**hox gene expression predicts tetrapod-like axial regionalization in the skate, *Leucoraja erinacea***

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Edited by Gunter Wagner, Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT; received August 6, 2021; accepted November 4, 2021

The axial skeleton of tetrapods is organized into distinct antero-posterior regions of the vertebral column (cervical, trunk, sacral, and caudal), and transitions between these regions are determined by colinear anterior expression boundaries of *Hox*\(^{5/6, -9, -10,\text{ and }-11}\) paralogy group genes within embryonic paraxial mesoderm. Fishes, conversely, exhibit little in the way of discrete axial regionalization, and this has led to scenarios of an origin of *Hox*-mediated axial skeletal complexity with the evolutionary transition to land in tetrapods. Here, combining geometric morphometric analysis of vertebral column morphology with cell lineage tracing of *hox* gene expression boundaries in developing embryos, we recover evidence of at least five distinct regions in the vertebral skeleton of a cartilaginous fish, the little skate (*Leucoraja erinacea*). We find that skate embryos exhibit tetrapod-like antero-posterior nesting of *hox* gene expression in their paraxial mesoderm, and we show that anterior expression boundaries of *hox5/6, hox9, hox10, and hox11* paralogy group genes predict regional transitions in the differentiated skate axial skeleton. Our findings suggest that *hox*-based axial skeletal regionalization did not originate with tetrapods but rather has a much deeper evolutionary history than was previously appreciated.

**Significance**

The tetrapod vertebral skeleton is subdivided into distinct regions that are determined by spatial expression of *Hox* genes in the early embryo. Fishes are thought to lack comparable regionalization, but the relationship between anatomy and *hox* expression has not been tested beyond tetrapods. By comparing vertebral morphology with embryonic *hox* gene expression in a cartilaginous fish, the skate (*Leucoraja erinacea*), we found that the skate vertebral column is regionalized, with regional transitions predicted by embryonic *hox* gene expression patterns. This discovery points to an origin of nested *hox* gene expression and *hox*-based vertebral regionalization at the common ancestor of jawed vertebrates.


The authors declare no competing interest.

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This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2114563118/-/DCSupplemental.

Published December 13, 2021.
morphological characteristics of vertebrae along the AP axis (32). However, evidence linking hox expression boundaries to changes in vertebral morphology along the axis in cartilaginous fishes is lacking. It therefore remains unclear what role Hox genes might have played in the evolution of vertebral complexity.

To test whether the tetrapod Hox code—and its role in establishing axial regions within the vertebral skeleton—has a deeper evolutionary origin within vertebrates, we investigated hox gene expression and axial regionalization in a cartilaginous fish, the little skate (Leucoraja erinacea). As a sister taxon to bony vertebrates, comparative studies of skate development facilitate inference of anatomical and developmental conditions in the last common ancestor of jawed vertebrates, and our previous work on skate axial skeletal development allows us to confidently relate somite to vertebral identity along the AP axis (33–35). Contrary to previous assumptions of relative axial skeletal homogeneity, our quantitative assessment of morphology reveals that the skate vertebral column possesses at least five distinct axial regions, with regional boundaries predicted by tetrapod-like patterns of hox gene expression within embryonic somitic mesoderm. This suggests that core elements of the mechanism governing tetrapod axial skeletal regionalization have been conserved for ∼500 million y from the origin of jawed vertebrates.

Results

Morphometric Analysis Reveals Five Axial Skeletal Regions in Skate. We first determined the extent of skate axial skeletal regionalization. The little skate vertebral column includes a synarcual, which is composed of ∼25 fused anterior vertebrae (determined by counts of spinal nerve foramina) that support the pectoral girdle and pectoral fin-based locomotion in skates (Fig. 1A) (36). Projecting laterally from the ventral midpoint of the synarcual are two articulations for the lateral stays, rectangular cartilages that stabilize the pectoral girdle. The pectoral arch extends laterally just posterior and dorsal to these processes to fuse to the scapulocoracoid of the pectoral girdle (37).

The fused nature of the synarcual prevented us from placing landmark coordinates on each individual vertebrae, and so our analysis of regionalization therefore began at the first vertebra posterior to the synarcual, vertebra 26. To test for regionalization, we performed Procrustes superimposition of microcomputed tomography (microCT)–rendered morphology for vertebrae caudal to the synarcual, up to vertebra 70 in three adult skates. We quantified vertebral morphology as homologous landmarks represented by three-dimensional (3D) Cartesian grid coordinates (Fig. 1B and SI Appendix, Table S1) and used principal-component (PC) scores of realigned landmark coordinates as shape variables. To test for region numbers and boundaries, we performed segmented linear regression on PC scores and used maximum-likelihood model selection to determine the best fit number of regions within the quantified axial skeleton, where individual regression slopes indicate regions and slope breaks represent region boundaries (SI Appendix, Fig. S1 and Table S2) (3, 38). PC analysis revealed a shape gradient along the AP axis of the vertebral column in the skate (Fig. 1C and SI Appendix, Fig. S1 A–C), with a four-region model having the best fit to the gradient, and an overall best model of five regions with boundaries at approximately vertebrae 26, 31, 46, and 51 when we included the synarcual as the first region (Fig. 1D and E and SI Appendix, Fig. S1 A–C and D and Table S2). To account for changes in shape not attributable to size-scaling developmental processes such as intracolumnar variation in growth rates, we conducted regionalization analyses using residuals from regression of PC scores against centroid size for each vertebra. We recovered nearly identical results, with average region boundaries within one vertebra of the original analysis (SI Appendix, Fig. S1 A′–C′ and E). Skates therefore appear to possess at least five distinct axial regions, hereafter referred to as R1 to R5: the synarcual; a short transitional region of approximately five vertebrae between the synarcual and the trunk; a trunk region of ∼15 vertebrae; a short transitional region of approximately five vertebrae between the trunk and the tail; and a tail region extending to vertebra 70 (the posterior-most element in our analysis).

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Nested Expression of \( hox \) Genes in Skate Somitic Mesoderm. We next tested for nested expression of \( hox \) genes during somitogenesis in skate embryos. Skates possess 34 \( hox \) genes, organized into three clusters (\( hoxa, hoxb, \) and \( hoxc \)) (39). We performed chromogenic whole-mount messenger RNA (mRNA) in situ hybridization (ISH) experiments for selected \( hox \) genes from paralogy groups 5 to 11 in embryonic stage 18 (S18), S20, S22, and S25. These stages were selected as they span the onset to termination of somitogenesis (Fig. 2 and SI Appendix, Fig. S2 and Table S3). Anterior expression boundaries for each \( hox \) ortholog were then determined by somite counts, assisted by DAPI staining (using both bright-field and fluorescence microscopy).

Expression boundaries for \( hoxa5, hoxb5, hoxa6, \) and \( hoxb6 \) were similar from S18 to S22, with the anterior expression limits ranging from somite 13 to 16 (SI Appendix, Fig. S2A–D and Table S3). By S25, anterior expression limits of \( hoxa5 \) and \( hoxb5 \) extend slightly anteriorly relative to \( hoxa6 \) and \( hoxb6 \), to the level of somite 10 (SI Appendix, Fig. S2A–D and Table S3). \( hoxa7 \) was expressed consistently up to somites 21 and 22 in all stages examined (SI Appendix, Fig. S2E and Table S3), while the anterior limit of \( hoxb7 \) expression aligned with somites 14 to 16 (SI Appendix, Fig. S2F and Table S3). \( hoxb8 \) and \( hoxd8 \) were both expressed up to somites 19 to 22 for all stages examined (SI Appendix, Fig. S2G and H and Table S3), while the anterior limit of \( hoxa9 \) expression aligned with somites 24 to 29 (SI Appendix, Fig. S2I and Table S3). \( hoxb10 \) was expressed up to the level of approximately somite 25 in S18 (SI Appendix, Fig. S2K and Table S3), with this anterior expression boundary shifting caudally to somites 31 to 34 from S20 to S25, while \( hoxa10 \) and \( hoxd10 \) were expressed consistently to the level of somites 31 to 34 in all stages (SI Appendix, Fig. S2J and L and Table S3). Finally, \( hoxa11 \) was expressed up to approximately somites 46 to 49 in S20 and S22 (which corresponds to the posterior margin of the cloaca), shifting to somites 42 to 47 in S25 (SI Appendix, Figs. S2M and S3 and Table S3), while \( hoxd11 \) was expressed up to somites 58 to 60 in all stages (SI Appendix, Fig. S2N and Table S3). Overall, these experiments reveal patterns of \( hox \) gene spatial nesting in skate somitic mesoderm that are more similar to the broadly spaced and nested AP expression of tetrapods (4, 11–13) than to the compacted expression boundaries seen in teleosts (22, 23, 26, 27).

**hox Expression Boundaries Correlate with Regional Transitions in the Skate Vertebral Column.** We tested whether the anterior \( hox \) expression boundaries noted above correspond to morphometric regional transitions in the differentiated vertebral skeleton. In the skate, relationships between somites and vertebrae have been established in the trunk and tail (35). However, the...
number of somites contributing to the chondrocranium is not known, and this hinders direct comparisons between somite number and eventual vertebral position. To more precisely test for relationships between anterior hox gene expression boundaries and axial skeletal regional transitions in skate, we conducted a series of somite fate-mapping experiments to serve as reference points between somites and the differentiated axial skeleton. Somites were labeled with the lipophilic dye CM-DiI at four positions along the AP axis: somites 13 to 15, 31 to 33, 48 to 50, and 58 to 60 (roughly corresponding to the anterior expression boundaries of hoxa6/hoxb6, hoxa10/hoxb10/hoxd10, hoxa11, and hoxd11). These positions were selected to test for potentially shared correspondence of hox expression boundaries and anatomical transitions between tetrapods and skate, and to span the AP axis of the skate embryo (thereby allowing us to confidently infer anatomical correlates of expression boundaries sitting rostral or caudal to our reference points). We then reared injected embryos to the point of axial skeletal differentiation and recovered and mapped labeled derivatives of our initial somite injections within the axial column (SI Appendix, Fig. S4).

We found that in the trunk, injection of a particular somite, counting from the first somite, led to labeling of nearly the same numbered vertebra, counting from the first vertebra posterior to the chondrocranium. This close correspondence suggests that, in contrast to tetrapods, there is limited somitic contribution to the chondrocranium, and that somite number therefore aligns closely with vertebral number (albeit with the half-somite shift resulting from resegmentation (35)). Our CM-DiI injection at the level of somites 13 to 15 (corresponding to the anterior boundary of hox expression) resulted in the recovery of CM-DiI-labeled chondrocytes in the middle of the synarcual, corresponding to the posterior margin of the lateral stay and anterior margin of the pectoral arch (Fig. 3 A and A'). Next, our CM-DiI injection at the level of somites 31 to 33 (corresponding to the anterior boundary of hox paralogy group 5 and 6 expression) resulted in the recovery of CM-DiI-labeled chondrocytes in the middle of the synarcual, corresponding to the posterior margin of the lateral stay and anterior margin of the pectoral arch (Fig. 3 A and A'). Further, our CM-DiI injection at the level of somites 31 to 33 (corresponding to the anterior boundary of hox paralogy group 10 expression) gave rise to CM-DiI-labeled chondrocytes in vertebrae 32 to 35 (i.e., at the R2–R3 boundary; Fig. 3 B and B'). Furthermore, by counting somites rostrally from this injection site, we were able to infer from this experiment that the anterior limit of hoxa9 expression (approximately somites 24 to 29) corresponds to the posterior margin of...
the synarcual (i.e., the R1–R2 boundary). Our CM-DiI injection at the level of somites 48 to 50 (corresponding to the posterior portion of the cloaca and the anterior limit of hoxa11 expression) gave rise to CM-DiI-labeled chondrocytes at the level of vertebrae 49 to 51 (i.e., at the R4–R5, or trunk–tail, boundary; Fig. 3 C and C’). Finally, CM-DiI injection at the level of somites 58 to 60 (corresponding to the anterior boundary of hoxd11 expression) resulted in CM-DiI-labeled chondrocytes within vertebrae 62 to 68 (Fig. 3 D and D’). The latter were located within the diplospodylous tail region, where each somite contributes to three vertebrae (35), and did not correspond to an axial skeletal regional transition. Taken together, our fate-mapping results show that three of the four region boundaries identified in our segmented linear regression analysis are predicted by anterior limits of hox gene or paralogy group expression within the somitic mesoderm—with an additional hox expression boundary corresponding to the projecting articulation for the lateral stays and pectoral arch within the synarcual (Fig. 4).

**Discussion**

The regionalization of the skate vertebral column that we report here stands in stark contrast to the conventional view of a simple distinction between trunk and tail vertebrae in fishes. Rather, our findings, along with reports of striking axial skeletal complexity and regionalization in some extinct and extant fishes (19–21, 32), point instead to greater ancestral axial skeletal complexity than is currently recognized for jawed vertebrates. While the tail region of the skate axial column is easily recognizable by the change in morphology from laterally projecting transverse processes to ventrally directed hemal arches, the more rostral vertebral regions vary more subtly in morphology. The morphometric approaches used here have recently resolved cryptic anatomical boundaries in the vertebral skeletons of tetrapod taxa previously thought to have lost axial regionalization (38), and have allowed for finer-scale resolution of regional boundaries in fossil lineages (3). If applied more widely, comparative morphometric approaches can help to detect cryptic regionalization in a much broader array of taxa, providing a more comprehensive picture of the evolutionary history of axial regionalization.

Skates also exhibit nesting of paraxial mesodermal hox gene expression consistent with characterization of hox expression in the dogfish shark, Scyliorhinus canicula (30), and resembling that of tetrapods, with expression boundaries spanning the AP axis of the embryo (4). Furthermore, we have found close qualitative associations between hox expression boundaries and anatomical transitions in the skate axial skeleton, with anterior expression limits of hox paralogy groups that pattern axial regions in tetrapods also predicting regional transitions in the skate vertebral column. In tetrapods, hox6, -9, -10, and -11 paralogs pattern the cervical–thoracic, thoracic–lumbar, lumbar–sacral, and sacral–caudal transitions, respectively. In the skate, the anterior expression limit of hox6 group genes corresponds to the position in the synarcual from which the lateral stay projects to articulate with the pectoral girdle (corresponding to spinal nerve foramina 13 to 17), while anterior expression limits of hox9, -10, and -11 group genes correspond, respectively, to transitions from R1–R2 (the back of the synarcual), R2–R3, and R4–R5 (the trunk–tail boundary) (Fig. 4).

Some regional distinctions within the skate vertebral column (the synarcual/R1, R3, and R5) are apparent based on qualitative morphological characteristics, while others (R2 and R4) are more subtle and are defined solely based on morphometry. Interestingly, the latter two regions both derive from somites that experience temporal shifts in the expression of single hox orthologs. While most of the hox genes investigated here show relatively static anterior expression boundaries across developmental stages, anterior limits of hoxb10 and hoxa11 expression exhibit conspicuous caudal and rostral shifts, respectively, from embryonic S18 to S25. Somites that experience the caudal shift in hoxb10 expression contribute to R2, while somites that experience the rostral shift in hoxa11 expression contribute to R4. Previous work in *Drosophila* has revealed several potential mechanisms in which subtle changes in expression of a single Hox gene can affect the patterning of multiple body segments. For example, near-constant expression of UBX protein is required to pattern parasegment 6 in *Drosophila* but, to arrive at the unique set of traits that make up parasegment 5, UBX expression must shift both in position and timing (40). Additionally, recent work in *Drosophila* has highlighted how small dosage-dependent effects of *Amp* and *Ubx* transcription can impact specification of appendage identity and morphology (41). We suggest that temporal shifts in hox transcription in skate could likewise lead to varying doses of hox protein within somites, and could reflect important roles for both qualitative and quantitative hox expression features in the establishment of vertebral regions exhibiting greater or lesser degrees of morphological disparity.
Hox genes impart positional identity onto serially homologous structures like body segments and vertebrae in animal groups as diverse as arthropods, annelids, and vertebrates (4, 42–47). This regionalization of segments, in turn, allows for functional specializations that have facilitated morphological change across the evolutionary tree of animals. The striking conservation of Hox expression with anatomical boundaries between tetrapods and skate indicates that an elaborate program of Hox-based vertebral regionalization did not arise along the tetrapod stem but rather has a much deeper origin, occurring within at least the last common ancestor of jawed vertebrates. Whether this conservation reflects homology of axial potential or simply approaches to identify axial regions across vertebrates has the potential to reveal the complex evolutionary history of this key feature of the vertebrate body plan.

Materials and Methods

Animal Collection and Husbandry. We acquired little skate (L. erinacea) adults and embryos from captive brood stock at the Marine Resources Center of the Marine Biological Laboratory (MBL). All animal work complied with protocols approved by the MBL (Institutional Animal Care and Use Committee Protocol No. 19-34). We killed adults and embryos for CT scanning and gene expression analyses, respectively, with an overdose of MS-222 (1 g/l) in seawater, fixed them in 4% paraformaldehyde (1 w/v for adults, overnight for embryos), and rinsed them in 1x phosphate-buffered saline (PBS). We then dehydrated adults into 70% ethanol and stored them at room temperature prior to CT scanning, and dehydrated embryos into 100% methanol and stored them at –20 °C prior to gene expression analysis. Skate embryo staging follows Ballard et al. (48) and Maxwell et al. (49).

Skate Vertebral Morphometrics. We scanned three adult little skates at the Cambridge Biotechnomorphometry Centre using a Nikon XITEK H.225 ST microCT scanner (see SI Appendix, Table S4 for scan parameters). We then segmented individual vertebrae up to number 70 (spanning expression boundaries of all potential hox genes investigated) from each scan in Mimics v23 (Materialise) or VGSTUDIO MAX v3.2.5 with the Coordinate Measurement module (Volume Graphics) and exported vertebral models as STL files. To collect 3D geometric morphometric data, we plotted a series of 12 homologous landmarks represented by 3D Cartesian grid coordinates on each vertebra using Checkpoint (Stratovan) (Fig. 1 and SI Appendix, Table S1). We exported landmarks plotted in Checkpoint as NTS files and combined them into a single file encompassing landmark coordinates for each vertebra in succession. We then imported NTS files into the geomorph R package (50, 51), aligned the coordinates using Procrustes superimposition to account for differences in size and position, and performed a PC analysis (Fig. 1 and SI Appendix, Fig. S1A–C).

Segmented Linear Regressions and Model Fitting. We tested for vertebral regionalization using a maximum-likelihood and segmented linear regression approach using the regions R package, as described in Jones et al. (3), modified from Head and Polly (38). This method fits iterative regression lines on the slopes of the PC scores from the morphometric analysis, for each potential region model (i.e., two regions, three regions, four regions, etc.), and then the most likely model is selected using the Akaike information criterion, which minimizes the overall residual sum of squares and penalizes against increasing numbers of regions (3, 38). We included the first five PCs in the regionalization score calculation (as in ref. 38).

These analyses tested for region boundaries that potentially align with known tetrapod regions, in the context of hox gene expression in skate. To compare region numbers in skate with Hox-associated boundaries in the tetrapod precardial axial skeleton, we selected a four-region maximum (five including the synarcal as the first region) for regression analysis (as in ref. 38).


27. K. E. Criswell, J. A. Gillis, Resegmentation is an ancestral feature of the gnathostome vertebral skeleton. eLife 5, e51690 (2020).


