Embryonic origin of the gnathostome vertebral skeleton

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Abstract

The vertebral column is a key component of the jawed vertebrate (gnathostome) body plan, but the primitive embryonic origin of this skeleton remains unclear. In tetrapods, all vertebral components (neural arches, haemal arches, and centra) derive from paraxial mesoderm (somites). However, in teleost fishes, vertebrae have a dual embryonic origin, with arches derived from somites, but centra formed, in part, by secretion of bone matrix from the notochord. Here, we test the embryonic origin of the vertebral skeleton in a cartilaginous fish (the skate, *Leucoraja erinacea*) which serves as an outgroup to tetrapods and teleosts. We demonstrate, by cell lineage tracing, that both arches and centra are somite-derived. We find no evidence of cellular or matrix contribution from the notochord to the skate vertebral skeleton. These findings indicate that the earliest gnathostome vertebral skeleton was exclusively of somitic origin, with a notochord contribution arising secondarily in teleosts.

Key Words

Vertebral skeleton, skate, somite, notochord, vertebrae, evolution

Introduction

The presence of vertebrae is a defining feature of the vertebrate body plan. A vertebral skeleton may consist of a series of paired neural arches that cover the spinal cord, paired haemal arches that enclose the caudal artery and vein, and, in many jawed vertebrates (gnathostomes), a series of centra that replace the notochord as the predominant support structure. Vertebral centra are highly variable in terms of morphology and tissue composition, and likely evolved independently in many different gnathostome lineages, including tetrapods, teleost fishes, and cartilaginous fishes [1]. This apparent evolutionary

convergence raises questions about the embryonic origin of vertebral skeletal elements across gnathostomes.

In tetrapods, all components of the vertebral skeleton derive from somites: transient, bilateral blocks of segmented paraxial mesoderm that form dorsally within the embryonic trunk. Somites are subdivided into dorsal and ventral subpopulations that give rise to trunk connective tissue and musculature ("dermomyotome") and skeletal tissues ("sclerotome"), respectively. Cell lineage tracing experiments using chick-quail chimaeras [2–5] and fluorescein-dextran injections or grafts from GFP-transgenic donor embryos in axolotl [6] have shown a fully somitic origin of the vertebral skeleton in these taxa, with somite-derived cells recovered in developing arches and nascent cartilage of the centra.

Conversely, in teleost ray-finned fishes, the vertebral skeleton appears to have a dual embryonic origin, with contributions from both paraxial mesoderm and the notochord. Teleost vertebral centra consist of an inner layer (the chordacentrum) and an outer layer, both composed of bone that forms by intramembranous ossification [7]. The chordacentrum of teleosts forms first, by secretion of bone matrix proteins (e.g. SPARC, type I collagen) from "chordoblast" cells that reside within the notochord epithelium [8–10]. In zebrafish, *in vitro* assays have shown that cultured notochord cells have the capacity to secrete bone matrix, and ablation experiments have demonstrated that in the absence of notochord, chordacentra fail to form [11]. Teleost chordacentra are subsequently surrounded by a relatively late-developing layer of paraxial mesoderm-derived membrane bone [7,12]. Additionally, zebrafish mutants with somite patterning defects possess normally-developing chordacentra, but exhibit profound neural and haemal arch defects, indicating the likely paraxial mesodermal origin of arch tissues [11,13,14].

To determine whether the dual origin of vertebral centra is a teleost-specific feature of the vertebral skeleton, or a general feature for gnathostomes that has been lost in tetrapods,

data on the embryonic origin of vertebrae from an outgroup to the bony fishes (i.e. Osteichthyes: the group that includes tetrapods and teleosts) are needed. Cartilaginous fishes (Chondrichthyes: sharks, skates, rays and holocephalans) occupy a key phylogenetic position as the sister group to the bony fishes, and data from this lineage may therefore be used to help infer primitive developmental conditions for the last common ancestor of gnathostomes. We have previously shown that vertebrae in the little skate (*Leucoraja erinacea*) each consist of a dorsal neural spine, two sets of dorsal cartilages that enclose the spinal cord (neural and intercalary arches), a single haemal arch and spine extending ventrally, and a tri-layered centrum (Figure 1) [15]. Here, we use somite and notochord fate mapping experiments, as well as mRNA *in situ* hybridization for genes encoding skeletal matrix proteins, to test the embryonic origin of the skate vertebral skeleton. We show that all components of the skate vertebral skeleton derive from paraxial mesoderm, with no evidence for cellular or matrix contributions from the notochord. When considered alongside data from bony fishes, our findings point to a general and likely primitive paraxial mesodermal origin of the vertebrate column in jawed vertebrates.

Materials and Methods

Somite fate mapping

L. erinacea embryos were obtained from the Marine Biological Laboratory (MBL) in Woods Hole, MA and kept in a flow-through sea table at ~16ºC until S24. A flap was cut in the egg case using a razor blade, and the embryo and yolk were transferred to a Petri dish. Embryos were anesthetized in a solution of MS-222 (100mg/L Ethyl 3-aminobenzoate methanesulfonate – Sigma-Aldrich) in seawater. CellTracker CM-DiI (Thermofisher) (5 µg/µL in ethanol) was diluted 1:10 in 0.3 M sucrose and injected into the ventral portions of the somites (1-3 injections per embryo) using a pulled glass capillary needle and a

Picospritzer pressure injector (Figure 2a). Embryos were then replaced in their egg cases and returned to the sea table to develop for approximately 7 or 12 weeks. Embryos were then fixed with 4% PFA, as described in Criswell et al. [15].

Notochord fate mapping

Embryos were kept as described above until S14, at which point a small window was cut in the egg case over the embryo. CM-DiI was microinjected into the notochord triangle as described above (Figure 2b). The window was then sealed with donor eggshell and Krazy Glue™ gel (Figure 2c), and eggs were returned to the sea table to develop for an additional 16-18 weeks prior to fixation (as described in Criswell et al. [15]).

Validation of CM-DiI injection placement

To verify the correct placement of CM-DiI injections, three somite-injected embryos were fixed immediately post-injection, and three notochord-injected embryos were fixed five days post-injection. Embryos were fixed in 4% paraformaldehyde in PBS overnight at 4ºC, rinsed 3X15 min in PBS, and stained with DAPI at 1ug/mL overnight at room temperature. Somite-injected embryos were imaged on a Zeiss lightsheet microscope and notochord-injected embryos were imaged on Zeiss lightsheet or LSM 780 confocal microscopes.

Histology and mRNA in situ hybridization

CM-DiI-labeled *L. erinacea* embryos were embedded in paraffin wax and sectioned at 8 μm thickness as described in O'Neill et al. [16] for histological analysis. Prior to embedding, embryos were demineralized in 10% EDTA (ethylenediaminetetraacetic acid) for 14 days. Histochemical staining was performed following the Masson's trichrome protocol of Witten and Hall [17]. *In situ* hybridization experiments for *Col1a1* (GenBank accession

number MG017616) and *SPARC* (GenBank accession number MG017615) were performed on sections as described in O'Neill et al. [16], with modifications according to Gillis et al. [18].

Results

Somitic contribution to all components of the skate vertebral skeleton

To test for somitic contribution to the skate vertebral skeleton, we microinjected CM-DiI into ventral portions of the somites (i.e. the presumptive sclerotome – Figure 3a) of skate embryos at stage (S) 24 (Ballard et al., 1993). Focal labeling of the somites (with no notochordal contamination) was confirmed by light sheet microscopy, in embryos fixed immediately post-injection (Figure 3b; n=3). By 50-52 days post-injection (dpi) (S31), spindle-shaped cells of the developing areolar tissue of the centrum surround the notochord, and preskeletal mesenchyme has condensed around the neural tube and caudal artery and vein. In all embryos analyzed at this stage (n=5), CM-DiI was recovered in the spindle-shaped cells of the developing areolar tissue (Figure 3c), indicating their somitic origin.

By 109dpi (S34), vertebrae are fully developed, with neural, intercalary and haemal arches, and a tri-layered centrum (Figure 1). In embryos analyzed at this stage (n=4), CM-DiI-positive cells were recovered throughout the vertebral skeleton. CM-DiI-positive cells were recovered in the cartilage of the neural (n=3 vertebrae in three embryos) and haemal arches (n=6 vertebrae in four embryos; Figure 3d, e), as well as in the inner layer of cartilage (Figure 3f; n=2 vertebrae in two embryos), the middle areolar tissue (Figure 3g; n=3 vertebrae in three embryos), and the outer cartilage of the centrum (Figure 3h; n=3 vertebrae in three embryos). Taken together, these findings demonstrate somitic contribution to all major components of the skate vertebral skeleton.

No evidence for notochordal contribution to the vertebral skeleton in skate

To test for cellular contributions of the notochord to the skate vertebral skeleton, we conducted a series of notochord fate mapping experiments. In cartilaginous fishes, the notochord derives from a small triangular region of progenitor cells (the "notochord triangle") that appears at the posterior margin of the blastodisc at S12 [19]. We focally labeled the notochord triangle of skate embryos with CM-DiI at S14 (Figure 4a), and we confirmed localization of the dye to the notochord at 5dpi (approximately S17) using confocal microscopy. In three embryos examined at S17, CM-DiI was found either only in the notochord (n=2), or in the notochord and neural tissue (n=1) (Figure 4b). In no cases were CM-DiI-labeled cells detected in the paraxial mesoderm.

We therefore labeled the notochord triangles of several skate embryos at S14, and reared these embryos to 116-129dpi (S34 – at which point the vertebral skeleton has fully differentiated). CM-DiI was recovered within the notochord (Figure 4c, c') and the notochord epithelium (Figure 4d, d') of the intervertebral regions of the axial column (n=5). In three embryos, CM-DiI-positive cells were recovered in the remnants of notochord epithelium that persist in the center of the centrum, where the notochord is almost completely replaced by inner layer centrum cartilage, but no CM-DiI-positive chondrocytes were recovered in the inner layer of cartilage itself. No CM-DiI labeled chondrocytes were observed in any other components of the axial column. These experiments, therefore, provide no evidence for a cellular contribution from the notochord to the vertebral skeleton.

In teleosts, chordoblast cells within the notochord epithelium secrete matrix components that make up the acellular bone of the chordacentrum. Though skates do not possess a chordacentrum, the areolar tissue of the skate centrum does mineralize, and at its origin, sits adjacent to the notochord epithelium [15]. To test whether notochord epithelial cells contribute matrix components to centrum tissue in skate, we characterised the expression of genes encoding the bone matrix proteins Col1a1 and SPARC in developing

skate centra. We did not detect transcription of *Col1a1* (Figure 5a) or *SPARC* (Figure 5b) in the notochord epithelium. Rather, these transcripts localized to the spindle-shaped cells of the areolar tissue (Figure 5a-b). These findings suggest that the paraxial mesoderm-derived cells of the areolar tissue itself – and not the notochord epithelium – are the source of extracellular matrix of the mineralized tissue of the skate vertebral centrum.

Discussion

Our somite fate mapping experiments demonstrate that presumptive sclerotome contributes to all components of the vertebrae in skate, including the neural and haemal arches, and all tissues of the tri-layered vertebral centrum. While it is possible that DiI could diffuse through the extracellular matrix after injection to contaminate tissues adjacent to the intended target (e.g. notochord), we have controlled for this possibility by imaging a subset of embryos shortly after injection to validate the precision of our labeling, and by performing complementary notochord fate mapping experiments. In the latter, we find that CM-DiI labeling of notochord progenitor cells resulted exclusively in labeling of the notochord and the notochord epithelium, with no contribution to vertebral tissues. In teleost fishes, chordoblast cells within the notochord epithelium express genes encoding the bone matrix proteins type I collagen and SPARC [10,20–22], and are likely the source of bone matrix for the earliest layer of the vertebral centrum [11,23–28]. As skates also possess a mineralized layer within their vertebral centra, we sought to test for expression of *Col1a1* and *SPARC* during skate vertebral development by mRNA *in situ* hybridization. We found these genes to be expressed exclusively within the somitically-derived spindle-shaped cells of the areolar tissue (the precursor to the mineralized middle layer of the centrum – Criswell et al. [15]), and not in the notochord epithelium. These findings suggest that the cells and matrix components of the skate vertebral centrum are entirely of paraxial mesodermal origin.

When considered alongside data from bony fishes, our demonstration of a somitic origin of the vertebral skeleton of skates suggests that this tissue was likely the sole, primitive source of vertebral skeletal tissues in gnathostomes, with a notochord contribution to centrum bone representing a derived condition of teleost fishes (Figure 6). Evidence from early fossil jawed and jawless fishes strongly suggests that the vertebral skeleton in the last common ancestor of gnathostomes consisted simply of a series of neural arches and a persistent notochord, with no centra [29–32]. Several gnathostome lineages, including elasmobranch cartilaginous fishes, teleosts, and tetrapods, subsequently evolved centra independently of one another [1]. At their origins, the vertebral centra of elasmobranchs and tetrapods derived entirely from paraxial mesoderm [3,6,12], but an inner layer of notochord-derived acellular bone was incorporated into the centrum with the independent origin of teleost centra.

It is not yet clear, however, if this specialized condition of teleosts is unique among ray-finned fishes. Despite recent changes to phylogenetic patterns [33] vertebral centra very likely evolved independently in multiple non-teleost ray-finned fish lineages (e.g. in gars and bichirs [1,34,35]. But, it is unclear whether the notochord contributes tissue to the different forms of centra observed in these taxa. Comprehensive analyses of the embryonic origins of vertebral tissues in strategically selected fish taxa are needed to better resolve the evolutionary and developmental assembly of the diverse array of axial skeletons, arguably the key characteristic, of vertebrates in general.

Ethics

All experimental work was done in compliance with protocols approved by the Animal Care and Use Committee at the Marine Biological Laboratory.

Data Accessibility

The sequence data associated with the genes in this study are available on GenBank (*Col1a1* accession number MG017616 and *SPARC* accession number MG017615).

Competing Interests

The authors declare no competing interests.

Author Contributions

KEC conceived of the study, performed histology, fate mapping, and *in situ* hybridization experiments, and drafted the manuscript; MIC coordinated the study and provided input on the manuscript; JAG designed portions of the study, coordinated the study, and helped to write the manuscript. All authors gave final approval for publication.

Acknowledgements

We thank H. Stinnett, R. Ho, M. Hale, A. Fleming, and M. Kishida for helpful discussions. We also acknowledge the support of R. Behringer, A. Sánchez-Alvarado, J. Henry, D. Lyons, the MBL Embryology community, and the staff of the MBL Marine Resources Center.

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Figures

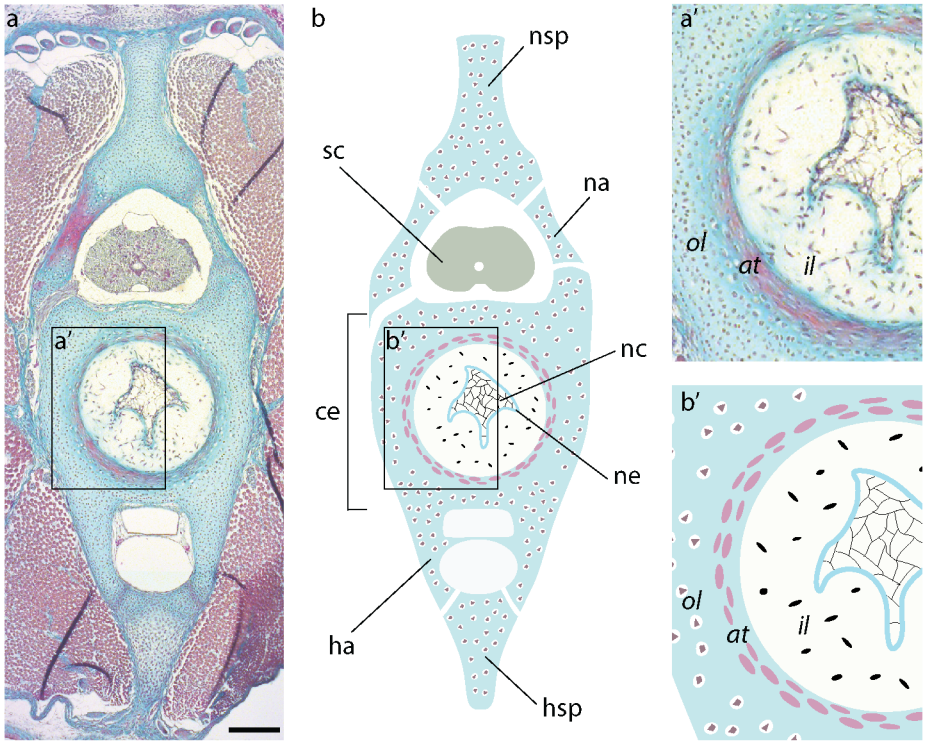

Figure 1. a, Cross section through a skate caudal vertebra (stained with Masson's trichrome); a', magnified cross section illustrating the three layers of the centrum; b, schematic

illustrating the components and tissues of the skate vertebra; b' schematic of the tri-layered centrum. at, areolar tissue; ce, centrum; ha, haemal arch; hsp, haemal spine; il, inner layer of the centrum; na, neural arch; nc, notochord; ne, notochord epithelium; nsp, neural spine; ol outer layer of the centrum; sc, spinal cord. Scale bar = 200 µm.

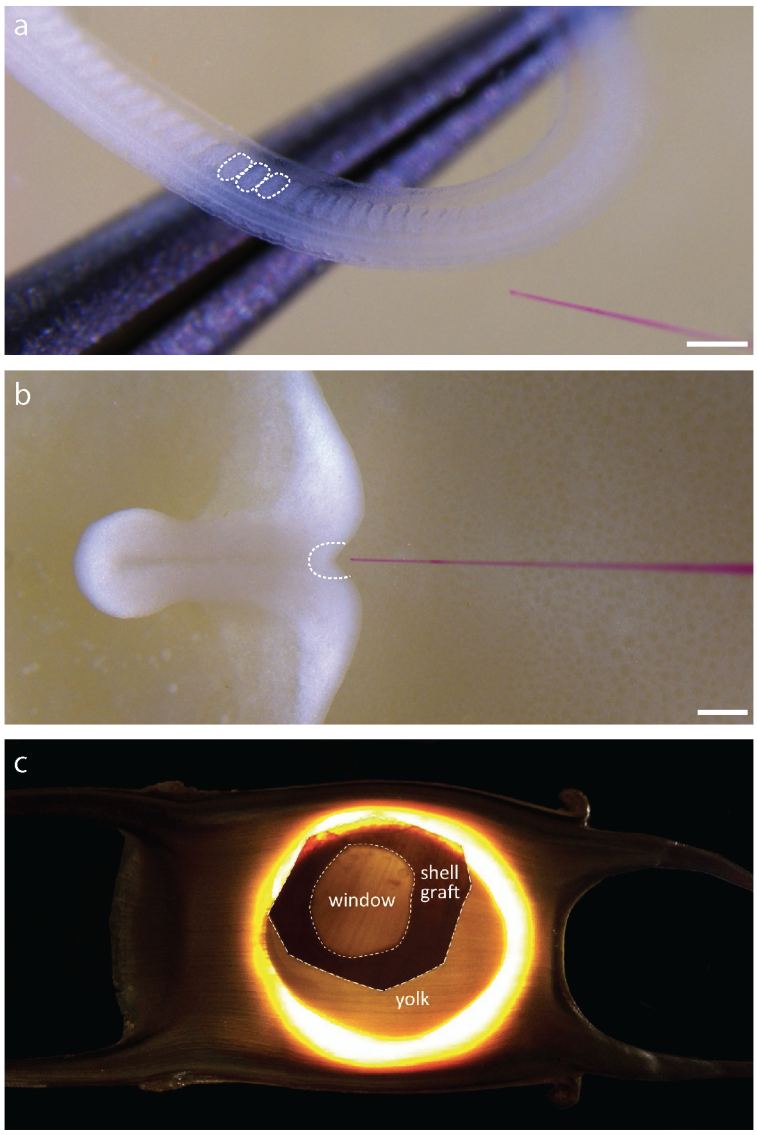

Figure 2. Microinjection of skate embryos with CM-DiI. CM-DiI labeling of a, somites at S24 (three somites are highlighted with dashed lines) and b, notochord progenitor cells at S14 (with the "notochord triangle" of Ballard et al. 1993 outlined). c, sealing of a windowed skate egg with donor egg shell. Scale bars = 200 µm.

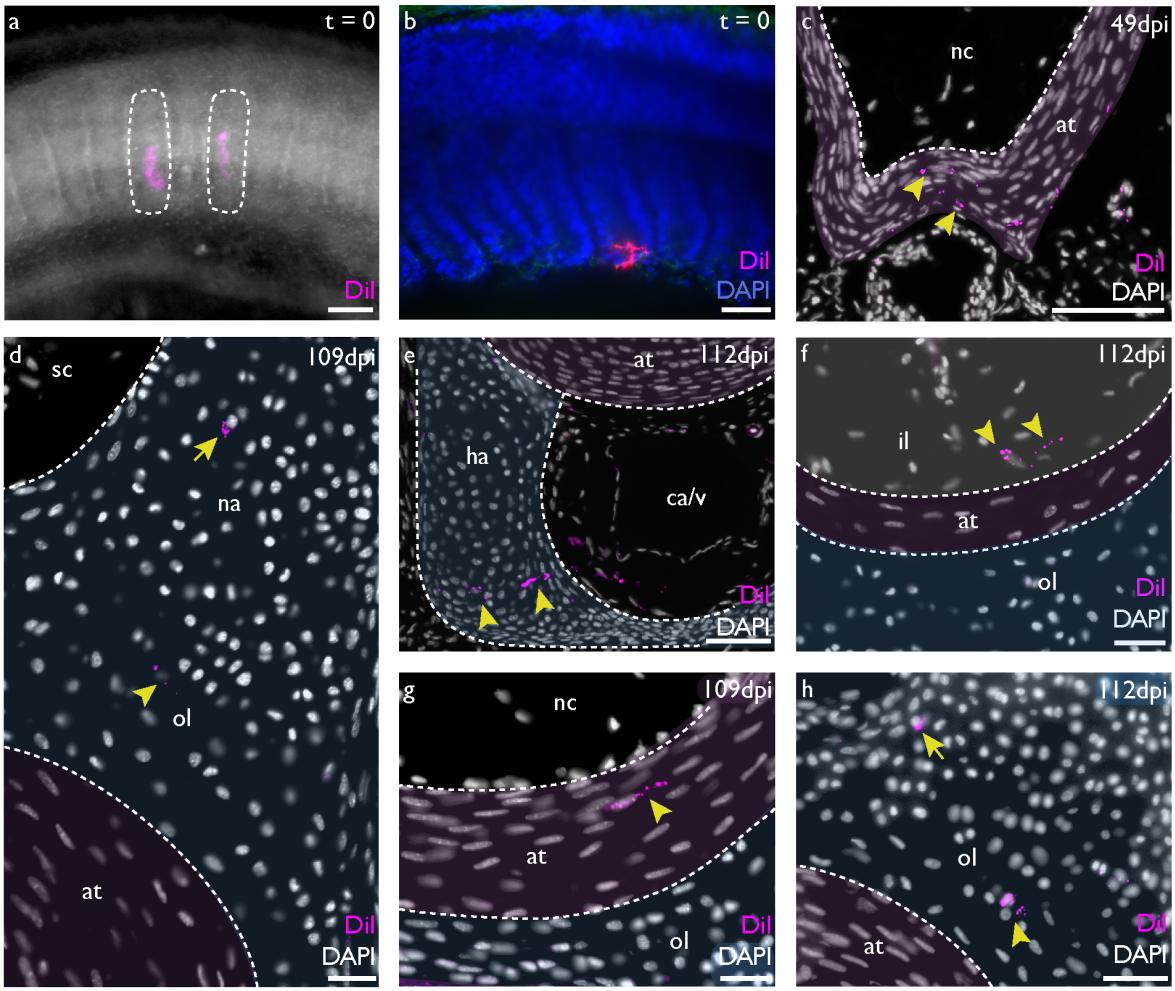

Figure 3. Somitic contribution to the skate vertebral skeleton. a, two CM-DiI injections in ventral somites; b, confocal image confirming the placement of the dye immediately post injection in sagittal section; c, CM-DiI labeled cells (indicated by yellow arrowheads) distributed within the spindle-shaped cells of the areolar tissue (at) at 49 dpi (false colored pink); d, CM-DiI labeled chondrocytes in the neural arch (na, indicated by yellow arrow) and outer layer of centrum cartilage (ol, indicated by yellow arrowhead) at 109dpi (cartilage false colored blue); e, CM-DiI labeled cells in the haemal arch at 112 dpi (ha, false colored blue); f, CM-DiI labeled chondrocytes (indicated by yellow arrowheads) in the inner layer of the centrum at 112 dpi (il, false colored white); g, CM-DiI labeled cells (indicated by yellow arrowhead) in the areolar tissue, the middle layer of the centrum at 109 dpi (at, false colored

368 pink); h, CM-DiI labeled chondrocytes in the outer layer of the centrum (ol, indicated by
369 yellow arrowhead) and in the neural arch (indicated by yellow arrow) at 112 dpi (na, false
370 colored blue). ca/v, caudal artery and vein; nc, notochord; sc, spinal cord. Scale bars = 100
371 µm.
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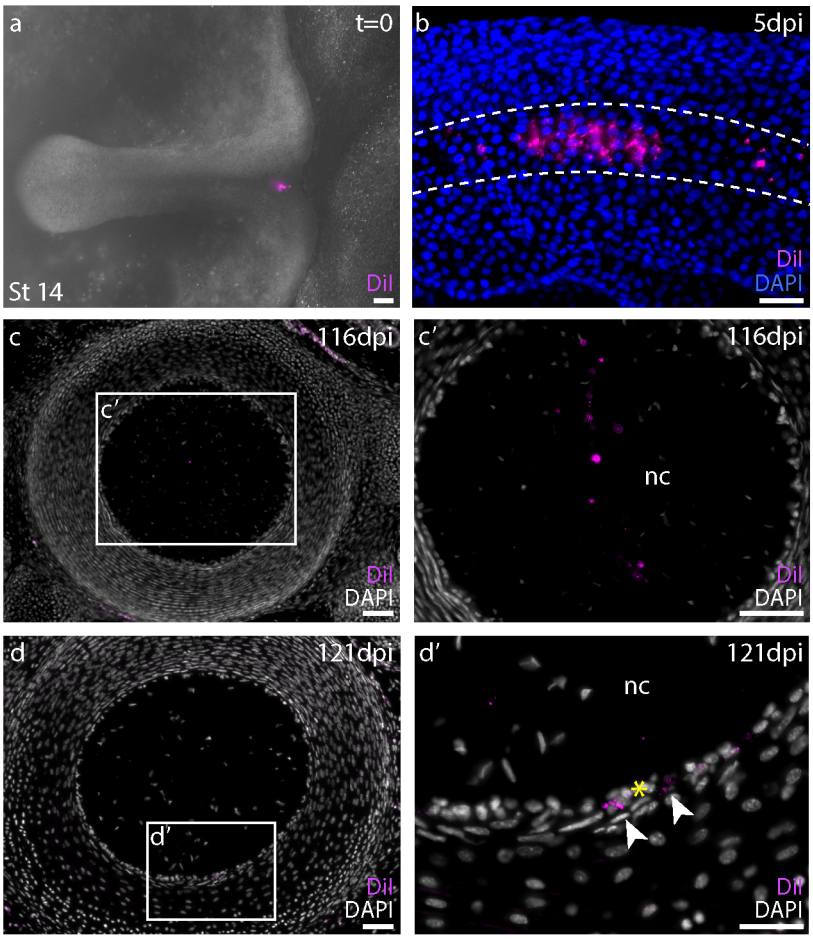

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374 **Figure 4. No cellular contribution from the notochord to the skate vertebral skeleton**. a,
375 CM-DiI injection of the notochord triangle of a skate embryo at S14; b, confocal image of a
376 skate embryo at 5dpi, showing CM-DiI-labeled cell in the notochord; c, a section through the
377 notochord at 116 dpi, showing CM-DiI positive notochord cells at 10x; c', higher
378 magnification view of the inset box in c; d, CM-DiI positive cells in the notochord
379 epithelium; d' higher magnification view of the inset box in d. Yellow asterisk indicates
380 notochord epithelium. Scale bars = 100µm.

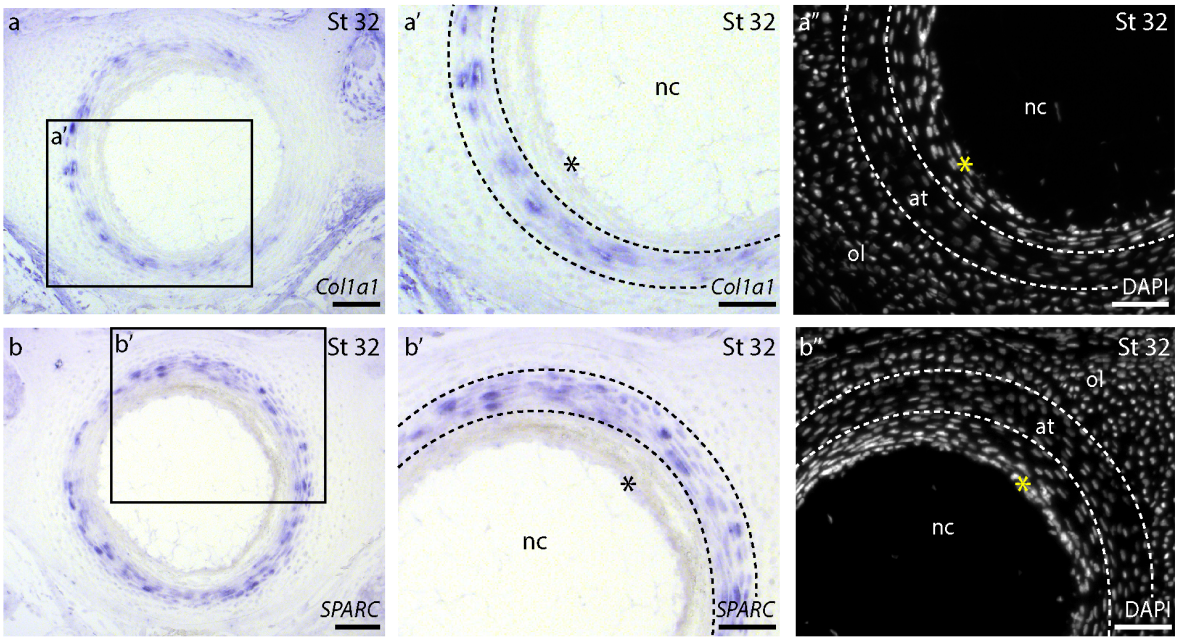
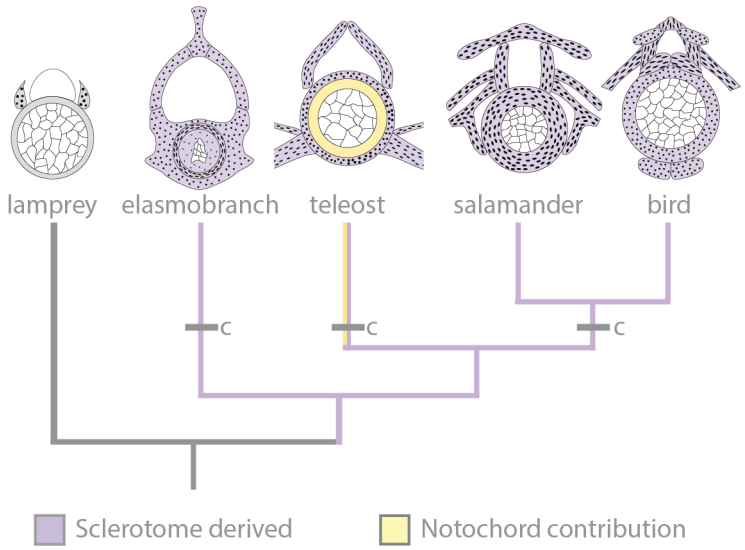


Figure 5. The notochord is not a source of bone-like tissue in skate vertebral centra. a, *Col1a1* is expressed in the areolar tissue of the developing centrum; a', a higher-magnification image of *Col1a1* expression; a'' DAPI staining of the same section as depicted in a', showing the boundary between areolar tissue and the notochord epithelium (yellow asterisk); b, *SPARC* is expressed in the areolar tissue of the developing centrum; b', a higher-magnification image of *SPARC* expression, and b'' DAPI staining of the same section as depicted in b', showing the boundary between areolar tissue and the notochord epithelium (yellow asterisk). at, areolar tissue; nc, notochord; ol, outer layer. Scale bars = 100 μm.



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393 **Figure 6. Embryonic origins of the vertebral skeleton across gnathostomes**.

394 Representative sections of lamprey, skate, teleost, salamander, and bird vertebrae, with

395 paraxial mesodermal derivatives indicated by purple, and notochord derivatives indicated by

396 yellow. Grey bars indicate independent originations of centra. Schematics redrawn after

397 Goodrich [36] (lamprey), Criswell [15] (skate), and MacBride [37] (teleost, salamander, and

398 bird).