Broken colinearity of the amphioxus Hox cluster

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Abstract

Background: In most eumetazoans studied so far, Hox genes determine the identity of structures along the main body axis. They are usually linked in genomic clusters and, in the case of the vertebrate embryo, are expressed with spatial and temporal colinearity. Outside vertebrates, temporal colinearity has been reported in the cephalochordate amphioxus (the least derived living relative of the chordate ancestor) but only for anterior and central genes, namely Hox1 to Hox4 and Hox6. However, most of the Hox gene expression patterns in amphioxus have not been reported. To gain global insights into the evolution of Hox clusters in chordates, we investigated a more extended expression profile of amphioxus Hox genes.

Results: Here we report an extended expression profile of the European amphioxus Branchiostoma lanceolatum Hox genes and describe that all Hox genes, except Hox13, are expressed during development. Interestingly, we report the breaking of both spatial and temporal colinearity for at least Hox6 and Hox14, which thus have escaped from the classical Hox code concept. We show a previously unidentified Hox6 expression pattern and a faint expression for posterior Hox genes in structures such as the posterior mesoderm, notochord, and hindgut. Unexpectedly, we found that amphioxus Hox14 had the most divergent expression pattern. This gene is expressed in the anterior cerebral vesicle and pharyngeal endoderm. Amphioxus Hox14 expression represents the first report of Hox gene expression in the most anterior part of the central nervous system. Nevertheless, despite these divergent expression patterns, amphioxus Hox6 and Hox14 seem to be still regulated by retinoic acid.

Conclusions: Escape from colinearity by Hox genes is not unusual in either vertebrates or amphioxus and we suggest that those genes escaping from it are probably associated with the patterning of lineage-specific morphological traits, requiring the loss of those developmental constraints that kept them colinear.

Keywords: Hox gene regulation, Hox cluster evolution, Amphioxus, Hox colinearity, Retinoic acid

Background

Hox genes code for a subfamily of homeodomain-containing transcription factors and have been found in all eumetazoans studied so far. Hox genes are responsible for giving the identity to morphological structures along the anterior-posterior (A-P) axis in most bilaterian animals [1-4]. Generally, these genes lie in the same genomic region and form gene clusters, usually one in invertebrates, and multiple clusters in vertebrates because of multiple rounds of genome duplication that took place at their origin (Figure 1) [5,6]. In most groups of animals, the position of Hox genes within any cluster corresponds with their mode of expression: genes placed more toward the 3′ end are expressed and pattern more anterior structures than do genes placed at the 5′ end. As a result, Hox genes are expressed along the A-P axis in a nested manner with more rostral limits for 3′ than for 5′ genes. This phenomenon is called spatial colinearity [7]. Moreover, in the case of vertebrates, the 3′ genes are expressed in earlier stages of the developing embryo than are 5′ genes in what is known as temporal colinearity [8,9]. The different combinations of Hox genes expressed in different structures along the A-P axis constitute what is called the Hox code [10]. It is believed that changes in the patterns of Hox expression are somehow responsible for the appearance of some vertebrate innovations, such as the elaboration of the segmentation of the hindbrain [11].
Chordates include the group olfactores (vertebrates and urochordates) and the cephalochordates [12] (Figure 1). However, urochordates, as a reflection of their highly reorganized genome and extensive gene losses, do not retain the typical clustered organization of Hox genes with only some genes linked, as in the ascidian *Ciona intestinalis* [13,14] (Figure 1), or as an atomized cluster, as is the case of the larvacean *Oikopleura dioica* [15] (Figure 1). Nonetheless, the cephalochordate amphioxus, representing the most basal branch of chordates, has a rather prototypical genome [16] and possesses a single cluster of 15 Hox genes, where all of them are transcribed in the same orientation, as in vertebrates [17]. Thus, amphioxus represents the best model to compare with vertebrates for illuminating the basal condition of chordates for both Hox content and regulation. However, the expression of amphioxus Hox genes is scarcely reported and studies have focused mainly on the anterior ones. The genes of the Floridian amphioxus *Branchiostoma floridae* Hox1-4 and Hox6 have been reported to be expressed during development in a colinear manner in the central nervous system (CNS), highlighting that, although it is not morphologically segmented as in vertebrates, the CNS in both groups to some extent conserves the same nested Hox system [18]. In addition, the expression patterns of Hox1, Hox3, and Hox4 have been reported in the epidermis and have been associated with the determination of different sensory neurons along the A-P axis [19]. Moreover, Hox1 mRNA is expressed in the middle part of the gut [20].

The acidic form of vitamin A, retinoic acid (RA), has an important role in the regionalization of morphological structures along the A-P axis of vertebrates, acting as the main posteriorizing factor during neural determination [21]. Its function is carried out in a gradient-dependent manner, with higher concentrations in posterior parts [22]. In the case of amphioxus, an excess of RA during development also causes changes of anterior into posterior identities, and the mouth and gill

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**Figure 1** Phylogenetic positions of cephalochordates, urochordates, and vertebrates, showing their Hox contents. The cephalochordate amphioxus represents the basal branch of chordates and possesses a single Hox cluster of 15 genes in both *B. floridae* and *B. lanceolatum*, although the whole genomic sequence of the latter has not been reported yet (indicated by dashed lines in the corresponding regions). Urochordates, the sister group of vertebrates, possess a disintegrated Hox cluster at different levels. Whereas the ascidian *Ciona intestinalis* has a highly disintegrated cluster with several reorganizations, the larvacean *Oikopleura dioica* has a completely atomized cluster where only two Hox9 genes remain linked, probably arising from an independent duplication. Vertebrates have multiple clusters, such as four in the mouse caused by two rounds of genome duplication (2R), or the seven clusters of zebrafish after a third teleost-specific round of genome duplication (3R). Squares represent Hox genes, with the same gray-scale colors indicating different paralogous groups (PG1 and PG2, PG3, central PG4-8, and posterior PG9-13/15). White boxes with dashed outlines represent pseudogenes.
slits do not form. Conversely, treatments with RA antagonists result in a caudal extension of the pharynx [23,24]. These effects caused by altered concentrations of RA during development are equivalent to those observed in vertebrate embryos and highlight that determination mechanisms of the structures along the A-P axis are somehow conserved in chordates. RA carries out its function by binding heterodimers of the RA receptor (RAR) and retinoid X receptor (RXR), which regulate the transcription of their target genes by binding to RA response elements (RAREs) in the regulatory regions of the genome. RAREs consist of two direct repeats (DRs) separated by a variable number of nucleotides. In the case of RAR/RXR heterodimers, they have been shown to bind DRs separated by one (DR1), two (DR2), or five (DR5) nucleotides [25]. RA has an important role in controlling Hox genes [26,27] via RAR/RXR binding to RAREs [28-30]. Besides, the anterior Hox genes in amphioxus are regulated by RA [18-20], and morpholino knock-down of Hox1 produces the same phenotype as treatment with an RA antagonist, indicating that Hox1 mediates the function of RA to establish the posterior limit of the pharynx [20]. Therefore, the regulation of anterior Hox genes by RA seems to be conserved between vertebrates and amphioxus, as suggested by heterologous reporter assays using regulatory regions of amphioxus Hox genes in both the mouse and chicken [31,32]. Again, most of these studies were focused on the anterior part of the cluster; hence, a general scenario for Hox cluster regulatory evolution is not yet available.

In this study, we report previously undescribed expression patterns of Hox genes of the European amphioxus Branchiostoma lanceolatum, and, surprisingly, find that some of them are not expressed in a colinear manner either in space or time, thus breaking the paradigm of Hox colinearity in amphioxus. We identified a different expression for B. lanceolatum Hox6 than that previously reported for the Floridian amphioxus [18,33] and detected Hox14 expression in the pharyngeal endoderm at the level of the endodystyle, the mid-hindgut, and notochord. Strikingly, it was detected in the cerebral vesicle, a part of the CNS where no Hox expression has been detected so far. Thus, this gene has escaped from the Hox coding pattern, as has been reported for vertebrate Hox14 genes [34,35]. We then investigated the regulation of these genes by RA and found that RA regulated the expression of Hox6 and the expressions of Hox14 in the gut and notochord. It also affected Hox14 expression in the cerebral vesicle, whereas the regulation of Hox14 in the pharyngeal endoderm seemed to be RA independent. The presence of RAREs near these genes, conserved between both the Floridian and European amphioxus species, makes these genes colinearity-breakers but still likely targets of RA regulation.

Methods

Embryonic culture and treatment with RA and the RA antagonist BMS009

Sexually mature amphioxus adults (B. lanceolatum) were collected in Argelès-sur-Mer (France) during the summer of 2009. Spawning was induced in the laboratory by heat shock [36]. After fertilization, embryos were reared in filtered seawater at 17°C. Treatments with RA (in DMSO), the RA antagonist BMS009 (in DMSO), or equivalent amounts of DMSO (as control) were carried out at the late blastula stage at a final concentration of 1 × 10^{-6} M as described [23,24]. At the early neurula stage, embryos were transferred to untreated filtered seawater, washed a few times, and kept in normal conditions. The control DMSO treatment did not affect development. Embryos and larvae were fixed at frequent intervals with 4% paraformaldehyde overnight at 4°C in a buffer containing 0.1 M MOPS, 0.5 M NaCl, 2 mM MgSO4, 1 mM EGTA, pH 7.4 for in situ hybridization.

Extension of the genomic region of B. lanceolatum Hox11

Because the coding sequences of B. floridanae and B. lanceolatum are extremely conserved, we decided to use primers based on the B. floridanae Hox11 exon 2 to amplify the first intron of B. lanceolatum’s one. Using a forward primer from the B. lanceolatum Hox11 exon 1 (5’-ATGGACGTTACTGGCTGTC-3’, [37]) and a reverse primer designed on the B. floridanae Hox11 exon 2 sequence (5’-CTGCCATATCCGAGGTTG-3’, [38]), we amplified a band of approximately 2.5 Kbp using B. lanceolatum genomic DNA as a template. We cloned it into pGEM-T Easy Vector (Promega) and sequenced it. The sequence corresponded to the first exon, first intron, and second exon of B. lanceolatum Hox11. The genomic sequence with the new annotation has been uploaded to the NCBI GenBank Database (http://www.ncbi.nlm.nih.gov/genbank/) under accession no. JX508623.

cDNA cloning, whole-mount in situ hybridization (WHISH), microscopy, and photography

A mix of embryo stages from gastrula to 2-day-old larvae of B. lanceolatum were fixed in RNAlater (Ambion) and total RNA was extracted using RNeasy Mini Kit (QIAGEN). The cDNA first strand was synthesized using Superscript III Reverse Transcriptase (Invitrogen) (1 h, 56°C). An embryo cDNA library was constructed using the CloneMiner Kit (pDNR222 vector; Invitrogen). Reverse transcription polymerase chain reaction (RT-PCR) amplification of the coding DNA sequence (CDS) and 5’- or 3’-rapid amplification of cDNA ends (RACE; Invitrogen) for all B. lanceolatum Hox genes (Hox1-15) were carried out with primers designed based on the sequences reported previously [37], except for Hox11, where we used the sequence of the exon 2 described...
here. We did not obtain any positive result for Hox13 and could not amplify the 3′-untranslated regions (UTRs) for Hox9, Hox11, and Hox15. For Hox11 we obtained the 5′-UTR. For Hox1, Hox3, Hox4, Hox6, Hox7, and Hox10 we obtained the 3′-UTR sequences. For Hox14 we obtained both 5′- and 3′-UTRs. For Hox9 we had only the 5′-UTR. We also searched for Hox13 in the recently published embryonic transcriptome of B. lanceolatum [39] but did not find any entry. Based on the sequences of the 3′-RACE clones, primers were designed to clone part of the 3′-UTR. CDS or 3′-UTR of each Hox gene was cloned into pBluescript SK II+, pCR II-TOPO Dual Promoter vector (Invitrogen), or pGEM-T Easy Vector (Promega), sequenced on both strands and used as templates for ribosynthesis of antisense digoxigenin-labelled probes. All primers used in this study are listed in Additional file 1: Table S1, and sequences of clones used in this study were uploaded to the NCBI GenBank Database under accession numbers JX088059-JX088072 and JX508612-JX508622. After WISH, the embryos were photographed as whole mounts. Several focus planes were merged using Helicon Focus software (d-Studio) for a more accurate identification of expression territories.

Identification of RAREs
Sequences of lambda phages containing either B. lanceolatum Hox6 and Hox5 (lambda phage no. λ4131 +λ4184 described in [37]) or Hox14 (lambda phage no. λ4100 in [37]) and their equivalent sequences from B. floridae [38] were analyzed by nuclear hormone receptor (NHR) scan using default parameter values [41]. We compared the results for sequences in both species and only those elements conserved in both B. floridae and B. lanceolatum were considered.

Results
B. lanceolatum Hox1, Hox3, and Hox4 expression patterns: genes that follow colinearity
The only amphioxus Hox genes for which the expression has been reported so far are Hox1 to Hox4 and Hox6 of the Floridian species B. floridae [18,42]. We used the sibling species B. lanceolatum to investigate the hitherto unknown expression of several Hox genes by WISH. As

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**Figure 2** 5′-UTR of B. lanceolatum Hox9 and probes used in this study. (A) Scheme of the B. floridae Hox cluster with Hox11, Hox10, and Hox9 represented. The B. lanceolatum Hox9 transcripts consist of two different versions: a large one, with three 5′-UTR (in green) exons far upstream of the Hox9 locus, with its splicing shown at the top. A shorter one, with a canonical 5′-UTR next to the first exon of Hox9, is shown with its splicing represented at the bottom. Black vertical lines represent exons of the different Hox genes. (B) The transcripts of all the B. lanceolatum Hox genes found to be expressed in this study. Color-coded boxes represent exons: beige, coding sequences; white, UTRs; and red, homeoboxes. The lines under the transcripts represent the probes used: red, a negative probe; blue, a positive probe. The third exon of B. lanceolatum Hox11 has not yet been described, so it is represented with a dashed line and fainter colors.
expected [43], we found that the expression patterns of *B. lanceolatum* Hox1, Hox3, and Hox4 in the CNS and mesoderm were very similar to those of their orthologous genes in *B. floridae* [18,42], in a clear colinear manner (Figure 3). However, while Schubert *et al.* [19] described a Hox nested expression of Hox1, Hox3, and Hox4 in scattered epidermal cells (likely involved in the patterning of developing sensory neurons) we found subtle differences in *B. lanceolatum*. The epidermal domain of *B. lanceolatum* Hox1 consisted of scattered cells in a mid-domain of the embryo, as described by Schubert *et al.* [19] (Figure 3, black arrows in Hox1). The most anterior limit of *B. lanceolatum* Hox1 in the CNS coincides with the most anterior limit in the epidermis (Figure 3). This epidermal expression of Hox1 is also clear from a dorsal point of view (see Additional file 2: Figure S1). However, in the case of Hox3, the high level of background obscured this pattern, although from a dorsal viewpoint there appeared to be epidermal expression at the late neurula stage (Additional file 2: Figure S1) as in *B. floridae*. Surprisingly, we were not able to detect epidermal expression of Hox4 (Figure 3), which was expressed only in the CNS.

A different expression pattern found for amphioxus Hox6 in the CNS: breaking spatial and temporal colinearity

Two different expression patterns have been reported so far for *B. floridae* Hox6. The first report described Hox6 as being expressed in all the neural tube posterior to the cerebral vesicle and in the endoderm up to the first gill slit, thereby breaking colinearity [33]. The second report showed a canonical expression in the CNS following colinearity with Hox1-4 [18]. To clarify this disparity, we studied the expression of *B. lanceolatum* Hox6. Surprisingly, we found a different pattern from those reported above. Unlike Hox1, Hox3, and Hox4, *B. lanceolatum* Hox6 was expressed in a restricted part of the neural plate at the mid-neurula stage, with very sharp anterior and posterior limits (Figure 4A). The anterior limit was at the level of the intersomitic cleft between somites 5 and 6 and the posterior limit was two somites behind (Figure 4A), unlike *B. floridae* Hox6, which Schubert *et al.* described to be expressed from the level between somites 6 and 7 (one somite behind the European amphioxus Hox6) rearwards to the tail bud [18]. It is remarkable that *B. lanceolatum* Hox6 was not expressed in the posterior CNS and tail bud. The anterior limit of Hox4 in amphioxus is at the level of the middle point of somite 6, half a somite behind Hox6, which means that Hox6 breaks spatial colinearity slightly. Regarding the timing, we have detected Hox6 exclusively at the mid-neurula stage (between 18 h and 21 h of development at 17°C), earlier than Hox4 expression (which is expressed from 24 h onwards, from late neurula stage), thus breaking temporal colinearity. *B. lanceolatum* Hox6 was not detected any later in development. The role of Hox6 must be very specific both in time and space in patterning of the CNS.

![Figure 3 B. lanceolatum Hox gene expression patterns.](http://www.evodevojournal.com/content/3/1/28)
European amphioxus *Hox7* and *Hox10* expression patterns

Apart from *Hox4* and *Hox6*, no other *Hox* gene expressions have been reported for the central group in cephalochordates. We detected a very weak expression of *Hox7* in the CNS, mesoderm, and tail bud (Figure 3). Due to its weak expression, we cannot rule out the possibility that *B. lanceolatum* *Hox7* was expressed in other tissues. At the mid-neurula stage, the anterior limit of *Hox7* was at a level equivalent to that of *Hox4*, but the anterior expression was so blurred that establishing a clear boundary was difficult; thus, we could not assess the colinearity relationship between *Hox4* and *Hox7* at this stage. However, from the pre-mouth stage the expression is more posteriorly restricted than for *Hox4*. On the other hand, it began to be expressed after more anterior *Hox* genes, from the late neurula stage, thus retaining temporal colinearity.

No expression of *Hox* genes from posterior groups has been characterized in cephalochordates so far. Here we investigated the expression patterns of *B. lanceolatum* *Hox10* and *Hox14*. We found that *Hox10* expression followed a similar pattern to that for *Hox7*, with very weak expression in the CNS and mesoderm (Figure 3). Again, the weak and blurred expression of *Hox10* makes difficult to exclude the possibility that is actually expressed in other tissues. As for *Hox4* and *Hox7*, the anterior limit of expression of *Hox10* was very diffuse, and although it seemed to be more rostral than the expressions of *Hox4* and *Hox7*, and thus breaking spatial colinearity, the diffuse anterior limit found makes evaluating the colinearity difficult.
Non-canonical expression of \textit{Hox14}

The most unexpected expression pattern was that of \textit{Hox14}. \textit{B. lanceolatum Hox14} is expressed from the pre-mouth larval stage. As for most abovementioned genes, a probe for the coding sequence produced high background and unspecific signals, so we decided again to clone the 3\textsuperscript{\prime}-UTR. We found that it was split into two exons, with a small intron of 45 bp. Amphioxus \textit{Hox14} was expressed in the mid-hindgut, in the posterior part of the notochord, and in the tail bud (Figure 4B\textquotesingle, C\textquotesingle). Strikingly, \textit{Hox14} was also detected in anterior structures such as the cerebral vesicle and the left side of the pharyngeal endoderm at the level of the endodystyle (Figure 4B\textquotesingle and inset).

Expression of \textit{Hox2}, \textit{Hox5}, \textit{Hox8}, \textit{Hox9}, \textit{Hox11}, \textit{Hox12}, and \textit{Hox15} detected by RT-PCR

Apart from the genes whose expression patterns we have been able to identify, other \textit{Hox} genes were detected by means of RT-PCR during \textit{B. lanceolatum} development. Among these genes are \textit{B. lanceolatum Hox2, Hox5, Hox8, Hox9, Hox11, Hox12}, and \textit{Hox15}. \textit{Hox13} was not identified either by RT-PCR or in the recently published embryonic transcriptome of \textit{B. lanceolatum} [39]. We obtained the CDSs for \textit{B. lanceolatum Hox2}, \textit{Hox5, Hox8, Hox9, Hox11, Hox12}, and \textit{Hox15}. \textit{Hox13} was not identified either by RT-PCR or in the recently discovered \textit{Hox15} [17,37], and 3\textsuperscript{\prime}-UTRs by using \textsuperscript{\prime}-RACE RT-PCR for \textit{B. lanceolatum Hox2}, \textit{Hox5}, \textit{Hox8}, and \textit{Hox12}. We also amplified the 5\textsuperscript{\prime}-UTR by means of \textsuperscript{\prime}-RACE RT-PCR of \textit{Hox2, Hox9}, and \textit{Hox11}. Nonetheless, we were not able to obtain any signal by WISH (Figure 2B).

The case of amphioxus \textit{Hox2} is quite similar to that of \textit{Hox6}. So far, two reports about its expression have shown two different expression patterns. The first one [42] reported the breaking of colinearity for \textit{Hox2}, while the second [18] reported a colinear expression with respect to \textit{Hox1} and \textit{Hox3}. As with \textit{Hox6}, we wanted to test which \textit{Hox2} expression pattern could be the correct one using \textit{B. lanceolatum}. However, although we performed WISH with different probes based on the sequences of the CDS or the 3\textsuperscript{\prime}-UTR, we were not able to obtain specific signals (Figure 2B).

We found that the 5\textsuperscript{\prime}-UTR of \textit{Hox9} had two different versions. One was shorter, with a canonical 5\textsuperscript{\prime}-UTR next to the start codon, and a second longer one was divided into four exons when aligned against the \textit{B. floridanae Hox} cluster (the complete sequence of the \textit{B. lanceolatum Hox} cluster is still not available): three of them were far upstream from \textit{Hox9}. The first exon was placed approximately 5.4 Kbp downstream of \textit{Hox11}, the second was approximately 9 Kbp downstream of \textit{Hox10}, and the third was approximately 25 Kbp upstream of \textit{Hox9}. The fourth corresponded to the canonical 5\textsuperscript{\prime}-UTR (Figure 2A).

For \textit{B. lanceolatum Hox11}, only the first exon has been annotated [37]. Here, we have extended the previously described genomic sequence up to exon 2 (see Methods), which allowed us to find \textit{Hox11} by \textsuperscript{\prime}-RACE RT-PCR. The 5\textsuperscript{\prime}-UTR was shorter than expected, which means that the previously automatically annotated exon 1 [37,38] was not correct and the actual exon 1 is shorter. As with the other \textit{Hox} genes, a probe based on the coding sequences of exons 1 and 2 gave no signal in WISH (Figure 2B).

RA and RA-antagonist treatments alter the expression of \textit{amphioxus Hox6} and \textit{Hox14}

The RA-\textit{Hox} system controls the patterning along the A-P axis during development of chordates (for a review see [1]), and such control has been reported widely for anterior \textit{Hox} genes in amphioxus [18-20,23,24,44]. Because we detected a different expression pattern for the \textit{B. lanceolatum Hox6} gene than that reported previously [18] and given that amphioxus \textit{Hox14} has been shown to have a non-canonical expression pattern, we treated embryos with RA, the RA antagonist BMS009, or with DMSO as an inert negative control, and carried out WISH experiments.

In RA-treated embryos, the anterior limit of \textit{Hox6} moved rostrally up to the level between somites 3 and 4 (compare Figure 4A with Figure 4A\textsuperscript{\prime}), whereas the posterior limit was unaltered. When treated with the antagonist, \textit{Hox6} expression disappeared. This can be explained by the anterior limit shifting posteriorly to the extreme of its fixed posterior extent, thus making \textit{Hox6} expression disappear (compare Figure 4A\textsuperscript{\prime} with Figure 4A\textsuperscript{\prime\prime}). Then, the level of the anterior limit would be that changed when taking the somites as a reference point, as in other more anterior \textit{Hox} genes [18] demonstrating that the changes in expression were regulated by RA (directly or indirectly) and not because of a general shift of internal structures.

In RA-treated larvae, the anterior limit of \textit{B. lanceolatum Hox14} expression (at least in the gut) was shifted anteriorly in a significant manner compared with the control, taking as a reference point the mid pigment spot of the CNS. However, it was not so clear for the expression in the notochord (in Figure 4, compare B\textsuperscript{\prime} and C\textsuperscript{\prime} with B and C, respectively). In contrast, when treated with BMS009 the expression of \textit{Hox14} in both the notochord and the gut shifted strongly to the posterior (in Figure 4, compare B\textsuperscript{\prime} and C\textsuperscript{\prime} with B\textsuperscript{\prime\prime} and C\textsuperscript{\prime\prime}, respectively). Surprisingly, expression of \textit{Hox14} in the pharyngeal endoderm did not disappear completely in either RA- or BMS009-treated embryos (black arrowheads in Figure 4). Although formation of the pharynx is strongly reduced in RA-treated larvae, with mouth and gill slits failing to form [23], we detected faint expression in the pharynx of \textit{Hox14} in the pre-mouth-stage larvae (Figure 4B, black
arrowhead), while Hox14 was detected clearly in the case of the 2-day-old RA-treated larvae (Figure 4C, black arrowhead). This suggests that regulation of Hox14 expression in the endostyle is RA independent. The amphioxus cerebral vesicle is a structure that is also reduced in RA-treated larvae but does not disappear, as has been shown using cerebral vesicle markers [18]. Interestingly, the expression of Hox14 in the cerebral vesicle was extremely reduced with both RA and BMS009 treatments (Figures 4B, 4B', 4C and 4C', white arrowheads), suggesting that the cerebral vesicle expression domain is somewhat very sensitive to variations in RA level.

RA regulates the expression of its target genes via heterodimers of RAR/RXR that bind RAREs. These heterodimers can bind DR1-, DR2-, and DR5-type RAREs [25]. Using an NHR scan [41], which has been shown to be effective in the prediction of DR2 and DR5 surrounding amphioxus ParaHox genes [45], we looked for RAREs near to amphioxus Hox6 and Hox14 genes, using the same genomic regions analyzed previously for non-conserved regions [37] (see Methods). We also screened the corresponding B. floribae genomic sequences used in our previous comparative regulatory analysis [37], to exclude predictions that have not been conserved between both amphioxus species, because they are probably not real and functional elements. We found that most of the predicted RAREs were not conserved between both species (see Additional file 1: Tables S2 to S5). Thus, we regarded these as false-positives and they were discarded. Using these criteria, we detected one DR2 and three DR5 elements near to Hox6, and two DR1 elements within the Hox14 locus: one within the second intron and the other located in the second exon of the 3'-UTR (Figure 4D).

### Discussion

**Different expression patterns between B. floribae and B. lanceolatum**

The expression patterns of Hox1, Hox3, and Hox4 in amphioxus epidermal neurons have been reported for B. floribae. However, we did not detect Hox6 expression in B. lanceolatum epidermis [19]. Therefore, our data are not consistent with the hypothesis of a 'skin brain' (similar to the diffuse net of neurons in hemichordates) in amphioxus [19,46].

As for Hox6, unlike the two patterns described previously in the Floridian amphioxus, we have found B. lanceolatum Hox6 only at the mid-neurula stage in a restricted stretch of the neural plate (Figure 4A'). The anterior limit of B. lanceolatum Hox6 is one somite level more rostral than that described for B. floribae by Schubert and colleagues (between somites 7 and 8, [18]) and much more caudal than the anterior limit found by Cohn [33].

One question arises from past and current data: what can explain such different expression patterns in three different experiments? One possibility is that one of the expression patterns of B. floribae (in the case of Hox6, most likely that reported by Schubert et al. [18] rather than that by Cohn [33], because the signal presented by the former seems more reliable than the faint one of the latter report) and those ones presented here for B. lanceolatum actually reflect a real species-specific difference. If so, it means that the expression of B. floribae Hox6 in the CNS in a colinear manner with the other Hox genes is not conserved in B. lanceolatum CNS patterning. On the other hand, it is possible that experimental consideration such as probe design may explain the differences. For example, since the nucleotide sequences of the homeobox regions of all central Hox genes are highly similar (see Additional file 2: Figure S2), a probe spanning this sequence might cause cross-hybridizations and thus partial mis-assignments of expression patterns. In fact, when we used a probe based in the CDS for most of the genes, we obtained either no signal or unspecific staining of the B. lanceolatum embryos (red lines in Figure 2B). Therefore, we decided to use 3'-UTR-based probes, which are unable to cross-hybridize with other Hox genes. The 3'-RACE RT-PCR using gene specific primers designed in the first exon gave only a single band in both Hox4 and Hox6 (see Additional file 2: Figure S3), indicating that alternative splicing does not lie behind the difference and that we were detecting only the expression of Hox4 and Hox6 transcripts. However, we cannot conclusively discard the presence of alternative transcripts that could account for the different expression patterns obtained upon the use of different probes.

We believe that a revisit of expression patterns in both B. floribae and B. lanceolatum and, essentially, in the Asian species Branchiostoma belcheri, will help to elucidate if the discrepancies reported come from truly species-specific differences or have an experimental nature.

### Escape from spatial and temporal colinearity

In B. lanceolatum, Hox6 was expressed slightly more rostrally than Hox4 and thus did not maintain spatial colinearity. It was also expressed in an earlier stage (mid-neurula) than that of the onset of Hox4 expression (late neurula), therefore, Hox6 also deviated from temporal colinearity. The function of Hox6 in amphioxus is not known, but it is likely involved in the patterning and regionalization of the CNS in a very specific domain of the neural plate at a very specific time in development. In vertebrates, Hox6 is expressed in the spinal cord behind the rhombencephalon to the caudal end of the spinal cord and also in the mesoderm. Therefore, the expression of Hox6 of amphioxus and vertebrates is not conserved. Given that the vertebrate Hox6 genes maintain both spatial and temporal colinearity, we believe...
that they represent the ancestral condition, while the expression of amphioxus Hox6 is probably more divergent. In addition, we have shown that Hox6 is still regulated (directly or indirectly) by RA, as are the more anterior Hox genes [18], suggesting that it is derived from the ancestral state of canonical nested expression together with its mode of regulation.

The expression of amphioxus Hox6 and the effects of RA and the RA antagonist are very similar to those of the amphioxus ParaHox gene Gsx [45]. Amphioxus Gsx is expressed in a few cells in the neuroectoderm, at the level of somite 5, just anterior to the Hox6 domain. Amphioxus Hox6 and Gsx likely participate in the A-P patterning of limited parts of the neuroectoderm in a similar manner, probably in combination with other Hox genes that overlap with them. RA treatments enlarge and shift the Gsx rostral expression limit anteriorly whereas RA antagonist treatments make the Gsx domain disappear, as in the case of Hox6. As with the posterior limit of Hox6, which is unaffected by RA treatment, the posterior limit of Gsx did not change dramatically with RA treatment. Therefore, as Osborne et al. [45] have suggested for Gsx, the anterior limit of Hox6 would be regulated by RA, but the posterior limit would not. Thus, in both Hox6 and Gsx, the loss of the domain following BMS009 treatment can be explained by a caudal shift of the anterior limit until it reaches its posterior one, making the expression to disappear. In vertebrates, it is not known whether Hox6 paralogs are direct targets of RA regulation. However, other central Hox genes such as HoxA7 and HoxC8 shift their anterior limit rostrally in the paraxial mesoderm of mouse embryos after RA treatment (Hox-1.1 and Hox-3.1, respectively, in [10]), and different Hox4 paralogs have been shown to be regulated directly by RA [47-49]. However, in other cases such as in the chicken neural tube, the expression levels of genes from HoxB6 to HoxB9 have been shown to be refractory to RA treatment [50]. Thus, further investigation is needed in both cephalochordates and vertebrates to understand the ancestral mode of regulation.

We have also detected the expression of three other Hox genes not studied so far: Hox7, Hox10, and Hox14. While the anterior limit of Hox7 expression is similar to that of Hox4 at the late neurula stage, it was more caudal from the pre-mouth larval stage onwards, thus keeping its spatial colinearity. However, the anterior limit of Hox10 expression in the CNS and mesoderm seemed to be more anterior than that of Hox4 and Hox7. Nonetheless, it is necessary to point out that the anterior limits of amphioxus Hox7 and Hox10 were very diffuse, unlike their vertebrate counterparts, which usually display sharp rostral limits, and their colinearity nature is then far from conclusive. Although this difference in the anterior limit between amphioxus and vertebrates might reflect different modes of regulation, we believe that this is the result of the clearly segmental nature of the vertebrate CNS (for example, rhombomeres) compared with the amphioxus CNS. Thus, the real anterior level up to where these genes are expressed in amphioxus might be different to that detected by WISH and thus their colinearity might differ, as discussed here. In vertebrates, Hox6 and Hox10 paralogs retain their colinearity and they have important opposite roles: Hox6 genes encode rib-promoting factors whereas Hox10 genes are rib-inhibiting [51]. Thus, the colinear expression of Hox6 and Hox10 genes in vertebrates is under strong developmental constraints that are not present in amphioxus, allowing these genes to escape from colinearity.

The most striking case of colinearity breakage is that of Hox14. Posterior Hox genes are involved in the appearance of morphological innovations and are also related to changes in the evolution of the vertebrate bauplan, such as the type of vertebrae [52] or morphological variability within squamates [53]. Interestingly, lamprey and shark Hox14 genes have non-canonical expression patterns. They are expressed only in the posterior part of the endoderm in the lamprey and in a very specific posteroventral area in the shark surrounding the cloaca [35]. Amphioxus Hox14 is expressed in anterior structures such as the cerebral vesicle. This is the first Hox gene to be detected in such an important organ. In vertebrates, no Hox genes are expressed in the midbrain or forebrain and all are excluded from Otx and Pax expression territories. However, what was thought to be a universal rule is broken in amphioxus. Likewise, the expression of Hox14 in the pharyngeal endoderm is an exceptional case, because no amphioxus Hox gene has been detected earlier in the pharynx. RA regulates the expression of Hox14 in the notochord and mid-posterior gut in the same manner as anterior genes (enlarged expression anteriorly when embryos are treated with RA, or posterior shift of the anterior limit when treated with an RA antagonist; arrows in Figure 4B-B'', C-C''). On the other hand, Hox14 seems not to be regulated by RA in the pharynx, because RA treatment did not make the expression disappear, and nor did treatment with the antagonist expand it posteriorly (Figure 4B-B", C-C", black arrowheads) as would be expected. Surprisingly, Hox14 regulation in the cerebral vesicle appeared to be very sensitive to RA, because both excess and a drop in RA level affected its expression strongly (Figure 4B-B", C-C", white arrowheads). Thus, the regulatory regions of Hox14 must be modular. Some expression domains, namely the gut and the notochord, would depend upon RA. Although we cannot discern from the data presented here whether this control is direct or indirect, the presence of DRI elements within amphioxus Hox14
locus could give clues for future experiments. Thus, an RA-independent module might regulate the pharyngeal endoderm domain. By contrast, a module sensitive to RA concentration might control the cerebral vesicle domain, perhaps from an indirect effect of RA treatment on some transcription factors that are directly regulated by RA. For vertebrates, a putative regulation of Hox14 by RA has not been studied. However, other posterior Hox genes respond in the opposite way to the anterior Hox by RA has not been studied. However, other posterior Hox genes respond in the opposite way to the anterior Hox. For example, HoxB9 is refractory to RA treatment in the neural tube of chicken embryos, as is HoxB6 [50], and in the mouse a putative function of RA seems to be to prevent the expression of posterior HoxD genes in the anterior domain [54,55].

The uncoupling of vertebrate and amphioxus Hox14 genes from a canonical Hox code is one sign of relaxation of the posterior part of the cluster, but not the only one. Posterior Hox genes of cephalochordates, urochordates, echinoderms, and hemichordates do not have clear orthologous relationships to the posterior paralogy groups of vertebrates [34], probably because of the higher evolutionary rate of this class of genes, a phenomenon named deuterostome posterior flexibility [56]. If the posterior Hox genes of amphioxus and vertebrates are true orthologs, our data imply that decoupling from the Hox code of Hox14 genes occurred in the last common ancestor of chordates. However, although not conclusively, phylogenetic analyses of deuterostome posterior Hox genes, including the recently reported amphioxus Hox15, suggest that the posterior Hox genes of amphioxus and vertebrates likely originated from independent duplications [17,34,35]. In line with the posterior flexibility hypothesis, the intergenic regions of the posterior Hox cluster are less conserved than the anterior ones in both amphioxus [37,38] and gnathostomes [57]. This trend for the posterior Hox cluster might be explained if the posterior genes had originated by specific expansions, for example, via tandem duplication, to give 14 Hox genes in the last common ancestor of vertebrates and 15 in Branchiostoma. Santini et al. [57] suggested that this lack of constraint among the posterior Hox cluster would have allowed these genes to be involved in the patterning of secondary axes in vertebrates, such as fins and limbs. In amphioxus, the same reasoning would apply to Hox14 and its unusual expression territories. If the origin of the posterior Hox cluster is truly independent, the decoupling of the Hox14 genes from the classical Hox code must have happened independently in the amphioxus and vertebrate lineages.

Conclusions

The escape of Hox genes from canonically nested expression is not unusual. For instance, the Hox1 and Hox2 paralogs of vertebrates also do not follow spatial colinearity, so that the anterior limit of Hox2 is more rostral than that of Hox1, which is expressed only in rhombomere 4. This delimited expression of Hox1 without extension to more caudal regions is similar to that of amphioxus Hox1 in the CNS. However, it is worth noting that this similarity cannot account for any homology between the Hox1 domain in amphioxus and rhombomere 4 in vertebrates. Furthermore, in lampreys, temporally colinear expression of Hox genes has not been detected [58]. Thus, different Hox genes, in different animals escape in one way or another from the colinearity ‘rule’. We believe that these escapes might be associated with the patterning of lineage-specific morphological traits that first requires a loss of the constraint that kept them colinear.

Additional files

Additional file 1: Table S1. Sequences of primers used in this study to clone B. lanceolatum Hox gene probes.

Additional file 2: Figure S1. Expression of B. lanceolatum Hox1 at early neurula (A), mid neurula (B) and late neurula (C) and Hox3 in the same stages (D, E, F) in dorsal view. The arrows mark the expression in the epidermis, from mid-neurula in the case of Hox1 and late neurula in Hox3.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

JP-A carried out the WISH experiments in amphioxus and performed the genomic DNA analysis for RAREs. NA carried out cloning experiments. SA synthesized and provided the RA antagonist BMS009. SK discussed the results critically. JP-A, SDA, and JG-F conceived the study, participated in the design and coordination of the project, and wrote the draft manuscript. All authors read, discussed, and approved the manuscript.

Acknowledgements

We thank the anonymous reviewers for constructive criticisms that improved the manuscript. We are indebted to Hector Escriva and the ASSEMBLE FP7 EU programme for providing space and support during amphioxus sampling in Laboratoire Aragó, Banyuls-sur-Mer, France. We thank Ina Amone and Rossella Annunziata of the Stazione Zoologica Anton Dohrn of Naples (Italy) for their kind help in RA experiments and Ángel R. de Lera for providing the RA antagonist BMS009. We also thank Beatriz Albuixech-Crespo for her kind help with preparing the figures. The authors thank Sena Jiménez-Delgado, Ignacio Maeso, Manuel Irimia, Beatriz Albuixech-Crespo, and Ildikó M. L. Somorjai for their fruitful suggestions and discussions. We also want to thank Ignacio Maeso and Manuel Irimia for critical reading of the manuscript. This work was supported by the Ministerio de Educación y Ciencia (Spain) BFC2008-03776 and BFU2011-23921 and the ICREA prize to JGF. JP-A held a ‘1F PhD fellowship of the Generalitat de Catalunya (Spain) and SDA a ‘Juan de la Cierva’ postdoctoral contract of the Ministerio de Educación y Ciencia (Spain).

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Received: 25 June 2012 Accepted: 4 October 2012 Published: 3 December 2012
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Cite this article as: Pascual-Anaya et al: Broken colinearity of the amphioxus Hox cluster. *EvoDevo* 2012 3:28.

doi:10.1186/2041-9139-3-28

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