Evolution of cyclostome Hox clusters

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Abstract

Hagfish and lampreys represent the only two lineages of agnathan (jawless) vertebrates that have survived to the present day, and both form the monophyletic group of cyclostomes, which is sister to the rest of the vertebrates, the gnathostomes (jawed vertebrates). Comparisons between cyclostome and gnathostome models are thus essential to infer and understand ancestral traits of vertebrates and their subsequent morphological, genomic, and physiological evolution. It is thought that Hox gene evolution had a major impact in vertebrate morphological diversification, but it has not been until the last decade or so that significant knowledge on the organization and regulation of the Hox clusters of cyclostomes has started to accumulate. In this chapter, we review current data on cyclostome Hox biology, presenting an integrated view of what we know thus far from studies of Hox genes of both the lamprey and hagfish, including their gene complements, cluster evolution, functional and expression diversification, and how this all relates to their body plans and the general vertebrate architecture. We present the similarities and differences with the most well-known gnathostome Hox genes, and infer common, ancestral traits. In conclusion, we propose a common ancestor radically different to previous reconstructions, with only two Hox clusters instead of four, but in which most of the well-known traits of Hox genes –spatial and temporal collinearity, association with cranial segmentation and posterior growth— were already established. This relatively simpler two-cluster ancestor was then independently modified in the cyclostome and gnathostome lineages, where although Hox characteristics have been broadly retained, lineage-specific differences arose over hundreds of millions of years of independent evolution, which could be behind the morphological diversification of each of the groups.

Introduction

Hagfish and lampreys are the only living representatives of jawless vertebrates, or agnathans, a group of vertebrates that diverged before the advent of the articulated jaw, a trait that defines the group of jawed vertebrates, or crown gnathostomes. Although hagfish and lampreys have traditionally been considered to form a paraphyletic group from a morphological point of view, with lampreys considered more closely related to gnathostomes and thus excluding hagfish from vertebrates, advances in both morphological and molecular phylogenetics have clearly shown that both form a monophyletic group, called Cyclostomata (from 'round mouth'), which is the extant sister group of all gnathostomes (Fig. 1A). Moreover, most of the morphological evidence used to support the paraphyly of hagfish and lamprey has been revised with the most recent works on hagfish development, mainly coming from the laboratory of Shigeru Kuratani (Higuchi et al., 2019; Oisi et al., 2013; Ota et al., 2011; Ota et al., 2007; Sugahara et al., 2016; see Kuratani, 2021, for a review). However, despite the monophyletic character of cyclostomes now being the most widely accepted view, the phylogenetic position of the hagfish is still surprisingly debated by some, and the previous morphology-based tree is still considered, although only occasionally in current studies (see Figure 1 in Theofanopoulou et al., 2021, for a relevant recent example). Clearly, a deep phylogenomic analysis taking advantage of whole genome data from the hagfish would likely solve the debate about the hagfish position definitively (currently, an early, fragmented version of a hagfish genome is publicly available at https://www.ensembl.org/Eptatretus_burgeri/; Pascual-Anaya et al., 2018). In either case, hagfish and lampreys are fundamental models in comparative analyses with gnathostomes to infer ancestral conditions of vertebrates from genomic, morphological, and evolutionary developmental perspectives (Shimeld and Donoghue, 2012).

Despite their key phylogenetic position, cyclostome models have been, probably together with chondrichthyans (sharks, rays and chimaeras), the least studied vertebrate groups, mainly due to the difficulty with accessing samples of both adults and eggs, especially from the hagfish. In the case of cyclostomes, the lamprey is relatively easier to access than the hagfish, and it has therefore been the model of choice by laboratories studying early evolution of vertebrates (see Shimeld and Donoghue, 2012, for a review). Lampreys can provide thousands of eggs in the laboratory – although seasonally and during a short period of the year—, and this has permitted, in recent years, the adaption of state-of-the-art functional techniques for application to their embryos, such as the

introduction of gene reporter constructs (Parker et al., 2014b), or more recently the application of CRISPR/Cas9 genome editing (Square et al., 2015).

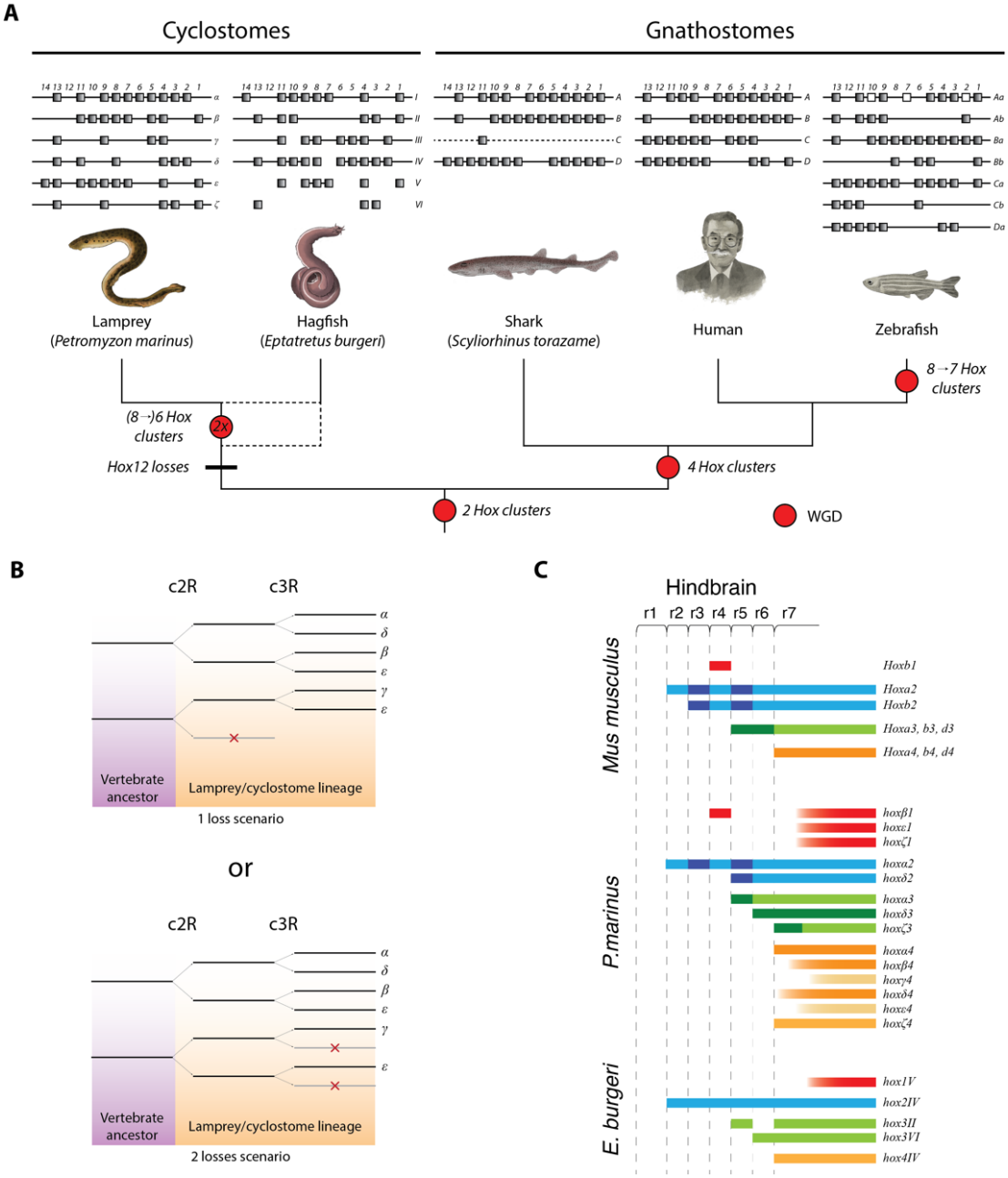

Figure 1. Hox evolution in cyclostomes (**A**) Schematic phylogenetic tree depicting selected representative species of the main vertebrate lineages (cyclostomes: hagfish and lamprey; gnathostomes: shark, human and fish) with representations of their Hox repertoires on top, not to scale. Hox genes are represented by gradient grey squares, with white squares representing known pseudogenes. Hox genes are oriented with 5' on the left to 3' on the right and all Hox genes are transcribed with the same orientation. Evolutionary events such as whole genome duplications (red circles) together with the increase or loss of Hox clusters are indicated. Solid lines depict genomic linkage; dashed line in shark represents the unknown nature of the HoxC cluster (Hara et al., 2018) in *Scyliorhinus torazame*; (**B**) Two

possible evolutionary scenarios to explain the presence of 6 Hox clusters, at least in the lamprey, after 3R of cyclostome-specific WGD events (c2R and c3R). Black lines indicate presence of a cluster, while grey lines represent losses (marked by a red cross); (**C**) Schematic representation of expression patterns of Hox1-4 genes in the hindbrains of the mouse (*Mus musculus*), sea lamprey (*Petromyzon marinus*) and inshore hagfish (*Eptatretus burgeri*).

Cyclostome and gnathostome lineages diverged over 500 million years ago (mya), with hagfish and lamprey lineages splitting from each other relatively soon after, around 400 mya (Kuraku and Kuratani, 2006). Despite having evolved for almost the same amount of time as gnathostomes, cyclostomes are generally wrongly considered more primitive than gnathostomes (Omland et al., 2008), which has contributed to the misperception that what is observed in cyclostomes is primitive while gnathostome characters display derived states. The striking differences we observe today between cyclostome and gnathostome body plans are, however, consistent with the long and independent evolution of both lineages, which include both primitive and derived character states in both groups. This is also the case between lampreys and hagfish, since both lineages are characterized by striking differences, the most conspicuous being that hagfish are direct developers, while all living lamprey species have a larval stage in their life cycles. Nonetheless, both the hagfish and the lampreys display some common features during development, suggesting a common ground plan that is consistent with their common ancestry (Kuratani, 2021). These long, independent evolutionary histories are likely behind the acquisition of radically different body plans, rather than one being ancestral and the other derived, and these differences must be somehow reflected in the genomes, including the Hox genes (Fig. 1A), as these are expected to have had an important contribution to the evolution of morphological diversity (Wagner et al., 2003). Here, we review and bring together all the current knowledge pertaining to Hox genes of cyclostomes: the gene complements, their expression during cyclostome development, and their regulation and integration into gene regulatory networks that are associated with key morphological traits of vertebrates. We conclude that, despite having retained conserved features, which originated in an ancestral vertebrate that possessed two rather than four Hox clusters, the independent expansion and evolution of Hox clusters in cyclostomes and gnathostomes are likely behind their morphological differences and their respective novelties (such as the jaw and paired appendages of gnathostomes) and, importantly, illustrates that there are two ways, rather than one, of making a vertebrate.

Evolution of cyclostome Hox complements

It has been proposed that the genomes of vertebrates have been shaped by several whole genome duplication (WGD) events early in their evolution (Fig. 1A). For instance, it is generally accepted that the gnathostome genome is the result of two ancestral tetraploidization events, classically known as the 2R Hypothesis (Dehal and Boore, 2005). As a result of these 2R events, the ancestral gnathostome genome contained four paralogous Hox clusters (Fig. 1A), which are more specifically ohnologous, in reference to Susumo Ohno, who was the first to propose these WGD events and their importance in genome karyotype evolution (Ohno, 1970). Invertebrates typically have one pre-duplicative Hox cluster (Pascual-Anaya et al., 2013). Most groups of jawed vertebrates have retained these four clusters, with distinct gene repertoires depending on differential Hox gene losses (Fig. 1A). The 2R are, however, not the only duplicative events that have contributed to shape vertebrate genome architecture, and some lineages, particularly fish, have experienced extra rounds of WGD in the past, the most well-known being that of teleost fishes (teleost-specific genome duplication or TGD; Amores et al., 1998; Taylor et al., 2001) (Fig. 1A). Due to this TGD, the teleost last common ancestor possessed 8 Hox clusters, and species deriving from that ancestor nowadays have retained >4 Hox clusters, with numbers ranging from 5 to 8, depending on whether one or more Hox clusters eroded or not: the African butterfly fish *Pantodon buchholzi* with 5 clusters (Martin and Holland, 2014); the zebrafish *Danio rerio*, 7 clusters (Amores et al., 1998) (Fig. 1A); and the European eel *Anguilla anguilla*, 8 clusters (Henkel et al., 2012), to mention some examples. An additional WGD after the TGD has occurred independently in the carp/goldfish (Luo et al., 2020; Xu et al., 2019) and salmonid (Berthelot et al., 2014; Lien et al., 2016) lineages, increasing their number of Hox clusters even further up to 13 (Moghadam et al., 2005a, b; Mungpakdee et al., 2008; Xu et al., 2014). Other non-teleost fish lineages have experienced an independent WGD event, namely paddlefish (Cheng et al., 2021) and sturgeon (Cheng et al., 2019; Du et al., 2020), and their genomes also contain 8 Hox clusters.

There thus seems to be a clear trend relating the number of WGD events to the number of Hox clusters in a lineage. This is important because whether the 2R happened before or after the cyclostome divergence has been, and continues to be, a matter of much debate. Several hypotheses have been proposed, based on different approaches. One that gained early, and the most recent support is that cyclostomes diverged after the 1R but before the 2R, making the 2R a gnathostome-

specific event (Escriva et al., 2002; Nakatani et al., 2021; Simakov et al., 2020). An alternative hypothesis considers that the 2R is ancestral to all vertebrates (Kuraku et al., 2009; Sacerdot et al., 2018). A minority view is that the 2R never occurred, and the vertebrate karyotype is the result of a 1R followed by extensive segmental duplications (Smith and Keinath, 2015; Smith et al., 2018). Before the current genomic era, describing the full complement of Hox clusters of cyclostomes provided early hints towards answering this long-standing question. The first studies aiming at describing the Hox clusters of cyclostomes utilized degenerate PCRs to find Hox genes in both hagfish and lamprey. In lampreys, these surveys were carried out in different species: *Petromyzon marinus* (Carr et al., 1998; Force et al., 2002; Irvine et al., 2002; Pendleton et al., 1993), *Lampetra planeri* (Sharman and Holland, 1998) and the Arctic lamprey *Lethenteron camtschaticum* (syn. *Lethenteron japonicum*: Kuraku et al., 2008; Takio et al., 2007; Takio et al., 2004). In the case of the hagfish, the first PCR-based assay (Stadler et al., 2004) described the presence of 33 Hox genes in the Pacific hagfish *Eptatretus stoutii*, which was one of the lowest counts of Hox genes for any vertebrate up to that date, but was still indicative of multiple clusters. Without whole genome sequence information, all these studies were far from conclusive and the 2R conundrum remained unsolved. Stadler and colleagues hypothesized, based on their very short sequence information, that cyclostomes diverged at least after the first WGD and that subsequent independent gene/cluster duplications expanded the Hox inventory. The analysis of the somatic genome of *P. marinus*, the first of a cyclostome species, did not solve this problem either, contributing only scattered information regarding Hox clusters (Smith et al., 2013). The description of the complete Hox repertoire of lampreys had to wait until the analysis of the genome of *L. camtschaticum* (Mehta et al., 2013), and was later confirmed with the analysis of the germ line genome sequence of *P. marinus* (Smith et al., 2018); in total lampreys have a conserved set of 42 Hox genes arranged in 6 intact clusters (Fig. 1A). The presence of more than 4 clusters suggested the existence of additional WGDs in lampreys, independently from whether the 2R was shared with gnathostomes or not (Mehta et al., 2013). Interestingly, the recent analysis of a preliminary draft genome assembly of the inshore hagfish *Eptatretus burgeri* together with its developmental transcriptome identified 40 Hox genes in at least 6 Hox clusters (Fig. 1A), very similar to the lamprey and thus implying the possibility that their origin preceded the divergence of the lamprey and hagfish lineages (Pascual-Anaya et al., 2018). Intriguingly, cyclostome Hox clusters are much larger in size than their gnathostome counterparts (with reported sizes ranging from 145 to 526 kb in *L.*

179 *camtschaticum*, compared to the 100-200 kb average size for gnathostomes) and contain higher
180 levels of interspersed repetitive elements (Mehta et al., 2013).
181 More recent, robust chromosome-level macrosynteny analyses of gnathostomes and
182 several lamprey genomes have eventually confirmed that cyclostomes diverged after a pan-
183 vertebrate ancestral 1R, but before the gnathostome-specific, classic 2R; secondarily, at least
184 lampreys have evolved after an independent 2R-3R (Nakatani et al., 2021; Simakov et al., 2020)
185 (Fig. 1A), which is supported by phylogenetic and synteny analyses, including linked non-Hox
186 genes, that suggest that lamprey Hoxβ and -ε clusters and –although less likely— Hoxα and -δ
187 clusters come from a lamprey duplication (Parker et al., 2019a; Smith et al., 2018). Due to the
188 generally weak phylogenetic signal between lamprey and hagfish Hox genes and the lack of a
189 hagfish chromosome-level genome assembly to use for a more robust macrosynteny analysis
190 (Pascual-Anaya et al., 2018; Smith et al., 2018), whether the hagfish shares these additional WGD
191 events with lampreys or not remains unclear (Fig. 1A). The presence of 6 Hox clusters implies
192 some losses during cyclostome evolution: either one cluster was lost after the
193 lamprey/hagfish/cyclostome-specific 2R, resulting in 3 clusters before a 3R, or two losses from an
194 8-cluster ancestor (Fig. 1B).
195
196 **Ancestral loss of Hox12 paralogs**
197 Analyses of both the lamprey and the hagfish Hox catalogues revealed the striking lack of all
198 members of the Hox12 paralogy group (PG). Given that orthology relationships between posterior
199 Hox genes of invertebrate chordates and vertebrate PGs do not correspond with each other
200 (Pascual-Anaya et al., 2013), a remote formal possibility is that at least two gnathostome Hox12
201 genes could have originated as independent tandem duplications from another posterior Hox gene
202 at least in the ancestral two Hox clusters of a stem gnathostome after divergence from cyclostomes,
203 and that a Hox12 would have never existed in the last common ancestor of vertebrates. However,
204 the paralogy of all gnathostome Hox12 genes is strongly supported by phylogenetic analyses,
205 which unequivocally implies the presence of a Hox12 gene in a pre-duplicative ancestral Hox
206 cluster. Therefore, the most plausible scenario is that at least two Hox12 genes were lost in the last
207 common ancestor of cyclostomes, one per each of the two ancestral Hox clusters. Phylogenetic
208 analyses done using Hox protein sequences from both hagfish and lamprey together with
209 gnathostomes have clearly shown that cyclostomes have members of the other posterior PGs,

strongly supporting the gene loss scenario (Pascual-Anaya et al., 2018). This is the only case in which one of the two major vertebrate groups lacks all members of a single Hox PG, and the impacts such losses could have had on the early divergence of cyclostomes remain unclear. Hox12 genes have important roles in patterning not only the posterior body, but also secondary structures like the distal parts of paired appendages of jawed vertebrates (fins and limbs). Since these paired appendages are not present in cyclostomes, this suggests that PG12 genes might have gained selective pressures in the gnathostome lineage while the genes could be lost in cyclostomes, potentially due to overlapping functionality with other posterior genes, for example in the tailbud (Kuraku et al., 2008; Takio et al., 2007).

Spatial collinearity and the *Hox* code in cyclostomes

In most animals, Hox genes are expressed following a nested pattern where the expression domain of each gene correlates with its position in the cluster, from 3'/rostral towards 5'/caudal (Gaunt, 2015). In this way, a distinct combination of Hox genes, or Hox code, is created along the anterior-posterior axis of the animal. In jawed vertebrates, this pattern is most clearly observed in the hindbrain and the neural crest-derived mesenchyme of pharyngeal arches, where the rostral border of anterior Hox gene expression sharply corresponds with or is restricted to these morphologically distinct segments (rhombomeres and pharyngeal arches), with the exceptions of rhombomere (r) 1 and the first pharyngeal arch (PA) 1, which are characterized by the absence of Hox expression. On the other hand, posterior Hox genes are involved in the patterning of more caudal regions of the embryo.

Most of the expression data in cyclostomes comes from studies using mainly two species of lampreys, *L. camtschaticum* (Kuraku et al., 2008; Murakami et al., 2004; Onimaru et al., 2011; Takio et al., 2007; Takio et al., 2004) and *P. marinus* (Parker et al., 2014a, 2019a). A comprehensive expression analysis of all PG1-4 genes in *P. marinus* has shown that these Hox genes are expressed similarly to their gnathostome counterparts, included segmentally restricted expression patterns of some of them in the hindbrain and the neural crest of pharyngeal arches (Parker et al., 2019a) (see Fig. 1C). For instance, the rhombomere-restricted expression of *hoxβ1* in r4 is reminiscent of that of *Hoxb1* in gnathostomes (Parker et al., 2014a, 2019a). In total, 8 out of 14 lamprey PG1-4 genes are expressed with a rhombomere registration, i.e., that their anterior borders of expression coincide with the anatomical borders of rhombomeres, like in gnathostomes

(Fig. 1C). For instance, at stage 23, lamprey *hoxα2* is expressed up to the r1/2 border with high levels of expression in r3 and r5, like gnathostome *Hoxa2* (Parker et al., 2014a, 2019a). Importantly, Parker and colleagues also found that some lamprey Hox4 genes (*hoxα4* and *hoxζ4*) are expressed from the r6/7 border, like in gnathostomes (Parker et al., 2019a) (Fig. 1C). In the hagfish, very similar patterns to those of the lamprey and gnathostome have also been found more recently, with *hox2.IV* expressed in the hindbrain with an anterior limit at the r1/2 border, *hox3.II* at the r4/5 border and *hox4.IV* at the r6/7 border (Pascual-Anaya et al., 2018) (Fig. 1C). Contrary to gnathostomes, however, this segmental registration of anterior Hox genes in the hindbrain seems to be transient and dynamic, at least in the lamprey, where the domain-restricted expression of some of the Hox genes seems to disappear as development progresses (Parker et al., 2019a). For instance, sea lamprey *hoxα3* has an initial rostral limit of expression at the r4/5 border, like gnathostome paralogues *Hoxa3*, *-b3* and *-d3*, but becomes more restricted into r4 at later stages, as also observed in the Arctic lamprey (Murakami et al., 2004; Parker et al., 2019a).

Five of the lamprey PG2-4 Hox genes are also expressed in nested domains within the pharyngeal arches, like in gnathostomes. Curiously, only genes from *hoxα* and *hoxδ* clusters are expressed in the pharyngeal arches' neural crest: *hoxα2*, *-δ*2, *-α*3, *-δ*3 and *-δ*4, but, unlike in gnathostomes, with little redundancy between genes of the same PG (Parker et al., 2019a; Parker et al., 2019b; Takio et al., 2007; Takio et al., 2004). In the hagfish, genes of the PG2-5 are also expressed colinearly in the pharynx, although the exact association of Hox genes with specific pharyngeal arches is not clear (Pascual-Anaya et al., 2018). In the lamprey, two Hox genes, *hoxδ2* and *hoxζ4*, are also expressed in the endostyle, a structure that during metamorphosis will give rise to the thyroid (Parker et al., 2019a).

Less it is known about the expression patterns of other central and posterior Hox genes and their possible functions in the morphological patterning of cyclostomes. Again, most of our knowledge comes from work with lampreys, in this case the Arctic lamprey. Several studies from the laboratory of Shigeru Kuratani on *L. camtschaticum* have reported the expression patterns of 13 out of 28 PG5-14 genes (*hoxα5*, *-γ5*, *-α6*, *-β7*, *-α8*, *-ε8*, *-β9*, *-β10*, -ε10, *-ε11*, *-γ13*, *-δ13* and *-ε14*; Kuraku et al., 2008; Takio et al., 2007; Takio et al., 2004). For most of these genes, they found colinear expression in the neural tube but not in the PA ectomesenchyme, as in gnathostomes. Posterior PG9-11 genes that were analyzed were expressed in the progressing tailbud and the neural tube, where they acquire a fixed anterior boundary, with the notable exception of *hoxε10*,

whose expression in the neural tube remains continuously restricted to the level of the tailbud, therefore displaying a dynamic anterior boundary that moves posteriorly throughout development, unlike in gnathostomes, where Hox genes have fixed rostral limits (Takio et al., 2007). *hoxδ13* is also expressed in the tailbud. Finally, *hoxγ13*, *-δ13* and *-ε14* are all found in the most posterior region surrounding the hindgut, where *hoxγ13* was found in the cloacal region (Kuraku et al., 2008). Consistent with its lamprey counterparts, hagfish *hox13.II* and *-13.VI* genes have been found in the cloacal region of the hagfish *E. burgeri*, although not *hox14.I*, at least in the stage and region assayed (Pascual-Anaya et al., 2018). These hindgut/cloacal-associated expression patterns have been found in gnathostomes as well (Theodosiou et al., 2007; Yokouchi et al., 1995), suggesting that this was the case for the most posterior Hox genes in the common ancestor of living vertebrates.

In conclusion, the broad similarities between gnathostomes, lampreys and hagfish strongly suggest that a nested expression of anterior Hox genes associated with the morphological segmentation of the hindbrain and the pharynx, including Hox absence from r1 and PA1 (Fig. 1C). In addition, colinear expression in the rest of the neural tube and the most posterior regions of the body are all traits that were established in the last common ancestor of crown vertebrates before cyclostomes and gnathostomes diverged. Interestingly, this implies that this integration occurred in a vertebrate that most likely possessed only two Hox clusters. This system then evolved independently in both cyclostomes and gnathostomes through lineage-specific duplications and divergence of paralogs, such as the dynamic expression boundaries observed in some cyclostome Hox genes, ultimately underlying the morphological differences we observe in both groups.

Regulatory evolution of Hox genes in the lamprey

Gnathostome Hox colinear expression is tightly integrated to the morphological segmentation of both the hindbrain and the pharynx through conserved gene regulatory networks (GRN; Alexander et al., 2009; Green et al., 2015; Parker et al., 2019b; Parker and Krumlauf, 2020), and the similarities found between gnathostome, lamprey and hagfish expression patterns of anterior Hox genes, together with the transient segmentation of the lamprey and hagfish hindbrain into rhombomeres (Kuratani et al., 1998; Oisi et al., 2013), strongly suggest the conservation of these GRNs in vertebrates. Indeed, reporter assays with gnathostome enhancers from Hox1-4 genes resulted in a temporally activated, segmental reporter activity in the hindbrain of lamprey and zebrafish embryos reflecting that of the genes these enhancers control endogenously (Parker et al.,

2014a). Importantly, this activation seems to be mediated by the same regulatory inputs, since mutations of specific transcription factor binding sites for known segmental regulators, such as Krox20 and Kreisler, eliminated or reduced the reporter expression of a *Hoxb3* r5 enhancer in both zebrafish and lamprey embryos (Parker et al., 2014a). Consistently, lamprey *kreisler* and *krox20* are expressed in hindbrain segments similarly to Hox genes and reminiscent of their gnathostome counterparts (Parker et al., 2014a). Furthermore, the dissection of the regulatory landscape of lamprey *hoxα2* revealed that, despite the lack of sequence conservation, this gene is controlled by discrete *cis*-regulatory elements equivalent to those of gnathostome *Hoxa2*, strikingly including an exonic/intronic element that controls the expression in r2 and r4 (Parker et al., 2014a; Tümpel et al., 2007; Tümpel et al., 2008; Tümpel et al., 2006). Further characterization of the lamprey *hoxα2* regulation found an enhancer with shared activity in r4 and its NC, like its *Hoxa2* mouse counterpart, which demonstrated that the NC GRN is also conserved to the base of vertebrates (Parker et al., 2019b). As in the hindbrain, this activity seems to be mediated by deeply conserved transcription factor binding sites in both the lamprey and gnathostomes that involves Meis and Pbx-Hox factors (Parker et al., 2019b). At the same time, some divergence of these regulatory elements seems to have occurred independently in paralogs in both jawed and jawless vertebrates. For instance, while the NC enhancer in lamprey *hoxα2* has a shared activity in r4, that of gnathostome *Hoxa2* is separated into distinct enhancers (but remains shared in *Hoxb2*) (Parker et al., 2019b). Taken together, the data by Parker and colleagues suggests that the implementation of the NC GRN in the lamprey is mediated, as in the hindbrain, by conserved regulatory inputs and cis-regulatory elements.

Interestingly, although the GRNs for the patterning of the vertebrate NC and hindbrain are well conserved in all major vertebrate lineages, none of the extant groups represent the ancestral condition particularly well, as these had to be established in a vertebrate that possessed only two Hox clusters, rather than the four that have usually been considered until now. There is no doubt that the independent WGDs taking place separately in the gnathostome and lamprey/hagfish/cyclostome lineages had a major impact in the divergence of the regulatory activity of these networks, while selective pressures and constraints on, for instance, cranial structures has broadly kept these GRNs intact.

More recently, an analysis of the global presence of active binding sites for CCCTC-binding factor (CTCF) in the lamprey has demonstrated their presence in the Hox clusters, like in

gnathostomes. However, unlike in gnathostomes, these seem to be abundant in the anterior regions of the clusters, which could be reflecting different modes of global regulation between amniotes and lampreys. However, the specific evolutionary implications of these differences remain unknown (Kadota et al., 2017; see Chapter 5).

Whole-cluster temporal collinearity of cyclostome Hox genes

Hox genes are also expressed following temporal collinearity (TC), meaning that genes in more anterior regions of the cluster are expressed at earlier developmental stages than genes in more posterior regions (Dollé et al., 1989; Izpisúa-Belmonte et al., 1991). In jawed vertebrates, this feature is usually observed across the whole cluster in order, which has recently been called whole-cluster temporal collinearity (WTC; Wang et al., 2017). In contrast, in some protostomes, subgroups of Hox genes are expressed according to TC in parallel, and this has been called sub-cluster temporal collinearity (STC; Wang et al., 2017). This raised the question of what mode of expression the Hox clusters of an ancestral vertebrates followed; either a more invertebrate-like mode, or WTC as seen in amniotes. Information from the cyclostomes could therefore help to resolve this. The first reports from the lamprey were unclear, since while the expression patterns observed from some genes belonging to the same cluster in the Arctic lamprey depicted no traces of temporal collinearity (Takio et al., 2007), reporter assays with anterior regulatory elements pointed to the presence of temporally ordered expression of Hox1-3 (Parker et al., 2014a). A recent comprehensive analysis of all Hox genes from the lamprey *L. camtschaticum* and the hagfish *E. burgeri*, in comparison with the catshark *Scyliorhinus torazame*, has demonstrated that, at least in the lamprey, Hox genes of several clusters are expressed following WTC during development, clearly suggesting that the last common ancestor of vertebrates possessed Hox genes that were expressed according to the WTC mode (Pascual-Anaya et al., 2018).

Again, it is noteworthy that vertebrate temporal collinearity had to be established in an ancestor with two Hox clusters, and therefore we can infer the presence of strong constraints that maintained it in all four Hox clusters of gnathostomes, while in the lamprey this seems to disappear from some gene-poor clusters (like *hoxy*), probably reflecting the disappearance of such constraints in some paralogous clusters after the extra duplications in the cyclostome lineage. The implications for the morphological evolution of the lamprey remains obscure, but future functional experiments altering the temporal collinearity in the lamprey will shed light on this question.

Perspectives on a putative Hox code in the anterior-posterior patterning of the vertebral column in lampreys

The anterior-posterior patterning along the animal's body axis is reflected in its vertebral column. Vertebrae are somite-derivatives (Ebner, 1889; Remak, 1855), and while somites are morphologically similar, they produce vertebrae with different morphologies. Each vertebra has a distinct form and function depending on its position along the anterior-posterior body axis (Gadow, 1933). The number of morphofunctional regions as well as the number of vertebrae that contribute to one region vary across vertebrates (e.g., Bergmann and Irschick, 2012; Berio et al., 2021; Bui and Larsson, 2020; Jawad, 2015; Johanson et al., 2005; Jones et al., 2018; Maxwell et al., 2021; Morin-Kensicki et al., 2002; Müller et al., 2010; Sallan, 2012; Scheyer et al., 2019; Ward and Mehta, 2014). Mostly, it has been assumed that vertebral regionalization is a gnathostome innovation, but there is evidence suggesting that it occurred earlier in vertebrate evolutionary history (Chevrinais et al., 2018; Tretjakoff, 1926).

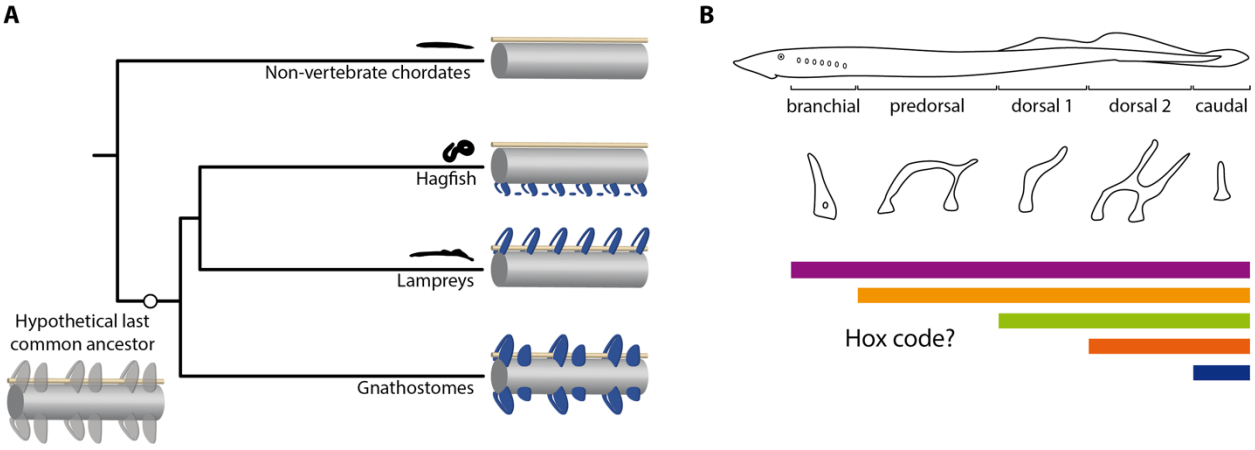

Figure 2. Vertebral column in agnathans. (**A**) Non-vertebrate chordates have a notochord (grey rod) lying ventrally to the neural tube (beige rod). The hypothetical last common ancestor of hagfish, lampreys and gnathostomes had cartilaginous vertebrae consisting of dorsal and ventral elements. Potentially, dorsal elements got lost during evolution in hagfish and ventral elements got lost in lampreys. After Ota et al. (2011). (**B**) Top, Schematic representation of a lamprey. The vertebral column is regionalized into five sections from head to tail. A vertebral arch representative for each region is shown. After Tretjakoff (1926) and Chevrinais et al. (2018). Bottom, open question regarding the possible existence of a Hox code underlying lamprey vertebral column regionalization.

Lampreys have cartilaginous vertebral arches that dorsally surround the spinal cord from head to tail (Tretjakoff, 1926) (Fig. 2A). Traditionally, hagfish were thought to lack true vertebrae.

However, a recent developmental study supports the observations made by Ayers and Jackson (1901) that they do have vertebra-like elements homologous to gnathostome vertebrae (Ota et al., 2011) (Fig. 2A). These cartilaginous elements lie ventral to the notochord but are restricted to the tail region in hagfish. It may be possible that the vertebral column in lampreys and hagfish is secondarily reduced because the extinct relative *Euphanerops* had vertebral elements at the dorsal and ventral aspect of the notochord (Janvier, 2011; Ota et al., 2014). Initially present in the common ancestor of all vertebrates, the ventral elements were then lost in lampreys and the dorsal elements lost in hagfish (Ota et al., 2011) (Fig. 2A). In contrast to hagfish, in which the vertebral elements are confined to the post-cloacal region, Tretjakoff (1926) noted that the vertebral column of lampreys is quite variable along its length, and identified 5 morphofunctional regions based on distinct characteristics (Fig. 2B): (1) The vertebral arches of the gill region ("Kiemengebiet") have an additional hole for the motor nerves; (2) In the cranial trunk region, the vertebral arches of the heart and liver region ("Herz-Lebergebiet") are characterized by being bipartite with a cranial and caudal half. The vertebral elements in this region are more or less vertical but become increasingly bent caudally; (3) The middle trunk region ("mittleres Rumpfgebiet") coincides with the cranial part of the dorsal fin. The vertebral arches are slightly S-shaped and always present as discrete structures; (4) In the caudal trunk region ("hinteres Rumpfgebiet"), the vertebral elements are taller compared to the preceding vertebrae. They are irregular in shape partly forming N- and H-formed structures; (5) The vertebral arches become increasingly smaller in the tail region ("Schwanzgebiet"). Chevrinais et al. (2018) later supported this observation, identifying a branchial, predorsal, dorsal 1, dorsal 2 and caudal region that correspond to the regions defined by Tretjakoff (1926). In total, there are about 130 vertebral arches forming the vertebral column in lampreys (Tretjakoff, 1926). The absolute number of segments is primarily controlled by the process of somitogenesis (e.g., Gomez et al., 2008; Schröter and Oates, 2010), whereas the morphofunctional regionalization is the result of the sequential expression of Hox genes (i.e., Hox code) in the somites (reviewed by Mallo et al., 2010). In tetrapods, for instance, the transition from the cervical vertebral region (neck) to the thoracic vertebral region (trunk) correlates with the anterior expression limit of the Hox PG 6 (Böhmer et al., 2015b; Burke et al., 1995; Mansfield and Abzhanov, 2010; Woltering et al., 2009). A longer neck with more cervical vertebrae is associated with a posterior shift of Hox-6 gene expression transposing the neck-trunk transition posteriorly. The correlation between regional identity of the vertebrae and the Hox gene expression pattern

even allows for inference of evolutionary modifications in the Hox code based on quantitative morphological differences in the vertebral column (Böhmer, 2017; Böhmer et al., 2018; Böhmer et al., 2015a; Böhmer and Werneburg, 2017; Guinard and Marchand, 2010; Head and Polly, 2015; Johnson and O'Higgins, 1996; Szczygielski, 2017). This prompts the question of whether there is a relationship between vertebral regionalization and a Hox code in the lamprey as well (Fig. 2B).

In lampreys, information on the somitic Hox gene expression is incomplete to date. Onimaru et al. (2011) reported on two Hox genes that are expressed in the primitive paraxial mesoderm: at stage 22, the anterior expression limit of *hoxα5* was found at somites 9-10 and that of *hoxα6* at somites 14-15. This data is clearly not sufficient to provide robust evidence, but it may hint at a spatial collinearity in the somitic expression of Hox genes in lampreys. To date, we can only hypothesize a correlation between a Hox code and regionalization in the vertebral column of lampreys (Fig. 2B).

Summary and future perspectives

In the last decade, significant efforts towards understanding cyclostome genomics have enabled unprecedented comparative analysis with jawed vertebrates, permitting probably the most accurate depiction of the last common vertebrate ancestor to date. It is important to note that this ancestor likely possessed two Hox clusters instead of the previously hypothesized four. Unfortunately, no lineage of vertebrates with this condition has survived to the present day, meaning that reconstructing this hypothetical animal is more complicated than previously thought. Here we have seen how cyclostome and gnathostome Hox clusters have originated following independent lineage specific WGDs, while the main features of the clusters have been broadly retained in both lineages, especially those involved in the patterning of cranial structures (e.g., conserved hindbrain and NC GRNs). There are also differences observed between the two groups, with distinct expression and regulatory divergence of paralogous Hox genes in each lineage. For instance, the dynamic nature of the anterior border of some cyclostome Hox genes versus the fixed pattern in gnathostomes, expression of Hox genes in lineage-specific organs (e.g., lamprey endostyle), and differences in paralog expression (e.g. functionally overlapping in the pharynx in gnathostomes but not in the lamprey). Whether these differences are behind the morphological divergence and the origin of novelties in each of the lineages (e.g., jaw versus cyclostome velum) is not clear. We anticipate that further analyses of the Hox code, especially in non-cranial structures, is still

necessary to better understand the Hox system of cyclostomes. This, together with more functional approaches using lamprey embryos, such as the mutation of specific Hox genes with the CRISPR/Cas9 system and high-throughput analyses (ChIP-seq, RNA-seq, ATAC-seq, in whole embryos and single cells), will enable an unprecedented dissection of the roles of cyclostome Hox genes. Ultimately, this will provide a deeper understanding of the ancestral roles of Hox genes in vertebrates, how these have changed and if those changes are responsible, at least in part, for vertebrate morphological diversification.

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