurons[8, 54]. The vertebrate homologs of *AmphiPOUIV*, *Brn3a/d*, are early markers of posterior placodes including the trigeminal, profundal, lateral line and otic, then later mark the olfactory placode. As mentioned above, amphioxus *POUIV/Brn3* appears to mark most epidermal sensory cells in the trunk, the oral epidermis which will form the mouth nerve plexus[51], and a few putative chemosensory cells in the rostrum. Vertebrate *Six3* and *Six6* are expressed in the anteriormost placodes, including the adenohypophysis and olfactory. Amphioxus *Six3/6* is coexpressed with *Pax6* in the putative chemosensory epithelium and preoral pit[55]. Vertebrate *Coe/Olf1* genes mark neural derivatives of the olfactory, trigeminal and otic placodes [56, 57]. In amphioxus, *Coe* does not mark the putative olfactory epithelium, though it does mark epidermal sensory cells scattered along the length of neurulae and early larvae. Thus, like their vertebrate cognates, different placode marker homologs label subsets of ectoderm-derived peripheral sensory cells in amphioxus (Figure 6). Furthermore, the rostral-caudal extent of their expression appears partially conserved in both vertebrates and amphioxus. In both groups, *Pitx*, *Pax6*, and *Six3/6* mark anteriormost cell types, *SoxB1* and *Brn3* mark posterior cell types, and various combinations of these factors and *Msx* and *Coe* are expressed in intervening epidermal cells [44].

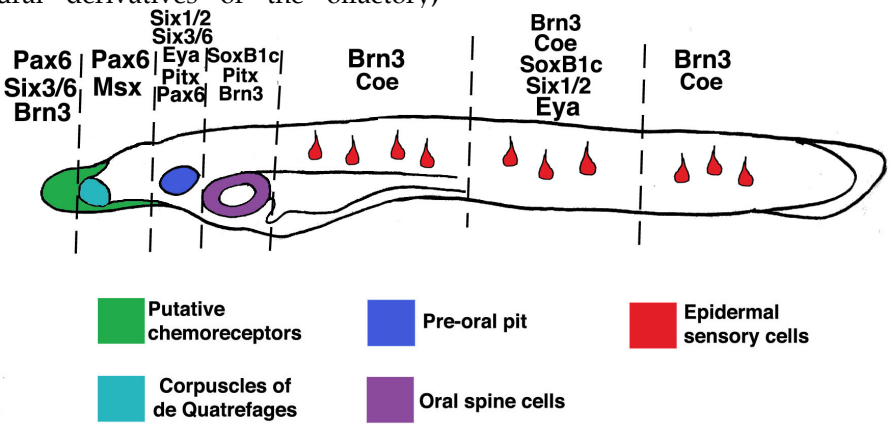

Figure 6. Expression of amphioxus placode marker homologs in subsets of epidermal sensory cells. *SoxB1c, Pitx, Msx, Pax6, Msx*, *Six1/2*, *Six3/6*, *Eya*, *Coe*, and *Brn3* homologs mark partially overlapping epidermal domains which give rise to putative sensory cells. Interestingly, the rostro-caudal extent of their expression corresponds roughly to that of their vertebrate cognates in cranial placodes. Though morphologically similar, epidermal sensory cells in the trunk (red) appear deploy different sets of genes. Taken together, these data suggest ancient functions for placode genes in sensory cell specification and a high level of sensory cell diversification in the first chordates.

A notable exception to this general trend is expression of *Six1/2* and *Eya* homologs. In vertebrates, *Six1*, *Six2*, and *Eya* mark virtually all placodes, while in amphioxus, these genes are only expressed in a subset of epidermal sensory cells and the preoral pit [55]. As in vertebrates, urochordate *Six1/2* and *Eya* homologs label broad ectodermal domains rather than individual cells[1, 2]. The expansion of *Six1/2* and *Eya* expression in urochordates and vertebrates relative to amphioxus may reflect expansion of generic placodal properties like the ability to thicken and invaginate. In the first chordates, these properties, and *Six/Eya* expression, may have been restricted to a pre-oral pit- like organ.

Similar expression of placode marker homologs implies a level of homology between sets of amphioxus epidermal sensory cells and some placodal derivates, though morphological evidence corroborating this is lacking. Most amphioxus epidermal sensory cells are non-descript ciliated mechanosensor-like cells grossly similar to several different vertebrate sensory cells[58, 59]. Others, like the corpuscles of de Quatrefages and oral spine cells, are highly derived and apparently unique to cephalochordates [15]. As previously noted [60], metazoan sensory cell morphology is highly plastic, leading to convergence and rapid divergence and confounding homology assignments. Thus, while placode marker expression in amphioxus and vertebrates suggests the activation of partially conserved sensory cell gene programs, it does not prove strict one-to-one homology between particular amphioxus and vertebrate cells. More accurately, subsets of amphioxus sensory cells likely represent cryptic homologs of particular placode-derived sense cells.

Conserved sensory cell gene programs are likely regulated by different patterning mechanisms in amphioxus and vertebrates.

In amphioxus, perturbing retinoic acid signaling causes changes in the antero-posterior position of epidermal sensory cells[61]. These changes mirror alterations in *Hox* gene expression caused by the same treatments, suggesting the antero-posterior patterning of amphioxus epidermal sensory cells may be

Hox-dependent. Consistent with this, epidermal sensory cells express different complements of *Hox* genes depending on their axial level, and *Hox1* perturbation mimicks retinoic acid perturbation in the central nervous system[61, 62]. It is possible that an epidermal *Hox* code activates expression of specific combinations of placode gene homologs which, in turn, drive differentiation of particular sensory cell subtypes at different axial levels in amphioxus. In vertebrates, level-appropriate placode specification does not appear to depend directly on retinoic acid or an epidermal *Hox* code. Rather, signals emanating from the neural tube and adjacent mesoderm, which are themselves patterned by retinoic acid and *Hox* genes, induce particular placodes at particular axial levels. Thus, though similar genetic machinery appears to drive sensory cell specification in vertebrates and amphioxus, different patterning mechanisms determine the final position of these cells in the ectoderm. It is likely that changes in the deployment of sensory cell specification programs in the vertebrate lineage involved changes in responsiveness to ectodermal patterning signals.

Diversification of cranial placodes by repeated recruitment of ancient sensory cell gene programs

Recent phylogenetic[3] and gene expression data from urochordates suggest this group diverged from vertebrates after the evolution of placodes. In both the ascidian *Ciona* and the larvacean *Oikopleura*, homologs of the placodal markers *Six*, *Eya*, and *Pitx* label ectodermal domains which undergo localized thickening, invagination, and give rise to sensory cells[1, 2]. The position of these proto-placodes in the larval head is consistent with the existence of two or three composite placodes putatively homologous to the adenohypophsial, olfactory, and otic placodes. In amphioxus, no region of thickened ectoderm appears to combine generic placodal properties with the ability to form sensory cells. However, amphioxus does possess non-neurogenic anterior ectoderm capable of invagination. The amphioxus pre-oral pit is an ectodermal structure formed by invagination. The pre-oral pit also expresses the pan-placodal markers *Six1/2* and *Eya*, and the adenohypophyseal placode markers *Pitx*, *Pax6*, and *Six3/6* as well as several adenohypophyseal hormones, leading to speculation that it is homologous to the adenohypophyseal placode[8-11]. Thus, anterior ectoderm displaying generic placodal properties, but incapable of generating sensory cells, was likely present in the first chordates.

Taken together, data from protochordates generally support the model proposed by Schlosser [10] whereby placodes evolved in an amphioxus-like ancestor with scattered epidermal sensory cells and anterior ectoderm capable of invagination (a putative adenohypophyseal placode). The recruitment of sensory cell specification programs to this anterior pre-placodal domain then occurred in the lineage leading to urochordates and vertebrates. Though it is controversial if urochordate and vertebrate placodes are homologous, gene expression and morphology suggest the full complement of vertebrate placodes evolved by partitioning of one or a few proto-placodes. The mechanism by which this may have occurred is unclear. Our data, along with previous studies, suggests placodal diversification arose via recruitment of pre-existing sensory cell specification programs to anterior pre-placodal ectoderm. We speculate that, in the common ancestor of vertebrates and amphioxus, epidermal retinoic acid and *Hox* patterning mechanisms activated placode marker homologs to specify particular sensory cell fates (Figure 7A). In the vertebrate lineage, these programs lost their dependence on epidermal patterning signals and gained responsiveness to inductive signals in the head. The recruitment of these primitive programs to anterior pre-placodal ectoderm, and their integration into novel gene regulatory networks, drove placodal diversification and the partitioning of proto-placodes (Figure 7B). In cephalochordates, these ancient programs were maintained in the epidermis to specify different sensory cell subtypes. In both lineages, divergence of sensory cell functionality and morphology occurred, obscuring overt evidence of common descent. Though similar expression of homologous genes in chordate sensory cells indicates conservation of some core genetic programming, it is unclear how deeply the gene networks are conserved across the three subphyla. Double-labeling is needed to show precisely which placode gene homologs are coexpressed in which amphioxus sense cells, and functional studies are needed to test if they interact in vertebrate-like gene networks. More rigorous correlation of amphioxus and ascidian placode marker homolog expression with sensory cell position, function, and morphology will also help define the sensory cell complement of the first chordates.

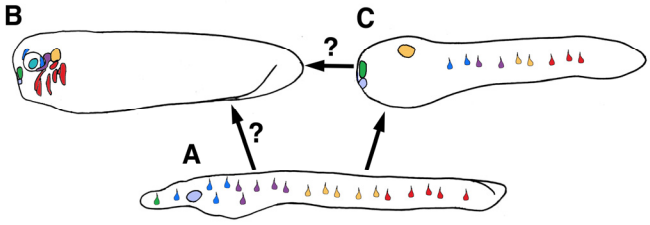

Figure 7. A model for the evolution of placodal diversity. Gene expression in amphioxus suggests placode marker homologs functioned primitively to specify an array of epidermal sensory cell fates in the first chordates (A). In the vertebrates lineage these genes were serially recruited to the anterior ectoderm, driving diversification of a pre-placodal primordium into the full complement of vertebrate placodes . This may have occurred by a loss of responsiveness to retinoic acid patternins mechanisms and a gain of responsiveness to inductive signals in the head. (B) Urochordates may have diverged at an intermediate phase in this process and thus possess some composite placodes putatively homologous to vertebrate cranial placodes, as well as epidermal sensory cells (C). Alternately, urochordate and vertebrate

placodes may represent parallel structures derived separately from similar sets of epidermal sensory cells.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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