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A Hox regulatory network of hindbrain segmentation is conserved to the base of vertebrates

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Abstract

A defining feature governing head patterning of jawed vertebrates is a highly conserved gene regulatory network that integrates hindbrain segmentation with segmentally-restricted domains of Hox gene expression. Although non-vertebrate chordates display nested domains of axial Hox expression, they lack hindbrain segmentation. The sea lamprey, a jawless fish, is poised to provide unique insights into vertebrate origins due to its phylogenetic position at the base of the vertebrate tree[1-3]. It has been suggested that lamprey may represent an intermediate state where nested Hox expression has not been coupled to the process of hindbrain segmentation[4-6]. However, little is known about the regulatory network underlying Hox expression in lamprey or its relationship to hindbrain segmentation. Using a novel tool that allows cross-species comparisons of regulatory elements between jawed and jawless vertebrates, we report deep conservation of both upstream regulators and segmental activity of enhancer elements across these distant species. Regulatory regions from diverse gnathostomes drive segmental reporter expression in the lamprey hindbrain and require the same transcriptional inputs (e.g. Kreisler, Krox20) in both lamprey and zebrafish. We find that lamprey Hox genes display dynamic segmentally-restricted domains of expression and have isolated a conserved exonic Hox2 enhancer from lamprey that drives segmental expression in rhombomeres 2 and 4. Our results show that coupling of Hox gene expression to segmentation of the hindbrain is an ancient trait with origin at the base of vertebrates that likely led to the formation of rhombomeric compartments with an underlying Hox code.

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Author Contributions

HJP, RK and MEB conceived this research program. HJP conducted the experiments. HJP, RK and MEB jointly analyzed the data, discussed the ideas and interpretations and wrote the manuscript.

The authors declare no competing financial interests.

The sequences for the lamprey *Hox1w* and *Kreisler* transcripts were deposited in Genbank under accession numbers KM087087 (Hox1w) and KM087088 (Kreisler). All original source data are deposited in the Stowers Institute Original Data Repository and available online at http://odr.stowers.org/websimr/.

The hindbrain of jawed vertebrates is a specialised region of the nervous system characterised by its subdivision into repetitive segments called rhombomeres (r)[7]. Anterior Hox genes are expressed in a nested pattern that is functionally coupled to this inherent segmentation program[8-10]. Non-vertebrate chordates possess patterned Hox gene expression along the body axis[11-14], likely regulated by conserved patterning signals in chordate evolution[15], but lack nervous system segmentation. Moreover, key segmental regulatory elements from jawed vertebrate Hox clusters are not conserved in amphioxus or ascidians[16-18]. In jawed vertebrates (gnathostomes), a well-characterised, highly conserved gene regulatory network (GRN) integrates hindbrain segmentation and Hox patterning[8,9]. The jawless (agnathan) vertebrate, lamprey, has been postulated to represent an intermediate state with rudimentary hindbrain segmentation, but lacking registration with motoneuron patterning or nested Hox expression[4-6]. However, little is known about gene regulatory events underlying Hox expression or coupling to hindbrain segmentation in lamprey. Here we address the nature of the agnathan hindbrain GRN and degree to which it has been evolutionarily conserved with that of gnathostomes.

To explore upstream GRN inputs regulating Hox expression, we first asked whether gnathostome hindbrain regulatory elements were functional in the sea lamprey, *Petromyzon marinus*, the only agnathan for which the genome is sequenced and experimental manipulation of early embryos is feasible[3,19]. Using transgenic methodologies[20,21], we developed a novel cross-species approach to compare activity of specific regulatory elements between jawed and jawless vertebrates, by creating a new construct that allows efficient transgenesis in both lamprey and zebrafish embryos. We chose a series of enhancers from different jawed vertebrates that mediate segmentally-restricted expression in their species of origin (Fig.1a; Extended Data Fig.1), focusing on elements that worked across multiple species and have well-characterised direct inputs from Krox20, Kreisler, retinoic acid and/or Hox auto/cross-regulation.

Analysis of F0 zebrafish embryos demonstrated that the majority of enhancers direct appropriate GFP reporter expression in segmental hindbrain domains (Fig.1b; Extended Data Fig.2 & Table1). The identities of segmental domains were determined by examining GFP expression in a zebrafish line expressing mCherry in r3/r5 under control of the endogenous *Krox20* locus. F1 lines were generated for many constructs and exhibited identical segmental expression patterns as F0s (Fig.1b;Extended Data Fig.2 & 3), confirming that analysis in F0 embryos accurately reflects enhancer mediated regulatory activities.

When tested for regulatory activity in lamprey, the same gnathostome constructs mediated segmental reporter expression reminiscent of that seen in their host species and/or zebrafish. The restricted stripes of GFP expression reflect an ordered series of domains (Fig.1b, Extended Data Fig.2 & Table2), implying that these gnathostome enhancers are activated by upstream lamprey factors to mediate reporter expression in a rhombomeric fashion. Reporter expression spans multiple developmental stages (st) with variable onset between elements (Extended Data Fig.4). The *Hoxb1* enhancer is active first (st18) in a broad domain that becomes restricted over time, followed by *Hoxb2* (st21), *Hoxb3* and *Hoxb4* (st22). These

data suggest that a similar underlying hindbrain GRN, with temporal colinearity reminiscent of gnathostomes, may be present in lamprey.

Gnathostome rhombomeric enhancers have known *cis*-regulatory inputs: Krox20 for EphA4[22] and *Hoxb2*[23,24] and Kreisler and Krox20 for *Hoxb3*[25,26]. We asked whether their homologues might play similar segmental regulatory functions in agnathans. To test this, we generated constructs with mutated Kreisler and/or Krox20 sites within the zebrafish *Hoxb3* r5 enhancer (Fig.1c,d). Mutation of the two Kreisler sites (*mut kr1+kr2*) completely eliminates reporter expression in both zebrafish and lamprey, whereas mutation of the Krox20 sites (*mut kroxA+kroxB*) modulates levels/efficiency of expression in both species (Extended Data Tables1 & 2). These results are consistent with roles for Kreisler and Krox20 in the mouse *Hoxb3* r5 enhancer[26], implying homologous roles in the lamprey hindbrain.

The data suggest that major components of the hindbrain GRN upstream of Hox genes are conserved in lamprey. Therefore, we characterised hindbrain expression patterns of lamprey *Kreisler* and *Krox20* across multiple developmental stages (19-26) (Fig.2). *Krox20* is expressed in two stripes in a manner reminiscent of its gnathostome counterpart[4] (Fig.2). We isolated a *Kreisler/MafB* homologous gene that is expressed in a single stripe in the lamprey hindbrain (Fig.2), similar to mouse *Kreisler*. The restricted expression of these key upstream regulators in lamprey supports our interpretation of their inputs to reporter activities.

We next examined whether lamprey Hox genes themselves display evidence of segmental expression. We previously identified two Hox clusters, Pm1 and Pm2, as well as several unassigned Hox genes in *P. marinus*[3]. These likely represent a subset of the total Hox gene complement; recent evidence from *Lethenteron japonicum* suggests up to six Hox clusters[27], two of which are homologous to Pm1 and Pm2. Lamprey Hox genes from paralogy groups 1-3, *Hox1* (Pm2), *Hox2* and *Hox3* (Pm1), display temporally dynamic hindbrain expression patterns. Early stages (21-23) reveal prominent stripes of restricted expression in the hindbrain for all three genes, apparently reflecting off-set segmental domains (Fig.2) temporally correlating with robust stripes of both *Krox20* and *Kreisler* expression. Later (st24-26), *Hox1* and *Kreisler* are progressively down-regulated in the hindbrain, while segmental expression for *Hox2* and *Hox3* become masked by their up-regulation in other regions (Fig.2). *Krox20* expression initiates at st20 and remains on throughout this developmental time-course. Although previous analysis of Hox gene expression, focusing on st26 in the Japanese lamprey, found no evidence for segmental expression[4,5], the potential links between Hox expression and hindbrain segmentation were presumably missed due to the dynamic and early nature of segmental expression of these genes.

To identify endogenous lamprey *cis*-regulatory regions that mediate these striking segmental Hox gene expression domains, we focused on the *Hox2* paralogy group, well-characterized from a regulatory perspective in jawed vertebrates[8,9]. We sequenced the *Hox2* locus and entire intergenic region between *Hox2-Hox3* of Pm1, as this genomic region in gnathostomes contains a series of enhancers that mediate hindbrain Hox expression (Fig. 3a;Extended Data Fig.1). Because no overt sequence conservation with known jawed vertebrate enhancers was detectable, we functionally tested sequences from -12kb upstream

to +1kb downstream of the lamprey Hox2 coding domain in lamprey embryos (Fig.3a-c). At st26, the -12kb intergenic region mediates GFP expression in the neural tube, pharynx (neural crest) and somites that closely resembles that of endogenous lamprey *Hox2* gene (Fig.3b). Deletion analyses demonstrate that *cis*-elements capable of mediating neural expression lie within the -9kb to -4kb intergenic region whereas those contributing to neural crest/somite expression lie in the -4kb fragment (Fig.3a).

Given that gnathostome *Hoxa2* is expressed in r2 and r4 via exonic and intronic regulatory elements[28-30] (Fig.3a;Extended Data Fig.1), we tested a comparable fragment of lamprey *Hox2* (*exon1-2*). Intriguingly, this fragment mediated restricted expression in two alternating stripes in the hindbrain from st22-26 (Fig.3c;Extended Data Fig.4). At st24, endogenous *Hox2* neural expression displays regions of varying intensity, apparently correlating with these stripes of GFP (Fig.3c). The anterior boundary of GFP expression in the hindbrain mediated by both the -12kb fragment and the *exon1-2* region appear to match that of the endogenous *Hox2* gene (Fig.3c). Hence, *Hox2*, as in jawed vertebrates, contains multiple enhancers with partially overlapping/shadow activities. The equivalent positions of rhombomeric enhancer(s) of *Hox2* and *Hoxa2* genes suggests that lamprey Hox genes may be coupled to hindbrain segmentation in part through conserved *cis*-elements.

The lack of apparent morphological hindbrain segmentation in lamprey makes it difficult to assign these gene expression patterns to specific features. To register these expression patterns, we performed multispectral analysis using co-injection of two fluorescent reporters. The *Hoxb3* enhancer was used to direct RFP in putative r5, allowing registration with other enhancer-mediated GFP expression (Fig.4a,b). *Hox2-exon1-2* mediates expression in r2 and r4; *EphA4* in r3; *Hoxb2* in r4; and *Hoxb4* with an anterior border of expression within r7 (Fig.4a,b). These segmental domains generally correlate with the activity of these *cis*-elements in gnathostomes, although the *EphA4* enhancer mediates expression only in r3 in lamprey as compared to r3/r5 in zebrafish (Fig.1b). The *Hoxb2* enhancer drives robust r3/r5 expression and weaker r4 expression in zebrafish (Fig.1b), whereas strongest expression in lamprey is in r4. Some embryos exhibit weaker r3/r5 expression, suggesting that the Krox20 sites in this enhancer are only moderately functional in lamprey. These data confirm that regulatory elements from both jawed and jawless vertebrates can mediate adjacent rhombomere-like segmental expression domains in the lamprey hindbrain.

To compare endogenous with enhancer-driven domains of expression we performed two-colour double *in situ* hybridisation (Fig.4c). Using *Krox20* as a reference for r3/r5, we mapped the site of *Hox1* expression to r4. Similarly, by comparison with *Krox20* and/or *Hox1*, we mapped *Kreisler* expression domains to r5, *Hox2* to r2-5 with elevated stripes in r3/r5, and the anterior stripe of *Hox3* expression to r5. Antisense GFP probes position expression directed by *Hoxb3* enhancer to r5. This analysis demonstrates that lamprey Hox genes are expressed in a nested pattern that corresponds to the same segmental territories as their gnathostome counterparts.

By taking advantage of the unique evolutionary position of lamprey at the base of vertebrates, we have resolved a fundamental question in vertebrate evolution concerning the

origin of segmental Hox patterning in the hindbrain. Our results reveal an amazing degree of conservation in both transcriptional inputs (Krox20, Kreisler) and regulatory element activity between jawed and jawless vertebrates (Fig.4d). Lamprey Hox genes display transient offset segmental expression domains, implying that the lamprey hindbrain, as in gnathostomes, is comprised of identifiable rhombomeric segments with an underlying Hox code. Thus, we conclude that the coupling of Hox gene expression to segmentation of the hindbrain via Krox20 and Kreisler is an ancient vertebrate trait that evolved prior to the agnathan/gnathostome split (Fig.4e).

METHODS

Enhancer elements

Enhancer elements were selected from the published data or identified based upon cross-species sequence alignments. DNA containing each element were amplified by PCR from genomic DNA templates, the size of the amplified fragments is indicated in (bp), using Phusion High-Fidelity DNA Polymerase (NEB) and the primers listed below were used for amplification. The sequences in bold represent homology to genomic DNA and adapter sequences are in non-bolded text:

Hoxb1(m)[31] (378bp)

F: 5’-AATTTGGGGCCC**TCTAATAATCCAAGAACCTATTGAAGG**-3’

R: 5’-TACAACCTCGA**GCAGTATGTCACAGAGCTGAAG**-3’

Hoxa2(m)[32] (808bp)

F: 5’-GATGCTGGGCCC**AGATCTGAATGCTGGAGCAGTCTCAG**-3’

R: 5’-CATAGCCTCG**AGGTACCTTCTCTCCCTCAAACC**-3’

Hoxa2b(zf) (2960bp)

F: 5’-**GGGTATTAAACAGGTATCTGAATGC**-3’

R: 5’-**AAATTCGCCGCTCTCAAAT**-3’

Hoxa2a(fr)[29] (1404bp)

F: 5’-ATCTGAGGGCCC**TGGCTTAATGCAAACGCTATATTT**-3’

R: 5’-GTACATCTCGAG**CCCTATTTCGAATACGACTCTG**-3’

Hoxa2b(fr)[29] (1263bp)

F: 5’-**TGCTGTAATGCCAAAACCTC**-3’

R: 5’-**CCTGCCTCGCCTTCGTGCCG**-3’

Hoxb2(m)[33] (2021bp)

F: 5’-ATGCGTGGGCCC**GGATCCCCACTTTAACACCCAAG**-3’

R: 5’-GTACAGCTCGA**GTCTCCGCCAATCGCTAGT**-3’

Hoxb2a(zf) (1488bp)

F: 5’-**TGACCCCATTCCGTAGTACC**-3’

R: 5’-**TATTTTGCGCTCCTGCTATG**-3’

EphA4(m)[22] (496bp)

F: 5’-AACTGAGGGCCC**AGCATGGAGCTCTCTTAGCGTA**-3’

R: 5’-TCATTACTC**GAGTTTCGGGCTCTAGATCTGC**-3’

Hoxb3(m)[25] (649bp)

F: 5’-AGCTCTCTCGA**GCAGTAGGATCCCAGGT**-3’

R: 5’-GCTAATCTC**GAGGAGGCCTGTAGGAGGAAG**-3’

Hoxb3a(zf) (928bp)

F: 5’-AATGGAGGGCCC**GTGTCCGGAAGTGTCGTTTC**-3’

R: 5’-AGGGAACTCG**AGCTCCAGTGAGTCCTGGTC**-3’

HoxB4(m)[34] (951bp)

F: 5’-AACTGAGGGCCC**TGGAATTGGTTGGGTTTTCT**-3’

R: 5-TATCTCCTCG**AGTGTCCATGGTGGAAAGC**-3’

HoxD4(m)[35] (582bp)

F: 5’-ACAAGTGGGCCC**TGGAGGAAGGGCTAGCTTAAA**-3’

R: 5’-AAAAAGCTC**GAGAAGGGTAGTTAAAGTCCAAAAGG**-3’

Lamprey *Hox2 exon1-2* (2625bp)

F: 5’-CGATGAGTCGAC**AGTTTGAGCGGGAAACTGG**-3’

R: 5’-CTAATCGTCGAC**CGAAATCTATTGCGCCTACA**-3’

Generation of reporter constructs

The HLC (Hugo’s Lamprey Construct) vector and its variants (*HLC-GW*, *HLC-RFP*) were created for this study (reagents and sequences are available on request). PCR-purified enhancer elements were cloned into HLC using either standard restriction enzyme-mediated methods or by first cloning PCR products into the *pCR8/GW/TOPO TA* vector (Invitrogen) followed by transfer into a Gateway-compatible variant of HLC (*HLC-GW*) via *in vitro* recombination using the Gateway LR-Clonase II enzyme (Invitrogen).

The 12kb intergenic region between lamprey *Hox2* and *Hox3* of the Pm1 cluster was cloned into HLC by homologous capture from lamprey *BAC 218A09 (L6)*[3] following previously described recombineering methods[36] and using the following homology arm sequences (homology arms indicated in bold):

Arm 1 5’-
GGGCCC**GTACACGGACCTGTCGTCTCATCACCACCCGACTCAGGAAGT**A
CTAGT-3’

Arm 2 5'-
ACACCCCCCCCCCTCCTCGCTCAGTGCTCCGTCAAGGCAGCCATGG-3'

Shorter fragments of this intergenic region were subsequently generated from the captured 12kb sequence by standard restriction enzyme-mediated cloning approaches. Site-directed mutagenesis was performed on the *Hoxb3(zf)* HLC construct using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) and the primers listed below. The bold underlined text refers to the mutated sequences which differ from wild type:

kr1mutF: 5'-
GTGTTTTCTGCATTTCGTTGCCTC**CTTG**CACGTGTTAGTTAATTAGTG-3'

kr1mutR: 5'-
CACTAATTAACTAACACGTG**CAAG**GAGGCAACGAAATGCAGAAAACAC-3'

kr2mutF: 5'-
CAATGCCGTTTAGTAAAAAGT**CAAG**GACACCTACATTTTTGCCTTG-3'

kr2mutR: 5'-
CAAGGCAAAAATGTAGGTGTC**CTTG**ACTTTTTACTAAACGGCATTG-3'

kroxAmutF: 5'-GCCTTCCTCCCA**GC**CCGTTGGTGATGC-3'

kroxAmutR: 5'-GCATCACCAACGG**GC**TGGGAGGAAGGC-3'

kroxBmutF: 5'-GTTGCAGACACC**G**ACATTTTTGCCTTGTGC-3'

kroxBmutR: 5'-GCACAAGGCAAAAATGT**C**GGTGTCTGCAAC-3'

Zebrafish reporter assay

The following zebrafish lines were used for embryo micro-injection experiments: Slusarski AB (wild-type); *egr2b:KalTA4BI-1xUASkCherry* (r3r5mCherry)[37]. Transient transgenic zebrafish embryos were generated for each reporter construct by Tol2-mediated transgenesis in fertilized eggs as described previously[38]. In general a minimum of 100 embryos were injected to monitor efficiency for each constructs due to mosaicism and position effects of integration. GFP-expressing transient transgenic embryos were raised to adulthood and crossed with either wild-type or r3r5mCherry fish to screen for germline transgene integration[38]. Embryos were screened for fluorescent reporter expression using a Leica M205FA microscope. Fluorescence and bright-field signals were captured with a Leica DFC360FX camera using LAS AF imaging software. Images were cropped and alterations to brightness and contrast were made using Adobe Photoshop CS5.1.

Lamprey reporter assay

Embryos were harvested from gravid lamprey (*Petromyzon marinus*) caught in the wild and provided by Hammond Bay Biological Station, Millersburg, MI, USA. Transient transgenic *Petromyzon marinus* embryos were generated by *I-SceI* meganuclease-mediated transgenesis as described previously[20]. Single-celled embryos at 4-6 hours post-fertilisation were injected with the digested construct at a concentration of 20ngμl^{-1}, maintained as described previously[19] and screened for reporter expression daily from st17 onwards. In general a minimum of 100 embryos were injected to monitor efficiency for each constructs

due to mosaicism and position effects of integration. The *HoxB3(zf)-HLC-RFP* construct, containing RFP rather than GFP, was created for the co-injection experiments. Co-injected constructs were mixed at a concentration of 15ngμl^{-1} each (resulting in a total DNA concentration of 30ngμl^{-1}) and digested for injection. Embryos were screened for fluorescence using a Zeiss SteREO Discovery V12 microscope and imaged with a Zeiss Axiocam MRm camera and AxioVision Rel 4.6 software. Images were cropped and altered for brightness and contrast using Adobe Photoshop CS5.1.

Cloning lamprey *in situ* hybridisation probes

Exonic probes were designed based on previously characterised/predicted gene sequences[3] and were amplified from *P. marinus* genomic DNA by PCR using Phusion High-Fidelity DNA Polymerase and cloned into the *pCR4-TOPO* vector. The size of each amplified fragment is indicated in (bp). For generating 5' and 3' UTR probes, RNA from st18-26 *P. marinus* embryos was extracted using the RNAqueous Total RNA Isolation Kit (Ambion) and used as a template for 5' or 3' RACE with the GeneRacer Kit and SuperScript III RT (Invitrogen). cDNA fragments were amplified by PCR using Phusion High-Fidelity DNA Polymerase and cloned into the *pCR4-TOPO* vector. The following primers were used for PCR:

krox20[4] (468bp, predicted exonic fragment)

F: 5'-CCACAAGCCCTTCCAGTG-3'

R: 5'-GGTGAGGACATCAGCGAGAG-3'

Kreisler (529bp, 5'UTR and partial exon)

F: Generacer 5' Nested Primer

R: 5'-GAGAGGGCCGCTCGGAGAACTTGA-3'

pm2Hox1 (949bp, partial exon and 3'UTR)

F: 5'-CAGAACCGGCGCATGAAGCAGAAGA-3'

R: Generacer 3' Nested Primer

pm1Hox2 (471bp, partial exon 2)

F: 5'-CAAGCGGCAGACTCAGTACA-3'

R: 5'-AGGTCCAGCGTGCTCTCTAA-3'

pm1Hox3 (661bp, partial exon 2)

F: 5'-GACGAGTTGAAATGGCCAAC-3'

R: 5'-TGAGACGACAGGTCCGTGTA-3'

eGFP (709bp)

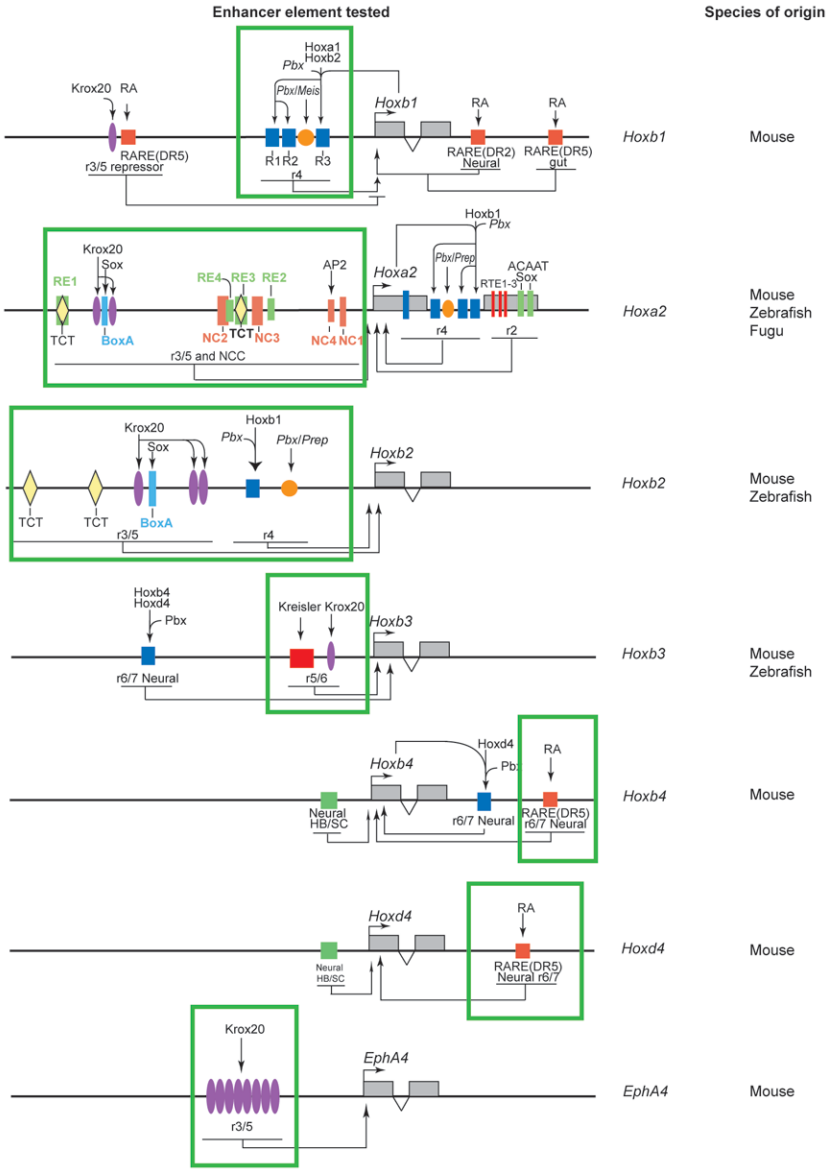
F: 5'-CAAGGGCGAGGAGCTGTT-3'

R: 5'-CTTGTACAGCTCGTCCATGC-3'

Lamprey *in situ* hybridisation

Digoxygenin- and fluorescein-labelled probes were generated by standard methods and used in single and double lamprey whole-mount *in situ* hybridisation as described previously[19]. Embryos were cleared in a solution of 75% glycerol prior to being imaged using a Leica MZ APO microscope with a Zeiss Axiocam HRc camera and Axiovision Rel 4.8 software. Images were cropped and altered for brightness and contrast using Adobe Photoshop CS5.1.

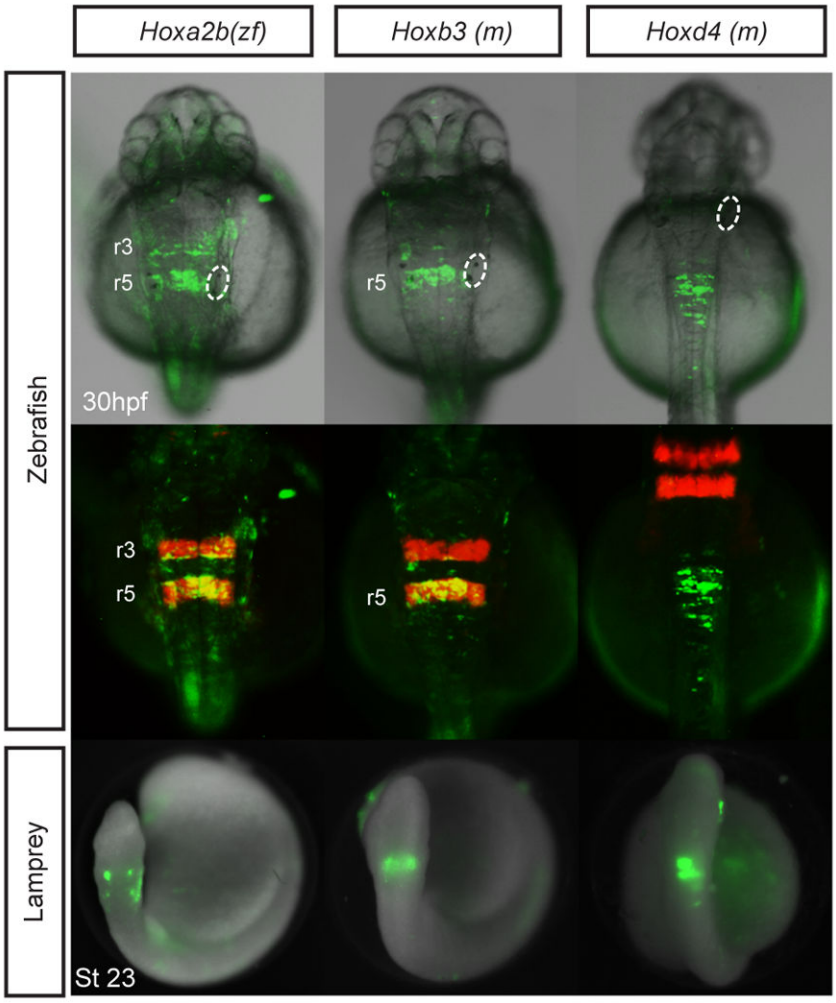
Extended Data



Extended Data Figure 1. Gnathostome enhancer elements selected for reporter analysis

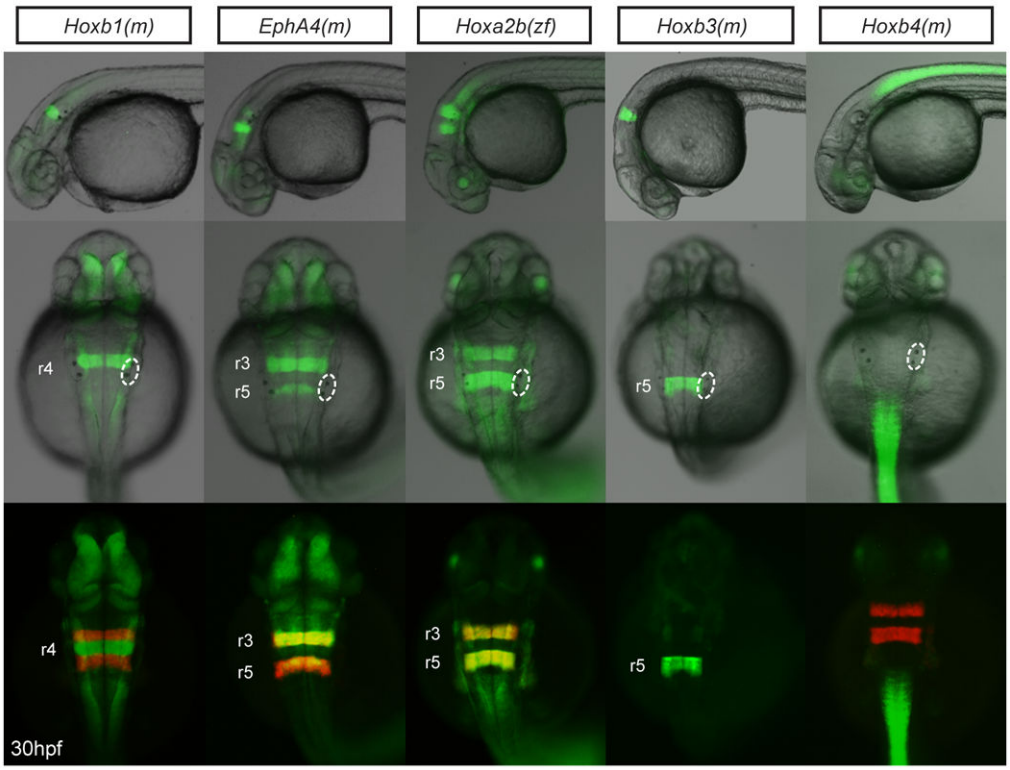
Schematic diagrams depicting the gnathostome enhancer elements assayed for activity in zebrafish and lamprey embryos in this study. The endogenous genomic positions of the enhancer elements (green boxes) are shown relative to the genes that they regulate. Known

trans-acting factors are listed above the elements, whilst the corresponding regulatory modules and their combined activity domains are detailed below the elements. For each element, the species from which it was cloned are listed on the right. Figure based on Fig. 4.2 from Tumpel *et al*. (2009)[9].



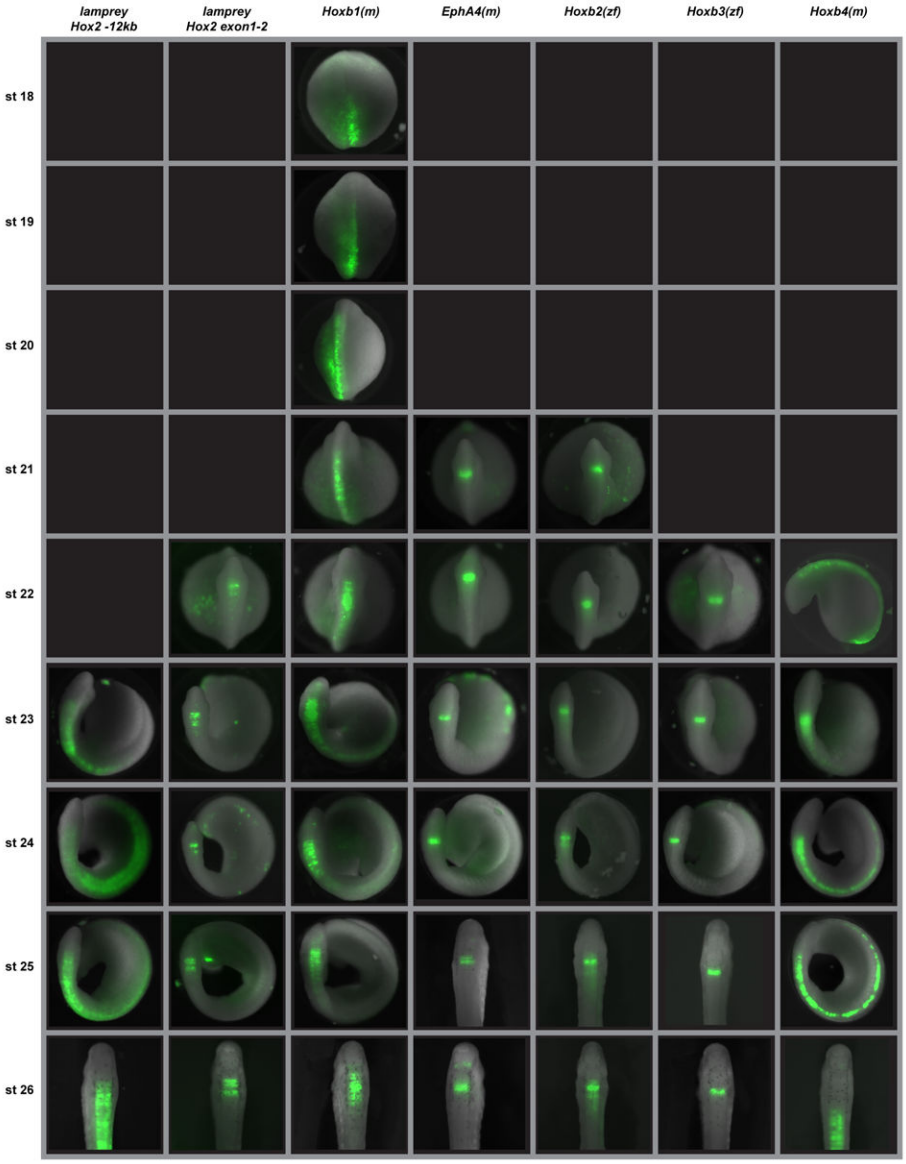
Extended Data Figure 2. Segmental activity of additional jawed vertebrate enhancers in zebrafish and lamprey

GFP reporter expression mediated by gnathostome enhancer elements in zebrafish and lamprey embryos. Dorsal views are shown, with anterior to the top. For zebrafish, two images of the same embryo are shown, presenting GFP plus brightfield (top) and GFP plus endogenous r3r5mCherry (middle) signals. The zebrafish otic vesicle is circled. Abbreviations: m, mouse; r, rhombomere; zf, zebrafish.



Extended Data Figure 3. Segmental patterns of GFP reporter expression in transgenic zebrafish lines

Lateral (top) and dorsal (middle) views of 30hpf transgenic (F1) zebrafish embryos show combined brightfield illumination and segmental GFP reporter expression in the hindbrain mediated by five different gnathostome enhancer elements. The corresponding transient transgenic GFP expression patterns mediated by these elements are shown in Fig. 1b and Extended Data Figure 2. When available, GFP lines were crossed with endogenous r3r5 mCherry reporter line as a reference (bottom). The otic vesicle is circled. Abbreviations: m, mouse; r, rhombomere; zf, zebrafish.



Extended Data Figure 4. Developmental time-course of GFP reporter expression mediated by lamprey and gnathostome regulatory elements in lamprey embryos

Stages 18 to 26 are shown. All embryos are positioned such that the hindbrain is viewed dorsally, with anterior to the top, except for *Hoxb4(m)* at stage 22, which is viewed laterally with anterior to the left. Black boxes indicate no GFP expression mediated by that element at that developmental stage. In both fish and lamprey, expression driven by the gnathostome *Hoxb1* enhancers appear to be temporally dynamic, starting broad and refining with time, likely caused by autoregulation within this element. However, we cannot rule out the possibility that the enhancers used may be missing some repressor elements that are required for fine-tuning.

Extended Data Table 1
Zebrafish reporter assay statistics

For each injected construct, the tissue-specific GFP expression domains are noted, along with the number and proportion of screened embryos exhibiting GFP expression in those domains. In each case, the numbers derive from individual rounds of injection, except for *Hoxb3a(zf)*, for which the data from three separate experiments (exp1-3), that were performed to ensure reproducibility, were combined. Letters in parentheses after the element names indicate the species of origin of the element: fr, fugu; m, mouse; zf, zebrafish. N/A numbers on efficiency not available.

	Element	Expression domain	# embryos	# specific expression	% specific expression
Gnathostome elements	*Hoxb1(m)*	hindbrain	230	218	94.8
	Hoxa2(m)	hindbrain	145	64	44.1
	Hoxa2b(zf)	hindbrain	125	46	36.8
	Hoxa2a(fr)	hindbrain	123	16	13.0
	Hoxa2b(fr)	no specific expression	N/A	N/A	N/A
	Hoxb2(m)	hindbrain	199	75	37.7
	Hoxb2a(zf)	hindbrain	147	95	64.6
	EphA4(m)	hindbrain	195	172	88.2
	Hoxb3(m)	hindbrain	98	70	71.4
	Hoxb3a(zf)	hindbrain	549	503	91.6
	Hoxb4(m)	spinal cord	160	125	78.1
	Hoxd4(m)	spinal cord	324	141	43.5
Hoxb3a(zf) dissection	*Hoxb3a(zf) exp 1*	hindbrain	194	161	83.0
	Hoxb3a(zf) exp 2	hindbrain	142	137	96.5
	Hoxb3a(zf) exp 3	hindbrain	213	205	96.2
	Hoxb3a(zf) kr12 mut exp 1	hindbrain	176	0	0.0
	Hoxb3a(zf) kr12 mut exp 2	hindbrain	220	0	0.0
	Hoxb3a(zf) kroxAB mut exp 1	hindbrain	162	14	8.6
	Hoxb3a(zf) kroxAB mut exp 2	hindbrain	219	55	25.1

Extended Data Table 2
Lamprey reporter assay statistics

For each injected construct, the tissue-specific GFP expression domains are given, along with the number and proportion of screened embryos exhibiting GFP expression in those domains. In each case, the numbers derive from individual rounds of injection, except for *Hoxb3a(zf)*, for which the data from three separate experiments (exp1-3), that were performed to ensure reproducibility, were combined. Letters in parentheses after the element names indicate the species of origin of the element: fr, fugu; m, mouse; zf, zebrafish. N/A numbers on efficiency not available.

	Element	Stage	Expression domain	# embryos	# specific expression	% specific expression
Gnathostome elements	*Hoxb1(m)*	22	neural tube	231	137	59.3
	Hoxa2(m)	24	neural crest	264	138	52.3
	Hoxa2b(zf)	23	neural crest	261	120	46.0
	Hoxa2a(fr)	23	hindbrain and neural crest	246	57	23.2
	Hoxa2b(fr)	24	pharynx	218	70	32.1
	Hoxb2(m)	N/A	no specific expression	N/A	N/A	N/A
	Hoxb2a(zf)	23	hindbrain	192	113	58.9
	EphA4(m)	23	hindbrain	695	100	14.4
	Hoxb3(m)	24	hindbrain	324	32	9.9
	Hoxb3a(zf)	23	hindbrain	1440	474	32.9
	Hoxb4(m)	24	hindbrain & spinal cord	590	169	28.6
	Hoxd4(m)	25	hindbrain & spinal cord	300	28	9.3
Hoxb3a(zf) dissection	*Hoxb3a(zf) exp 1*	23	hindbrain	435	247	56.8
	Hoxb3a(zf) exp 2	23	hindbrain	557	93	16.7
	Hoxb3a(zf) exp 3	23	hindbrain	448	134	29.9
	Hoxb3a(zf) kr12 mut exp 1	23	hindbrain	407	0	0.0
	Hoxb3a(zf) kr12 mut exp 2	23	hindbrain	437	2	0.5
	Hoxb3a(zf) kr12 mut exp 3	23	hindbrain	446	0	0.0
	Hoxb3a(zf) kroxAB mut	23	hindbrain	522	47	9.0
Lamprey elements	*Hox2 -12kb*	24	neural tube, pharynx, somites	N/A	N/A	N/A
	Hox2 -9kb	23	neural tube, pharynx, somites	N/A	N/A	N/A
	Hox2 -4kb	23	pharynx, somites	N/A	N/A	N/A
	Hox2 exon1-2	23	hindbrain	406	123	30.3

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References

1. Putnam NH, et al. The amphioxus genome and the evolution of the chordate karyotype. Nature. 2008; 453:1064–1071. [PubMed: 18563158]
2. Shimeld SM, Donoghue PC. Evolutionary crossroads in developmental biology: cyclostomes (lamprey and hagfish). Development. 2012; 139:2091–2099. [PubMed: 22619386]
3. Smith JJ, et al. Sequencing of the sea lamprey (Petromyzon marinus) genome provides insights into vertebrate evolution. Nat Genet. 2013; 45:415–421. 421e411–412. [PubMed: 23435085]
4. Murakami Y, et al. Segmental development of reticulospinal and branchiomotor neurons in lamprey: insights into the evolution of the vertebrate hindbrain. Development. 2004; 131:983–995. [PubMed: 14973269]
5. Takio Y, et al. Evolutionary biology: lamprey Hox genes and the evolution of jaws. Nature. 2004; 429:1–262. [PubMed: 15154395]
6. Takio Y, et al. Hox gene expression patterns in Lethenteron japonicum embryos-Insights into the evolution of the vertebrate Hox code. Dev Biol. 2007
7. Lumsden A. Segmentation and compartition in the early avian hindbrain. Mech Dev. 2004; 121:1081–1088. [PubMed: 15296973]
8. Alexander T, Nolte C, Krumlauf R. Hox genes and segmentation of the hindbrain and axial skeleton. Annu Rev Cell Dev Biol. 2009; 25:431–456. [PubMed: 19575673]
9. Tümpel, S.; Wiedemann, LM.; Krumlauf, R. HOX Genes, Current Topics in Developmental Biology. Pourquié, Olivier, editor. Vol. 88. Academic Press; 2009. p. 103-137.
10. Wilkinson DG, Bhatt S, Cook M, Boncinelli E, Krumlauf R. Segmental expression of Hox-2 homeobox-containing genes in the developing mouse hindbrain. Nature. 1989; 341:405–409. [PubMed: 2571936]
11. Wada H, Garcia-Fernandez J, Holland PW. Colinear and segmental expression of amphioxus Hox genes. Dev Biol. 1999; 213:131–141. [PubMed: 10452851]
12. Seo HC, et al. Hox cluster disintegration with persistent anteroposterior order of expression in Oikopleura dioica. Nature. 2004; 431:67–71. [PubMed: 15343333]
13. Ikuta T, Yoshida N, Satoh N, Saiga H. Ciona intestinalis Hox gene cluster: Its dispersed structure and residual colinear expression in development. Proc Natl Acad Sci U S A. 2004; 101:15118–15123. [PubMed: 15469921]
14. Lowe CJ, et al. Anteroposterior patterning in hemichordates and the origins of the chordate nervous system. Cell. 2003; 113:853–865. [PubMed: 12837244]
15. Pani AM, et al. Ancient deuterostome origins of vertebrate brain signalling centres. Nature. 2012; 483:289–294. [PubMed: 22422262]
16. Schubert M, Holland ND, Laudet V, Holland LZ. A retinoic acid-Hox hierarchy controls both anterior/posterior patterning and neuronal specification in the developing central nervous system of the cephalochordate amphioxus. Dev Biol. 2006; 296:190–202. [PubMed: 16750825]
17. Natale A, et al. Evolution of anterior Hox regulatory elements among chordates. BMC Evol Biol. 2011; 11:330. [PubMed: 22085760]
18. Manzanares M, et al. Conservation and elaboration of Hox gene regulation during evolution of the vertebrate head. Nature. 2000; 408:854–857. [PubMed: 11130723]
19. Sauka-Spengler T, Meulemans D, Jones M, Bronner-Fraser M. Ancient evolutionary origin of the neural crest gene regulatory network. Dev Cell. 2007; 13:405–420. [PubMed: 17765683]

20. Parker HJ, Sauka-Spengler T, Bronner M, Elgar G. A reporter assay in lamprey embryos reveals both functional conservation and elaboration of vertebrate enhancers. PLoS One. 2014; 9:e85492. [PubMed: 24416417]
21. Parker HJ, Piccinelli P, Sauka-Spengler T, Bronner M, Elgar G. Ancient Pbx-Hox signatures define hundreds of vertebrate developmental enhancers. BMC Genomics. 2011; 12:637. [PubMed: 22208168]
22. Theil T, et al. Segmental expression of the EphA4 (Sek-1) receptor tyrosine kinase in the hindbrain is under direct transcriptional control of Krox-20. Development. 1998; 125:443–452. [PubMed: 9425139]
23. Sham MH, et al. The zinc finger gene *Krox-20* regulates *Hoxb-2 (Hox2.8)* during hindbrain segmentation. Cell. 1993; 72:183–196. [PubMed: 8093858]
24. Nonchev S, et al. The conserved role of *Krox-20* in directing *Hox* gene expression during vertebrate hindbrain segmentation. Proc Natl Acad Sci USA. 1996; 93:9339–9345. [PubMed: 8790331]
25. Manzanares M, et al. Segmental regulation of *Hoxb3* by *kreisler*. Nature. 1997; 387:191–195. [PubMed: 9144291]
26. Manzanares M, et al. Krox20 and kreisler co-operate in the transcriptional control of segmental expression of Hoxb3 in the developing hindbrain. EMBO J. 2002; 21:365–376. [PubMed: 11823429]
27. Mehta TK, et al. Evidence for at least six Hox clusters in the Japanese lamprey (Lethenteron japonicum). Proc Natl Acad Sci U S A. 2013; 110:16044–16049. [PubMed: 24043829]
28. Tümpel S, Cambronero F, Sims C, Krumlauf R, Wiedemann LM. A regulatory module embedded in the coding region of Hoxa2 controls expression in rhombomere 2. Proc Natl Acad Sci U S A. 2008; 105:20077–20082. [PubMed: 19104046]
29. Tümpel S, Cambronero F, Wiedemann LM, Krumlauf R. Evolution of *cis* elements in the differential expression of two *Hoxa2* coparalogous genes in pufferfish (*Takifugu rubripes*). Proc Natl Acad Sci U S A. 2006; 103:5419–5424. [PubMed: 16569696]
30. Tümpel S, et al. Expression of *Hoxa2* in rhombomere 4 is regulated by a conserved cross-regulatory mechanism dependent upon *Hoxb1*. Dev Biol. 2007; 302:646–660. [PubMed: 17113575]
31. Pöpperl H, et al. Segmental expression of *Hoxb1* is controlled by a highly conserved autoregulatory loop dependent upon *exd/Pbx*. Cell. 1995; 81:1031–1042. [PubMed: 7600572]
32. Nonchev S, et al. Segmental expression of Hoxa-2 in the hindbrain is directly regulated by Krox-20. Development. 1996; 122:543–554. [PubMed: 8625806]
33. Maconochie MK, et al. Cross-regulation in the mouse *HoxB* complex: the expression of *Hoxb2* in rhombomere 4 is regulated by *Hoxb1*. Genes Dev. 1997; 11:1885–1896. [PubMed: 9242495]
34. Gould A, Itasaki N, Krumlauf R. Initiation of rhombomeric *Hoxb4* expression requires induction by somites and a retinoid pathway. Neuron. 1998; 21:39–51. [PubMed: 9697850]
35. Zhang F, Nagy Kovacs E, Featherstone MS. Murine hoxd4 expression in the CNS requires multiple elements including a retinoic acid response element. Mech Dev. 2000; 96:79–89. [PubMed: 10940626]
36. Nolte C, Jinks T, Wang X, Martinez Pastor MT, Krumlauf R. Shadow enhancers flanking the HoxB cluster direct dynamic Hox expression in early heart and endoderm development. Dev Biol. 2013; 383:158–173. [PubMed: 24055171]
37. Distel M, Wullimann MF, Koster RW. Optimized Gal4 genetics for permanent gene expression mapping in zebrafish. Proc Natl Acad Sci U S A. 2009; 106:13365–13370. [PubMed: 19628697]
38. Fisher S, et al. Evaluating the biological relevance of putative enhancers using Tol2 transposon-mediated transgenesis in zebrafish. Nature protocols. 2006; 1:1297–1305. [PubMed: 17406414]

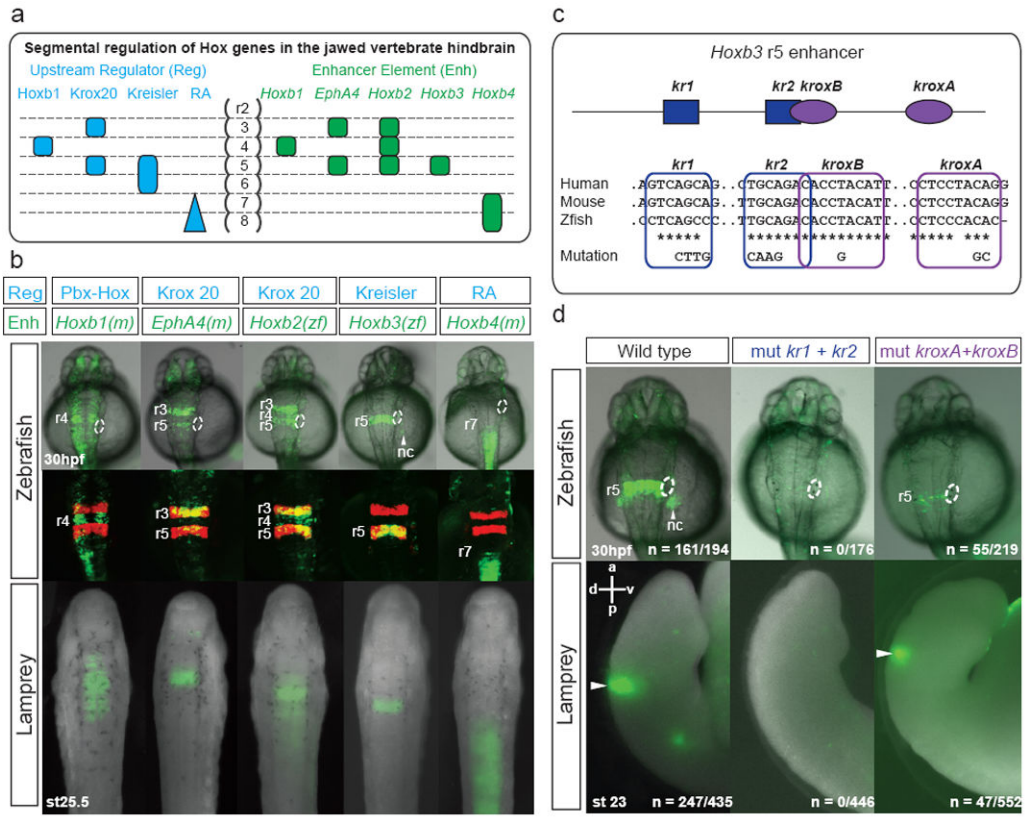

Figure 1. Conserved segmental activity of jawed vertebrate enhancers in zebrafish and lamprey

a, Schematic depicting components of the GRN for segmental *Hox* expression in the gnathostome hindbrain. The rhombomeric (r) expression of upstream segmental regulators (Reg: blue) and the activity domains of known enhancer elements they control (Enh: green) are shown. **b**, GFP reporter expression in dorsal views of zebrafish and lamprey hindbrains mediated by enhancers from panel **a**. For zebrafish, two images of the same embryo are shown, GFP plus brightfield (top) and GFP plus endogenous r3r5mCherry (middle) signals. The otic vesicle is circled and GFP^{+} rhombomeres indicated. Letters in parentheses indicate the species of origin of the element: m, mouse; zf, zebrafish. **c**, The zebrafish *Hoxb3* r5 enhancer contains conserved Kreisler (*kr*: blue) and Krox20 (*krox*: purple) binding sites (asterisks). Mutations known to influence activity are detailed below the aligned sites[26]. **d**, GFP reporter expression of wild type and mutated (mut) versions of the r5 enhancer in zebrafish (dorsal views) and lamprey (lateral views) embryos. Numbers (n) denote the proportion of embryos exhibiting segmental reporter expression. Extended Data Tables 1 and 2 provide the number of embryos and efficiency of specific expression for all constructs. Arrowheads indicate segment-like reporter expression in the lamprey hindbrain. Abbreviations: a, anterior; d, dorsal; hpf, hours post fertilization; nc, neural crest; p, posterior; r, rhombomere; st, stage; v, ventral.

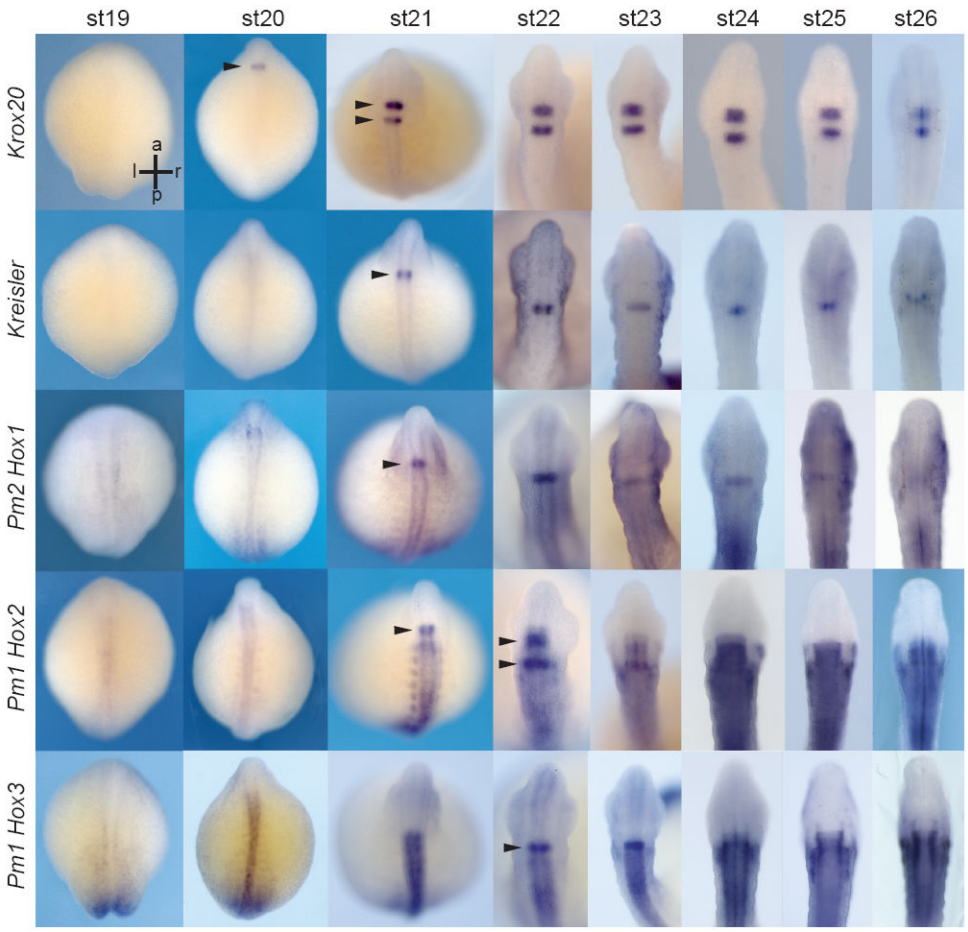

Figure 2. Expression of segmental regulators and Hox genes in the lamprey hindbrain

Gene expression visualized by *in-situ* hybridization in lamprey embryos at developmental stages (st) 19-26. Dorsal views are shown, with anterior to the top. Arrowheads indicate the onset of segmental-like gene expression in the developing hindbrain. Abbreviations: a, anterior; l, left; p, posterior; r, right.

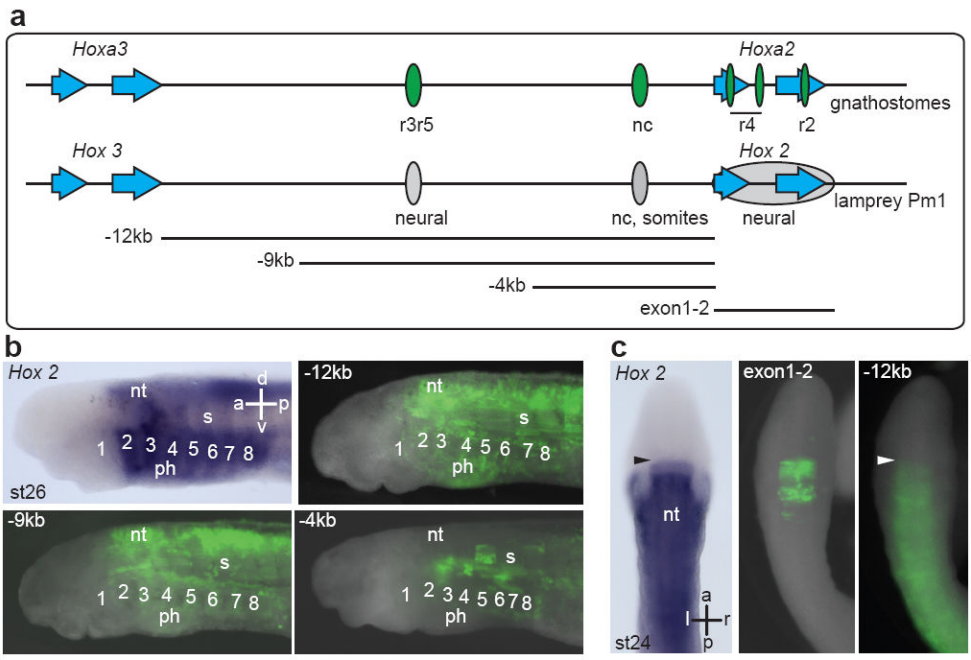

Figure 3. Identification of enhancers from the lamprey Hox2 locus

a, The *Hoxa2-Hoxa3* genomic region from gnathostomes and the equivalent region from the lamprey Pm1 Hox cluster. Hox gene exons (blue arrows) and relative positions of previously characterized enhancer elements in gnathostomes (green ovals) are shown[9]. Hox2 enhancers identified in this study are denoted as grey ovals. Fragments of Pm1 tested in lamprey reporter assays are shown below. **b**, Lateral views of st26 lamprey embryos comparing the endogenous expression of *Pm1 Hox2* with GFP reporter expression mediated by fragments of Pm1. Pharyngeal arches are numbered. **c**, Dorsal views of st24 lamprey embryos showing endogenous expression of Pm1 *Hox2* compared to GFP reporter expression. The *exon1-2* region mediates two stripes of segmental expression (Extended Data Table2 provides information on number of embryos and efficiency of specific expression for the *exon1-2* region). Arrowheads indicate anterior extent of expression in the neural tube. Abbreviations: nc, neural crest; nt, neural tube; ph, pharynx; s, somites.

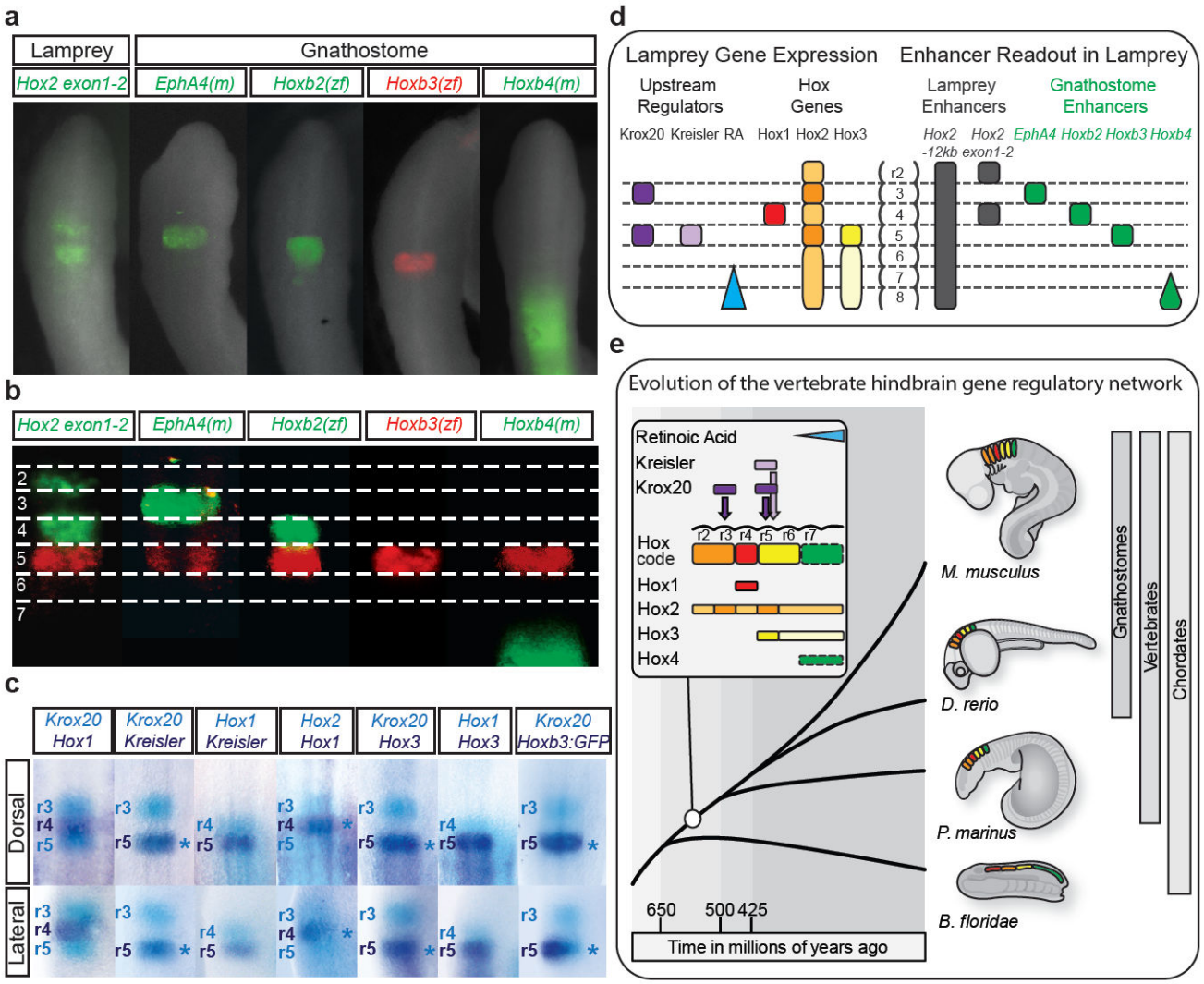

Figure 4. Comparison of enhancer activity and segmental gene expression in lamprey supports an origin of the hindbrain GRN at the base of vertebrates

a-b, The register of segmental domains of GFP expression mediated by lamprey and gnathostome enhancers in st24 lamprey embryos (**a**) are mapped to putative rhombomeres (2-7) by direct comparison with a co-injected r5 enhancer from zebrafish *Hoxb3* linked to RFP(**b**). **c**, Double *in-situ* hybridization reveals that endogenous Hox gene expression and GFP reporter expression align with segmental regulators in the lamprey hindbrain. Dorsal (top) and lateral (bottom) views of st23-24 embryos are shown with anterior to the top and the inferred rhombomeric expression domains annotated. Asterisks indicate overlapping domains of *in-situ* signal. **d**, Schematic summary of segmental gene expression and enhancer activity in the lamprey hindbrain at st23-24. For *Hox2* and *Hox3*, darker colour shades indicate stronger levels of gene expression. **e**, An evolutionary model based on our data, indicating that the GRN coupling the Hox code in the neural tube to hindbrain segmentation (rhombomeres) via Krox20 and Kreisler evolved prior to the split between jawed and jawless vertebrates.