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, can provide unique insights into vertebrate origins owing to its phylogenetetic position at the base of the vertebrate tree. 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. However, little is known about the regulatory network underlying Hox expression in lamprey or its relationship to hindbrain segmentation. Here, 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 (for example, Kreisler (also known as Mafba), Krox20 (also known as Egr2a)) in both lamprey and zebrafish. We find that lamprey hox genes display dynamic segmentally restricted domains of expression; we also isolated a conserved exon 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 probably led to the formation of rhombomeric compartments with an underlying Hox code.

The hindbrain of jawed vertebrates is a specialized region of the nervous system characterized by its subdivision into repetitive segments called rhombomeres. Anterior Hox genes are expressed in a nested pattern that is functionally coupled to this inherent segmentation program. Non-vertebrate chordates possess patterned hox gene expression along the body axis, which may be regulated by conserved patterning signals in chordate evolution, but lack nervous system segmentation. Moreover, key segmental regulatory elements from jawed vertebrate Hox clusters are not conserved in amphioxus or ascidians. In jawed vertebrates (gnathostomes), a well-characterized, highly conserved gene regulatory network (GRN) integrates hindbrain segmentation and Hox patterning. The jawless (agnathans) lamprey, has been postulated to represent an intermediate state with rudimentary hindbrain segment, but lacking registration with motoneuron patterning or nested Hox expression. 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 the 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 in both lamprey and zebrafish lineages. To this end, we generated constructs with mutated Kreisler and/or Krox20 sites within the zebrafish hoxb3 r5 enhancer. Mutation of the two Kreisler sites (mut kro1 + kro2) 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. These results are consistent with roles for Kreisler and Krox20 in the mouse Hoxb3 r5 enhancer, implying homologous roles in the lamprey hindbrain.

These data suggest that major components of the hindbrain GRN upstream of Hox genes are conserved in lamprey. Therefore, we characterized hindbrain expression patterns of lamprey kreisler and krox20 across multiple developmental stages (st19–26). krox20 is expressed in two stripes in a manner reminiscent of its gnathostome counterpart. We isolated a kreisler homologous gene that is expressed in a single stripe in the lamprey hindbrain, similar to mouse Kreisler. The expression of these key upstream regulators in lamprey supports our interpretation of their inputs to reporter activities.
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 expression of upstream segmental regulators (blue) and the activity domains of known enhancer elements they control (green) are shown. RA, retinoic acid. b, GFP reporter expression in dorsal views of zebrafish and lamprey hindbrains mediated by enhancers from a. For zebrafish, two images of the same embryo are shown, GFP plus brightfield (top) and GFP plus endogenous r3r5-mCherry (middle) signals. The otic vesicle is circled and GFP localized rhombomeres are indicated. Letters in parentheses indicate the species of origin of the element: m, mouse; zf, zebrafish. Enh, enhancer; hpf, hours post-fertilization; nc, neural crest; Reg, regulator. c, The zebrafish hoxb3 r5 enhancer contains conserved Kreisler (kr; blue) and Krox20 (kro; purple) binding sites (asterisks). Mutations known to influence activity are detailed below the aligned sites. 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.

Figure 2 | Expression of segmental regulators and hox genes in the lamprey hindbrain. Gene expression visualized by in situ hybridization in lamprey embryos at st19–26. Dorsal views are shown, with anterior to the top. Arrowheads indicate the onset of segmental-like gene expression in the developing hindbrain. a, anterior; l, left; p, posterior; r, right.

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. These probably represent a subset of the total hox gene complement; recent evidence from Lethenteron japonicum suggests up to six Hox clusters, two of which are homologous to Pm1 and Pm2. Lamprey hox genes from paralogous groups 1–3, hox1 (Pm2), hox2 and hox3 (Pm1), display temporally dynamic hindbrain expression patterns. Early developmental stages (st21–23) 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 downregulated in the hindbrain, while segmental expression for hox2 and hox3 become masked by their upregulation 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, the potential links between Hox expression and hindbrain segmentation were presumably missing owing 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 expression domains, we focused on the hox2 paralogous group, well-characterized from a regulatory perspective in jawed vertebrates. We sequenced the hox2 locus and entire intergenic region between hox2 and hox3 of Pm1, as this genomic region in gnathostomes contains a series of enhancers that mediate hindbrain Hox expression (Fig. 3a and Extended Data Fig. 1). Because no overt sequence conservation with known jawed vertebrate enhancers was
Deletion analyses demonstrate that expression in the neural tube, pharynx (neural crest) and somites that embryos (Fig. 3a–c). At st26, the region mediates two stripes of segmental expression (Extended Data Table 2 expression of Pm1. Pharyngeal arches are numbered. nt, neural tube; s, somites; of Pm1 tested in lamprey reporter assays are shown as grey ovals. Fragments of Pm1 tested in lamprey reporter assays are shown9. asin 1–2 mediated 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 with r3/r5 in zebrafish (Fig. 1b). The hoxb2 enhancer drives robust r3/r5 expression and weaker r4 expression in zebrafish (Fig. 1b), whereas the strongest expression in lamprey is in r4 and there is weak expression in r6. 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 hybridization (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

**Figure 3 | Identification of enhancers from the lamprey hox2 locus.** a. The hoxa2–hox3 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 shown9. 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. nt, neural tube; s, somites; ph, pharynx. c. Dorsal views of st24 lamprey embryos showing endogenous expression of Pm1 hox2 compared with GFP reporter expression. The exon 1–2 region mediates two stripes of segmental expression (Extended Data Table 2 provides information on number of embryos and efficiency of specific expression for the exon 1–2 region). Arrowheads indicate the anterior extent of expression in the neural tube.

detectable, functionally tested sequences from −12 kb upstream to +1 kb downstream of the lamprey Hox2 coding domain in lamprey embryos (Fig. 3a–c). At st26, the −12 kb intergenic region mediates GFP expression in the neural tube, pharynx (neural crest) and somites that closely resembles that of the endogenous lamprey hox2 gene (Fig. 3b). Deletion analyses demonstrate that cis-elements capable of mediating neural expression lie within the −9 kb to −4 kb intergenic region whereas those contributing to neural crest/somite expression lie in the −4 kb fragment (Fig. 3a).

Given that gnathostome Hoxa2 is expressed in r2 and r4 via exonic and intronic regulatory elements2–8, (Fig. 3a and Extended Data Fig. 1), we tested a comparable fragment of lamprey hox2 (exon 1–2). Intriguingly, this fragment mediated restricted expression in two alternating stripes in the hindbrain from st22 to st26 (Fig. 3c and 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 −12 kb fragment and the exon 1–2 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 red fluorescent protein (RFP) in putative r5, allowing registration with other enhancer-mediated GFP expression (Fig. 4a, b). hox2 exon 1–2 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 with r3/r5 in zebrafish (Fig. 1b). The hoxb2 enhancer drives robust r3/r5 expression and weaker r4 expression in zebrafish (Fig. 1b), whereas the strongest expression in lamprey is in r4 and there is weak expression in r6. 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 hybridization (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

**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, 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). For hoxb2 a weaker r6 stripe begins to appear at st23. 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 before the split between jawed and jawless vertebrates.
kresler expression domains to r5, hox2 to r2–5 with elevated stripes in r3/r5, and the anterior stripe of hox3 expression to r5. An antisense GFP probe positions expression directed by the hox3 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 composed 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 before the agnathan/gnathostome split (Fig. 4e).

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions H.J.P., R.K. and M.E.B. conceived this research program, H.J.P. conducted the experiments, H.J.P., R.K. and M.E.B. jointly analysed the data, discussed the ideas and interpretations and wrote the manuscript.

Author Information The sequences for the lamprey hox1w and kresler transcripts have been deposited in GenBank under accession numbers KM087087 (hox1w) and KM087088 (kresler). All original source data have been deposited in the Stowers Institute Original Data Repository and are available online at http://odr.stowers.org/ web/simr/. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.K. (rek@Stowers.org).
METHODS

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

Mouse hoxb1a (ref. 31) (378 bp), F: 5'-AACTGAGGGCCCAGCTTACG-3'; R: 5'-GTACATCTCGAGGCAGTTAC-3'.

Mouse hoxb2a (1,488 bp), F: 5'-TATTTTTGCCTTGTGCATGTACGAC-3'; R: 5'-AACTGAGGGCCCAGCTTACG-3'.

Mouse hoxb3a (2,021 bp) F: 5'-AACTGAGGGCCCAGCTTACG-3'; R: 5'-GTACATCTCGAGGCAGTTAC-3'.

Mouse hoxb3b (1,404 bp) F: 5'-AACTGAGGGCCCAGCTTACG-3'; R: 5'-GTACATCTCGAGGCAGTTAC-3'.

Mouse hoxb3c (949 bp, partial exon and untranslated region (UTR) probes, RNA isolation Kit (Ambion) and used as a template for 5'-CCACAAGCCCTTCAAGTG-3'; R: 5'-GGTGGACACACGAGAGAGAGAG-3'.

Lamprey hox2a1 (928 bp) F: 5'-AACTGAGGGCCCAGCTTACG-3'; R: 5'-GTACATCTCGAGGCAGTTAC-3'.

Lamprey hox2a2a (1,263 bp) F: 5'-GGCTGTAATGCAAACGCTAATGACAC-3'; R: 5'-GGCTGTAATGCAAACGCTAATGACAC-3'.

Lamprey hox2b (582 bp) F: 5'-GGCTGTAATGCAAACGCTAATGACAC-3'; R: 5'-GGCTGTAATGCAAACGCTAATGACAC-3'.

Mouse hox3a (710 bp) F: 5'-AACTGAGGGCCCAGCTTACG-3'; R: 5'-GTACATCTCGAGGCAGTTAC-3'.

R: 5'-AACTGAGGGCCCAGCTTACG-3'.

P. marinus embryos were extracted from gravid lamprey (P. marinus) caught in the wild and provided by Hammond Bay Biological Station. Transient transgenic P. marinus embryos were generated by 1-Sce meganuclease-mediated transgenesis as described previously. Single-celled embryos at 4-6 h post-fertilization were injected with the co-injection constructs. Co-injected constructs were mixed at a concentration of 15 ng µl⁻¹ each (resulting in a total DNA concentration of 30 ng µl⁻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 hybridization probes. Exonic probes were designed based on previously characterized/predicted gene sequences 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' untranslated region (UTR) probes, RNA from st18–26 P. marinus embryos was extracted using the RN Aquasol Total RNA Isolation Kit (Ambion) and used as a template for 5' or 3' rapid amplification of cDNA ends (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 (ref. 4) (468 bp, predicted exonic fragment) F: 5'-CCACAAGCCCTTCAAGTG-3'; R: 5'-GGTGGACACACGAGAGAGAGAG-3'.

Lamprey hox2a1 (471 bp, partial exon 2) F: 5'-CACAACGGCGGCTAAGCGGAGACG-3'; R: 5'-ATGCGTGGGCCCCGACCGTGGTGG-3'.

Lamprey hox2a2 (246 bp) F: 5'-AACTGAGGGCCCAGCTTACG-3'; R: 5'-GTACATCTCGAGGCAGTTAC-3'.

Lamprey hox2b (297 bp) F: 5'-AACTGAGGGCCCAGCTTACG-3'; R: 5'-GTACATCTCGAGGCAGTTAC-3'.

Lamprey hox3a (195 bp) F: 5'-AACTGAGGGCCCAGCTTACG-3'; R: 5'-GTACATCTCGAGGCAGTTAC-3'.

Zebrafish and lamprey experiments. Animals of the National Institutes of Health and protocols were approved by the Institutional Animal Care and Use Committees of the Stonerow Institute (zebrafish, RK Protocol #2013-0110) and California Institute of Technology (lamprey, MEB Protocol #1436-11).

Zebrafish reporter assay. The following zebrafish lines were used for embryo micro-injection experiments: Slusarski AB (wild type); eg2b KalTA4Bi-1×UCASCherry (ref. 35). Transient transgenic zebrafish embryos were generated for each reporter construct by To2-mediated transgenesis in fertilized eggs as described previously. In general a minimum of 100 embryos were injected to monitor efficiency for each construct due to mosaicism and position effects of integration. GFP-expressing transient transgenic embryos were raised to adulthood and crossed with either wild-type or tr35-mCherry fish to screen for germine transgene integration. 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 lamprey in situ hybridization. Digoxigenin- and fluorescein-labelled probes were generated by standard methods and used in single and double lamprey whole-mount in situ hybridization as described previously. Embryos were cleared in a solution of 75% glycerol before being imaged using a Leica MZ.APC camera and Axiovision Rel 4.6 software. Images were cropped and altered for brightness and contrast using Adobe Photoshop CS5.1.
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, while 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 adapted with permission from figure 4.2 in ref. 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 r3r5-mCherry (middle) signals. The zebrafish otic vesicle is circled. m, mouse; zf, zebrafish.
Extended Data Figure 3 | Segmental patterns of GFP reporter expression in transgenic zebrafish lines. Lateral (top) and dorsal (middle) views of 30 hpf 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 Fig. 2. When available, GFP lines were crossed with the endogenous r3r5-mCherry reporter line as a reference (bottom). The otic vesicle is circled. m, mouse; zf, zebrafish.
| lamprey Hox2 -12kb | lamprey Hox2 exon1-2 | Hoxb1(m) | EphA4(m) | Hoxb2(zf) | Hoxb3(zf) | Hoxb4(m) |
|-------------------|----------------------|----------|----------|-----------|-----------|-----------|
| st 18             |                      |          |          |           |           |           |
| st 19             |                      |          |          |           |           |           |
| st 20             |                      |          |          |           |           |           |
| st 21             |                      |          |          |           |           |           |
| st 22             |                      |          |          |           |           |           |
| st 23             |                      |          |          |           |           |           |
| st 24             |                      |          |          |           |           |           |
| st 25             |                      |          |          |           |           |           |
| st 26             |                      |          |          |           |           |           |
Extended Data Figure 4 | Developmental time course of GFP reporter expression mediated by lamprey and gnathostome regulatory elements in lamprey embryos. Developmental stages st18–26 are shown. All embryos are positioned such that the hindbrain is viewed dorsally, with anterior to the top, except for mouse Hoxb4 at st22, which is viewed laterally with anterior to the left. For hoxb2 a weaker r6 stripe begins to appear at st23. 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 appears to be temporally dynamic, starting broad and refining with time, which is probably 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. m, mouse; zf, zebrafish.
### 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 zebrafish hoxb3a, for which the data from three separate experiments (exp 1–3), which were performed to ensure reproducibility, were combined.

Letters in parentheses after the element names indicate the species of origin of the element: fr, *Fugu rubripes*; m, mouse; zf, zebrafish. N/A, numbers on efficiency not available.

| Element                        | Expression domain | # embryos | # specific expression | % specific expression |
|--------------------------------|-------------------|-----------|-----------------------|-----------------------|
| 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) 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                  |

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 zebrafish hoxb3a, for which the data from three separate experiments (exp 1–3), which were performed to ensure reproducibility, were combined. Letters in parentheses after the element names indicate the species of origin of the element: fr, *Fugu rubripes*; m, mouse; zf, zebrafish. N/A, numbers on efficiency not available.
### Extended Data Table 2 | Lamprey reporter assay statistics

| Element   | Stage | Expression domain               | # embryos | # specific expression | % specific expression |
|-----------|-------|--------------------------------|-----------|-----------------------|-----------------------|
| \(\text{Hoxb1(m)}\) | 22    | neural tube                    | 231       | 137                   | 59.3                  |
| \(\text{Hoxa2(m)}\) | 24    | neural crest                   | 264       | 138                   | 52.3                  |
| \(\text{Hoxa2b(zf)}\) | 23    | neural crest                   | 261       | 120                   | 46.0                  |
| \(\text{Hoxa2a(zf)}\) | 23    | hindbrain and neural crest     | 246       | 57                    | 23.2                  |
| \(\text{Hoxa2b(fr)}\) | 24    | pharynx                        | 218       | 70                    | 32.1                  |
| \(\text{Hoxb2(m)}\) | N/A   | no specific expression         | N/A       | N/A                   | N/A                   |
| \(\text{Hoxb2a(zf)}\) | 23    | hindbrain                      | 192       | 113                   | 58.9                  |
| \(\text{EphA4(m)}\) | 23    | hindbrain                      | 695       | 100                   | 14.4                  |
| \(\text{Hoxb3(m)}\) | 24    | hindbrain                      | 324       | 32                    | 9.9                   |
| \(\text{Hoxb3a(zf)}\) | 23    | hindbrain                      | 1440      | 474                   | 32.9                  |
| \(\text{Hoxb4(m)}\) | 24    | hindbrain and spinal cord      | 590       | 169                   | 28.6                  |
| \(\text{Hoxd4(m)}\) | 25    | hindbrain and spinal cord      | 300       | 28                    | 9.3                   |

#### Gnathostome elements

| Element | Stage | Expression domain               | # embryos | # specific expression | % specific expression |
|---------|-------|--------------------------------|-----------|-----------------------|-----------------------|
| \(\text{Hoxb3a(zf) exp 1}\) | 23    | hindbrain                      | 435       | 247                   | 56.8                  |
| \(\text{Hoxb3a(zf) exp 2}\) | 23    | hindbrain                      | 557       | 93                    | 16.7                  |
| \(\text{Hoxb3a(zf) exp 3}\) | 23    | hindbrain                      | 448       | 134                   | 29.9                  |
| \(\text{Hoxb3a(zf) kr12 mut exp 1}\) | 23    | hindbrain                      | 407       | 0                     | 0.0                   |
| \(\text{Hoxb3a(zf) kr12 mut exp 2}\) | 23    | hindbrain                      | 437       | 2                     | 0.5                   |
| \(\text{Hoxb3a(zf) kr12 mut exp 3}\) | 23    | hindbrain                      | 446       | 0                     | 0.0                   |
| \(\text{Hoxb3a(zf) kroxAB mut}\) | 23    | hindbrain                      | 522       | 47                    | 9.0                   |

#### Lamprey elements

| Element | Stage | Expression domain               | # embryos | # specific expression | % specific expression |
|---------|-------|--------------------------------|-----------|-----------------------|-----------------------|
| \(\text{Hox2 -12kb}\) | 24    | neural tube, pharynx, somites  | N/A       | N/A                   | N/A                   |
| \(\text{Hox2 -9kb}\) | 23    | neural tube, pharynx, somites  | N/A       | N/A                   | N/A                   |
| \(\text{Hox2 -4kb}\) | 23    | pharynx, somites               | N/A       | N/A                   | N/A                   |
| \(\text{Hox2 exon1-2}\) | 23    | hindbrain                      | 406       | 123                   | 30.3                  |

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 zebrafish hasb3a, for which the data from three separate experiments (exp 1–3), which were performed to ensure reproducibility, were combined. Letters in parentheses after the element names indicate the species of origin of the element: fr, Fugu rubripes; m, mouse; zf, zebrafish. N/A, numbers on efficiency not available.

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