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## Development and regeneration of the crushing dentition in skates (Rajidae)

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### Abstract

Sharks and rays (elasmobranchs) have the remarkable capacity to continuously regenerate their teeth. The polyphyodont system is considered the ancestral condition of the gnathostome dentition. Despite this shared regenerative ability, sharks and rays exhibit dramatic interspecific variation in their tooth morphology. Ray (batoidea) teeth typically constitute crushing pads of flattened teeth, whereas shark teeth are pointed, multi-cuspid units. Although recent research has addressed the molecular development of the shark dentition, little is known about that of the ray. Furthermore, how dental diversity within the elasmobranch lineage is achieved remains unknown. Here, we examine dental development

and regeneration in two Batoid species: the thornback skate (*Raja clavata*) and the little skate (*Leucoraja erinacea*). Using *in situ* hybridization and immunohistochemistry, we examine the expression of a core gnathostome dental gene set during early development of the skate dentition and compare it to development in the shark. Elasmobranch tooth development is highly conserved, with *sox2* likely playing an important role in the initiation and regeneration of teeth. Alterations to conserved genes expressed in an enamel knot-like signalling centre may explain the morphological diversity of elasmobranch teeth, thereby enabling sharks and rays to occupy diverse dietary and ecological niches.

### **Key words**

Tooth development, elasmobranchs, dental lamina, *sox2*, evo-devo, gnathostome evolution

### **Introduction**

Sharks and rays have the remarkable ability to continuously regenerate their teeth, following a conveyor-belt like replacement system<sup>1-4</sup>. This condition is termed polyphyodonty and is considered to be the ancestral condition of all vertebrate dentitions<sup>5,6</sup>. However, this regenerative ability has subsequently been lost in many widely studied vertebrate models (including the zebrafish, chicken and mouse). To gain a comprehensive understanding of vertebrate tooth evolution and development, it is essential to examine groups that retain this regenerative ability, such as sharks and rays.

Sharks, skates and rays collectively form the subclass of Elasmobranchii, belonging to the class Chondrichthyes (the cartilaginous fishes), which comprises the ancient sister lineage to Osteichthyes (the bony fishes). Extant Batoids (rays) comprise over half of all chondrichthyans and display considerable morphological and taxonomic diversity<sup>7,8</sup>.

Adaptation to bottom feeding is reflected by their characteristic dorso-ventrally flattened bodies, with enlarged pectoral fins and ventrally positioned jaws. This diversity is further

highlighted by several divergent trophic phenotypes, such as the sawfish snout and cephalic feeding apparatus of eagle and manta rays. However, the majority of batoids have evolved flattened, pavement-like teeth, adapted to a durophagous diet of hard-shelled prey<sup>8</sup>.

Within the superorder of Batoidea, individual genera possess vast dental diversity. The spotted eagle ray (*Aetobatus narinari*) develops individual rows of broad fusiform teeth, forming a continuous crushing surface<sup>9</sup>. The upper jaw dentition of bat eagle ray (*Myliobatis californica*) constitutes a large central row of hexagonal flattened teeth, flanked by lateral rows of smaller teeth, while in the lower jaw numerous teeth extend anteriorly beyond the jaw margin, forming a spade-like appendage used to excavate prey from the sandy substrate<sup>8,10-12</sup>. This morphological variation in Batoid dentition has facilitated the adoption of diverse ecological niches.

The batoid dentition contrasts with the typically elongate and blade-like teeth of sharks, which are adapted for piercing and sawing at both hard and soft bodied prey. Recent research has addressed important distinctions and similarities of dental phenotypes between batoid taxa and shark outgroups<sup>3</sup>. One key distinction is that unlike all known shark clades, batoid teeth lack distinct multiple cusps. Despite recent progress towards understanding batoid tooth morphology and taxonomy, our knowledge of the molecular mechanisms underpinning dental development and regeneration is lacking<sup>3</sup>. Molecular developmental studies are essential for determining how the batoid dentition is established, and how such incredible dental diversity is generated.

Recent studies have shed light upon the evolution and development of the dentition and dermal denticles of an emerging model elasmobranch: the small-spotted catshark (*Scyliorhinus canicula*)<sup>4,13-15</sup>. Following initial expression studies<sup>2,16-18</sup>, comprehensive gene expression analysis identified that conserved gene representatives of the hedgehog (Hh),

wingless (Wnt), bone morphogenetic (BMP) and fibroblast growth factor (FGF) signalling pathways are observed throughout development of the shark dentition<sup>4</sup>. Due to their early divergence in the gnathostome phylogeny (relative to the tetrapod lineage), the use of elasmobranch models for studying the ancestral polyphyodont condition has become increasingly prominent. Recent studies on shark tooth morphogenesis suggest that a core set of genes, largely homologous to those found in the mammalian enamel knot (EK)<sup>19–21</sup>, has been conserved and redeployed throughout over 450 million years of gnathostome tooth evolution and development<sup>4</sup>. However, whether this important tooth shape signaling centre (EK) directs the morphogenesis of flattened cusps in rays is unknown. Given the conservation of the EK among toothed gnathostomes<sup>4</sup> the loss of this organising unit would seem unlikely. Although, there indeed is a dramatic shift in the shape of the ray teeth compared to the EK-present shark teeth, we might expect to see a related shift in the activity of the EK, if present at all.

During early odontogenesis, the oral epithelium invaginates to give rise to the dental lamina (DL). Molecular interactions between the DL and underlying mesenchyme regulates dental development and regeneration<sup>22</sup>. The DL is short lived in diphyodont mammals, degrading after the development of permanent adult teeth<sup>23</sup>. However, in polyphyodont gnathostomes, the DL is permanent and transiently elongates to the aboral location of the free end of DL, known as the successional lamina (SL). The SL, in turn provides a source of transit-amplifying cells necessary to sustain continuous tooth regeneration<sup>24–26</sup>. In contrast, some polyphyodont teleosts (such as the rainbow trout, *Oncorhynchus mykiss*, and cichlids) replace their teeth via a population of epithelial cells closely associated with the preceding tooth<sup>27,28</sup>. Similar to reptiles, the shark develops a deeply embedded SL. During the transition from primary to SL, the leading epithelial tip of the lamina extends inwards, enabling the

continuous development of multi-generational teeth<sup>4</sup>. This system may represent the ancestral state of gnathostome dentition, although this remains uncertain.

The thornback ray/skate (*Raja clavata*) and the little skate (*Leucoraja erinacea*) possess contrasting adult dental morphologies: flattened, pavement-like teeth and pointed cusped teeth on a flattened base, respectively. However, these differences appear later in ontogeny, and the developmental stages examined here show comparable early tooth development between both species. Using *in situ* hybridization (ISH) and immunohistochemistry (IHC), we examine the early molecular development and regeneration of these skate dentitions and compare it to dental development of the shark (*S. canicula*). We ask whether shifts in the expression of core genes responsible for shark tooth development and regeneration can explain the diversity that differentiates the shark and skate dentition.

## Materials and methods

### *Fish husbandry*

Shark species used in this study include the small-spotted catshark (*Scyliorhinus canicula*) and large-spotted catshark (*Scyliorhinus stellaris*). Ray/Skate species used include the thornback ray (*Raja clavata*) and the little skate (*Leucoraja erinacea*). *S. stellaris* and *R. clavata* were supplied by the Native Marine Centre, UK, *L. erinacea* were supplied by Woods Hole Marine Biological Laboratory, USA, and *S. canicula* were supplied by Station Biologique de Roscoff, France, and North Wales Biologicals, Bangor, UK. Embryos were raised to the desired developmental stages<sup>29</sup> in an aerated saltwater aquarium at the University of Sheffield, at 12°C. Prior to analysis, embryos were anesthetized with MS-222 (tricaine) before overnight fixation in 4% paraformaldehyde (PFA) at 4°C. Embryos were dehydrated and stored in methanol (MeOH).

### *Staging of individuals*

For staging of embryos, due to the absence of a published series of normal stages for *R. clavata* and *L. erinacea*, individuals were staged using external morphology in accordance with Ballard<sup>30</sup> *et al.*, (*S. canicula*) and Maxwell<sup>31</sup> *et al.*, (Winter Skate; *L. ocellata*), cross-referenced. However, in this study, guidance is drawn primarily from Ballard<sup>30</sup>. Based upon this current literature, the developmental window from onset of dental competence (odontogenic band; OB) to early tooth replacement is proposed to occur between Stages 28-29; (T1-T3) 31-32; (T3-T4) 32-33. However, the authors note this to be a guide rather than a conclusive series of normal stages. Maxwell *et al.*<sup>31</sup> consider ontogenetic progression in *L. ocellata* to be largely in accordance with *S. canicula* with some notable interspecific variation; for example, jaw reshaping, which occurs at otherwise equivalent Stages 27 and 28, respectively.

#### *Clearing and staining*

Fixed embryos were rehydrated and placed in a 1% trypsin solution for 1 hour. Calcified tissue was then stained using Alizarin red dissolved in potassium hydroxide (KOH). Embryos were then cleared in KOH, before grading into 100% glycerol, with thymol as a biocide.

#### *Micro Computed Tomography (MicroCT)*

Rendered X-ray computerized tomography (microCT) scans of an adult *Raja clavata* and *Scyliorhinus canicula* (Figure 1), and *Leucoraja erinacea* (Figure 2) were scanned using the Metris X-Tek HMX ST 225 CT scanner at the Imaging and Analysis Centre, Natural History Museum, London, and rendered using VG studio max 2.0. CT scans from Figure 2 were rendered using Drishti.

#### *Serial thin sectioning*



Embryos were dissected and graded into paraffin (Paraplast, Sigma). Thin serial sections were cut at 10-14  $\mu\text{m}$  with a Leica Microtome. Sections were washed in Xylene (Sigma) before rehydration to phosphate buffered saline (PBS), and use in *ISH* and *IHC*.

#### *Design of RNA probes*

Digoxigenin-labelled antisense riboprobes were designed using partial *L. erinacea*, *S. canicula* and *Scyliorhinus torazame* expressed sequence tag (EST) assemblies<sup>32</sup> (SkateBase, skatebase.org; VTcap, transcriptome.cdb.riken.go.jp/VTcap; and an unpublished *S. canicula* transcriptome, ScanRNAseq). The riboprobes used in this study were cloned from *S. canicula* cDNA using the following primer sequences, sequence databases and in some cases published GenBank accession numbers:  *$\beta$ -cat* (forward GGTGAAAATGCTTGGGTCT and reverse GGACAAGGGTTCCTAGAAGA; GenBank accession number: AF393833.1)<sup>33</sup>, *bmp4* (forward TGTGGAGTTCACCGAATTG and reverse GATTCCTGGTAACCGAATGC; SkateBase), *lef1* (forward GGGCTTTCTGCTGACTGATG and reverse CGTAAGGAGCGGCAACTTC; VTcap), *MK* (forward GACAGGGTCCTCTGAAGCTG and reverse TTAGGGTTCCATTGCGAGTC; VTcap), *pitx1* (forward GGTCGGGTGAAAGCAGAG and reverse GATGTTGCTGAGGCTGGAG; SkateBase), *pitx2* (forward GACGGAAGCTGGAAACAGTC and reverse TTTGCAAACCTGGGTGTCAAG; GenBank accession number AB625610.1)<sup>34</sup>, *shh* (forward AGTGGCAGATACGAAGGAAG and reverse AGGTGCCGGGAGTACCAG; GenBank accession number: HM991336.1)<sup>35</sup>, *sox2* (forward GGAGTTGT CAGCCTCTGCTC and reverse TGTGCTTTGCTGCGTG TAG; Skate-Base), *fgf4* (forward: ATGTTGATCAGGAAGCTGCG, reverse: GTATGCGTTGGATTTCGTAGGC; ScanRNAseq), *fgf3* (forward: CTTGCTCAACAGTCTTAAGTTATGG, reverse: CGGAGGAGGCTCTACTGTG; ScanRNAseq), and *fgf10* (forward: TGGATACTGACAAAGGGTGCC, reverse:

GACATCGTGTCTCACCACTATTGG; ScanRNAseq). Digoxigenin-labelled anti-sense riboprobes were generated using the Riboprobe System Sp6/T7 kit (Promega).

#### *Whole mount and section in situ hybridization (ISH)*

Whole mount and section ISH was undertaken as previously described<sup>4,14</sup>. Sections were imaged using an Olympus BX51 microscope and Olympus DP71 Universal digital camera attachment and whole mount samples were imaged using a Nikon SMZ15000 stereomicroscope. False colour processing of section images was undertaken using Adobe Photoshop CS5 Extended (v. 12.0 x 64). The brightness and contrast of wholemount samples was adjusted using Fiji<sup>36</sup>.

#### *Immunohistochemistry (IHC)*

IHC was undertaken as previously described<sup>4</sup>. Sections were imaged using an Olympus BX51 Upright Compound Microscope and Olympus DP71 Universal digital camera attachment. False colour processing of sections images was undertaken using Adobe Photoshop CS5 Extended (v. 12.0 x 64).

## **Results**

### *Elasmobranchs exhibit diverse dental morphologies*

Despite the shared ability of elasmobranchs to continuously regenerate their teeth<sup>4</sup>, skates and sharks possess vastly different tooth morphologies (Fig. 1A-F). The adult skate (*R. clavata*) dentition typically consists of multiple interlocking rows, forming a continuous crushing surface adapted to process hard prey such as shellfish (Fig. 1A, C-D)<sup>3</sup>. The adult shark (*S. canicula*) dentition is comprised of multiple generations of pointed, multi-cuspid teeth, capable of grasping both hard and soft prey, including both shellfish and fishes (Fig. 1B, E-F). In both rays (including skates) and sharks, replacement teeth initiate from the successional

lamina (SL), which is continuous with the dental lamina (DL) (Fig. 1G-J) (Stages 31-32). The developing jaws of both sharks and rays are rich in taste buds, developing lingually to early generations of mineralising teeth (Fig. 2A-D)<sup>4</sup>. Despite clear disparity in their dental morphologies, there are developmental similarities of tooth development between rays and sharks, each forming from a continuous dental lamina with new teeth developing through a conveyor belt-like replacement system.

*sox2 demarks tooth competent epithelium during initiation of the skate dentition*

Studies describing rajiform (skate) embryonic development suggest elasmobranchs share a series of common early stages prior to ontogenetic divergence<sup>37</sup>. Comprehensive observational studies of shark (*S. canicula*) development have provided a useful reference guide for stage matching with other elasmobranchs, based upon shared characteristics<sup>29,37,38</sup>. Consequently, it can be deduced that similar to the shark, dental development in the skate begins at Ballard stage 28-29<sup>4,29</sup>.

*sox2* is a highly conserved marker of dental competence and progenitor cells required for gnathostome tooth regeneration, meaning it can be considered a marker of cell populations with progenitor-like properties<sup>4,13,25,39</sup>. Whole mount *ISH* was used to investigate the expression of *sox2* throughout early development of the skate (*R. clavata*) dentition. Prior to dental development, *sox2* demarks sensory organs known as ampullae of Lorenzini (Fig. 3A-B, AL). These electroreceptors cover the ventral surface of the skate head, with a higher density of units located towards the rostrum. Additionally, we noted two rows of ampullae located beneath the lower jaw (Fig. 3B), which likely play a role in electroreception whilst seeking prey. At Stage 28-29<sup>29</sup>, the odontogenic band is visible demarking a tooth competent region of the upper jaw (Fig. 3C-D, OB). The OB subsequently divides into individual units

that give rise to first and second-generation teeth (Fig. 3E-F, T, T1, T2) with the free-end of the lamina (SL) reserved for the initiation of subsequent generations. Section *ISH* revealed that *sox2* is expressed in restricted dorso-ventrally opposing domains of oral epithelium in both the upper and lower jaws, during initiation of the skate dentition (Fig. 4A-F). In the shark, *sox2* is expressed in equivalent domains of the oral and dental lamina at the equivalent stage (28-29) (Fig. 4G-I), implying a conserved role in the initiation of dental development between elasmobranchs.

*sox2 is continually expressed in the regenerative skate dentition*

We next examined expression of *sox2* throughout later development of the skate dentition, during development of the second dental generation (Ballard stage 31-32<sup>29</sup>). Additionally, we undertook IHC of proliferating cell nuclear antigen (PCNA), to examine cell proliferation dynamics throughout first and second-generation tooth development<sup>40</sup>.

In the thornback skate (*R. clavata*), PCNA marks the extent of the DL in both the epithelium and underlying mesenchyme. The DL forms a comparatively compact and acutely angled invagination of the dental epithelium (Fig. 5A-C) (Stage 31-32). Although first-generation tooth germs develop as vestigial units and are later replaced by functional teeth, they already show signs of a characteristic flattened morphology compared to the shark (Fig. 5B-C).

During development of second-generation teeth, PCNA continues to label the oral-dental epithelium and underlying mesenchyme (Fig. 5C).

Additionally, *sox2* is expressed in epithelial domains of the lower and upper jaw, at the first-generation tooth stage (Fig. 5D). This *sox2*<sup>+</sup> stripe of oral epithelium extends to the posterior tip of the DL, lingual to developing teeth, with co-localisation of mRNA and protein expression (Fig. 5E-F). This pattern of co-localisation is maintained throughout second-

generation tooth development, with expression terminating a short distance within the adjoining oral epithelium (Fig. 5G-I).

In the shark, PCNA immunoreactivity marks similar domains of the oral-dental epithelium and mesenchyme of first-generation teeth (Fig. 5J-L). Additionally, PCNA marks the dental and adjoining oral epithelium extending outwards along the maxillary valve (Fig. 5J), a structure associated with the regulation of water intake that is absent at these stages from the ray/skate oral apparatus, although they may appear later in ontogeny. PCNA immunoreactivity is reduced in more advanced replacement teeth (Fig. 5L, T1), which subsequently become mineralised units. Successive replacement teeth also show a distinct lack of PCNA in the medial epithelial tip (Fig. 5L). This shares notable similarity to mammalian tooth development, during which a conserved signalling centre called the enamel knot (EK) activates to direct tooth cusp morphogenesis, prior to apoptosing<sup>20,41</sup>. In contrast, successional teeth of the skate show no corresponding reduction in DAB-immunoreactivity (Fig. 5C). This, however, could be an artefact of either DAB-overstaining or timing of the transient appearance of the EK-like structure, which interestingly is shown by immunofluorescence of PCNA (Figure 6A-C) in subsequent generations.

*sox2* expression in first generation teeth of the shark<sup>4</sup> matches the expression in the skate (Figure 5). *sox2* expression overall follows a similar pattern to PCNA, with mRNA localising to the oral epithelium of the maxillary valve (Fig. 5J,<sup>4</sup>), continuous with the dental lamina and terminating lingually to first generation teeth (Fig. 5). IHC for Sox2 in the lower jaw revealed expression in similar oral-dental epithelial domains (Fig. 5,<sup>4</sup>). These expression patterns were maintained throughout subsequent tooth replacement (Fig. 5 and 6), with further strong localisation to taste buds developing along the maxillary valve<sup>4</sup>. We also observed a subsurface pocket of *sox2*+ epithelial cells at the junction between the oral and dental epithelium (Fig. 5F, <sup>4</sup>). These cells may constitute a distinct population of quiescent

progenitors maintained during tooth development and replacement, to sustain this regenerative capacity<sup>4,13,25</sup>. Overall, expression of *sox2* and PCNA is broadly conserved throughout early tooth development of both skates and sharks. Notably, we did observe a reduction in PCNA immunoreactivity in the medial epithelial tip of successive skate teeth (Figure 6A and C), showing that an EK-like structure is present, albeit reduced, in the flattened skate dentition.

#### *A conserved dental gene set is expressed in the developing skate dentition*

We next sought to determine whether developmental genes expressed in the dentitions of the shark and other gnathostomes are conserved during skate tooth development<sup>4</sup>. Using *ISH*, we examined expression of paired-like homeodomain 1 and 2 (*pitx1*, *pitx2*), sonic hedgehog (*shh*), midkine (*MK*), bone morphogenetic protein 4 (*bmp4*) and wingless (Wnt)-associated signalling, throughout first and second-generation tooth development in the skate (equivalent to Ballard stage 31-32<sup>29</sup>) (Fig. 7).

*Pitx1* and *Pitx2* are conserved throughout mammalian, teleost and reptilian dental development<sup>26,42-47</sup>. Additionally, we observed these genes in the developing shark dentition<sup>4</sup>. We observed *pitx1* in the upper and lower jaw dental epithelium and adjoining inner oral epithelium. Expression was noted throughout the epithelium of the DL and was largely absent from the anlagen itself (Fig. 7A-C) (Stage 31-32). *pitx2* was expressed in the dental epithelium and mesenchyme in the lower and upper jaws. Some expression was also observed in the inner oral epithelium (Fig. 7D-F). In either jaw, expression was specific to the lingual DL and underlying mesenchyme. Expression of *pitx2* is epithelial throughout the tooth development of most gnathostomes<sup>45,48</sup>, although in the upper and lower dentition of the shark it also occupies the mesenchyme<sup>4</sup>. In the skate, we observed dental expression of *pitx2*

in the epithelium and mesenchyme of both jaws. This indicates a clearly conserved pattern of *pitx2* expression among divergent elasmobranch species. Therefore, the transcription factor *pitx2* is a gene that can exhibit spatial expression variation between different vertebrate species<sup>4</sup>, however, the specific impact that this shift in expression has is unknown. Whether this broader expression pattern in elasmobranchs is important for continuous tooth regeneration is currently unclear. However, it is intriguing to speculate that this may have been the primitive condition for early emerging gnathostomes, later becoming reduced to the dental epithelium as tooth generations became restricted and tooth shape became more complex.

*Shh* is a conserved ligand of the hedgehog (Hh) signalling pathway, and has been widely studied throughout gnathostome tooth development<sup>2,46,49,50</sup>. In mammalian tooth morphogenesis, *shh* and *MK* are both expressed in the EK, where they coordinate cell behaviour to define overall tooth cusp shape<sup>20,51</sup>. The conservation of an EK-like signalling centre in sharks remains subject to debate<sup>52</sup>, although detection of *shh*, *MK* and additional markers in shark dental development is indicative of potential conservation throughout gnathostomes<sup>4</sup>. Similar to the shark, *shh* was expressed in the epithelial tip of the anlagen during development of the primary skate dentition, albeit in a less restricted pattern (Fig. 7G-I). Additional expression was noted in a restricted domain of the inner oral epithelium, continuous with the DL (Fig. 7H). *MK* was observed in a similar domain, with expression observed in the medial epithelium of the anlagen in either jaw, and in the inner oral epithelium continuous with the DL (Fig. 7J-L).

*Bmp4* belongs to the transforming growth factor beta (TGF- $\beta$ ) superfamily, and is widely conserved throughout gnathostome tooth development<sup>46,53-55</sup>. In the shark, *bmp4* is expressed in the epithelium and mesenchyme at different stages of dental development. Expression of *bmp4* during shark tooth morphogenesis is considered indicative of an EK-like signalling

centre<sup>4</sup>. In the skate, we observed similar epithelial and mesenchymal expression of *bmp4* throughout early tooth development (Fig. 7M-O). In either jaw, *bmp4* expression labelled the lingual DL and underlying mesenchyme, indicating a potential role in the development of early replacement teeth. We noted further expression in a focused band of cells in the directly overlying oral mesenchyme (Fig. 7M). This band of *bmp4*<sup>+</sup> cells extends outwards into the inner oral mesenchyme, directly underlying the *pitx1/2*, *shh* and *bmp4* expressing inner oral epithelium. This region may be linked to the ‘taste-tooth field’, a domain of oral epithelium rich in taste buds expressing a similar set of genes, hypothesised to be linked to the evolutionary origin of the regenerative shark, and therefore elasmobranch, dentition<sup>13</sup>.

The canonical Wnt signalling pathway is also highly conserved throughout gnathostome tooth development.  $\beta$ -catenin ( *$\beta$ -cat*) and lymphoid enhancer binding factor (*lef1*) are widely used readout genes of Wnt signalling<sup>56-60</sup>. Previous research has indicated that interactions between the Wnt, Hh and Bmp pathways, and the presence of slow cycling tooth progenitor cells, can control reptilian tooth replacement<sup>26,55</sup>. In the shark, expression of  *$\beta$ -cat* and *lef1* provide similar evidence to support the conservation of Wnt circuits throughout gnathostome dental development<sup>4</sup>. In the skate,  *$\beta$ -cat* was expressed within the dental epithelium of first and second-generation teeth in either jaw, and further expression was noted in the adjoining oral epithelium (Fig. 8A-C) (Stage 31-32). *lef1* was expressed in a similar pattern, with some additional expression in the underlying dental mesenchyme of first and second-generation teeth (Fig. 8D-F).

Next, to obtain a better spatial understanding gene expression patterns throughout the dental lamina, we undertook ISH for *pitx1* and *sox2*, and IHC for PCNA and Sox2, from coronal sections of the skate (*L. erinacea*) jaw during tooth replacement (Fig. 8G-L) (Stage 32-33). Expression patterns of *pitx1* were consistent with those from a sagittal plane (Fig. 7A-C), with extensive expression throughout the inter-tooth epithelium of the SL (Fig. 8G-H).



Immunoreactivity of PCNA revealed high levels of generic proliferation throughout the dental epithelium, mesenchyme and surrounding SL. In accordance with sagittal expression profiles, *sox2* was extensively expressed in the lingual SL but absent from teeth, with *sox2*<sup>+</sup> cells extending into and terminating within the medial dental epithelium of the inter-tooth region (Fig. 8J-L). Overall, we observed conserved expression patterns of a dental gene set in the developing and regenerating skate dentition, considered to be widely important throughout dental development of diverse gnathostome clades.

*The enamel knot signalling centre likely directs flattened tooth shape in skates*

The typical ray (Batoidea) dentition is characterised by the development of flattened tooth cusps. Here in both *Leucoraja* and *Raja* (Rajidae) the developing dentition presents the characteristic flattened yet rounded teeth, however, these taxa also develop a small cusplet on the posterior aspect of the main cusp (Figure 1D, 2B, 9A). The early development of this enameloid cusplet appears to coincide with the posterior expression of a selection of genes (*fgf3*, *fgf4*, *fgf10*; Figure 9) and non-proliferation (with a lack PCNA protein expression; Figure 6A and C) known to be conserved and associated with the enamel knot (EK) signalling centre, important for regulating tooth shape in other vertebrates, including mammals<sup>61</sup>, fishes<sup>28</sup>, and sharks<sup>4</sup>. This posteriorly positioned cusplet in *Raja* and *Leucoraja* (Figure 1D) appears as a small point of enameloid associated with the development of the main flattened tooth, and the expression of EK genes during the development of this cusplet suggests that the EK signalling centre is present and highly conserved in all gnathostome teeth, regardless of cusp type. It is possible that the restricted EK-like structure (Figure 6, 7 and 9) in Rajidae is necessary to direct the development of the flattened cusp away (anterior) from the posterior cusplet. We consider this EK-like structure in *Raja* and *Leucoraja* to be

transient and of less influence than that observed in sharks<sup>4</sup>, where pointed, blade-like teeth form with multiple cusps (up to 7 cusps per tooth in *Scyliorhinus*).

Interestingly, the presence of a small cusplet on the rounded teeth of *Raja* and *Leucoraja* (Figure 1D; Figure 9A) may allow the dentition to transition during maturation and breeding in males, which can develop pointed, gripping-type cusps, reminiscent of the shark teeth. Whether an EK-like signalling centre is necessary for the development of the ‘cusp-less’ pavement-like teeth present in other members of the batoidea superorder (e.g. Myliobatiformes), is unknown, however, future comparative developmental studies will be required to fully appreciate the extent of EK signalling diversity.

## Discussion

*sox2 is a conserved marker of dental competence and regeneration throughout gnathostomes*

Our gene expression analysis shows that early stage skate and shark tooth development follow remarkably similar mechanisms, despite divergence in their adult form. Either dentition is derived from a restricted, infolded, tooth-competent oral epithelium (DL), defined by expression of *sox2*. The maintenance of tooth regenerative capacity in elasmobranchs has previously been shown to result from *sox2*<sup>+</sup> quiescent progenitor cell populations within the dental lamina<sup>4,13</sup>. *sox2* is likely a conserved vertebrate marker of tooth competence, initiation and regenerative capacity, throughout evolutionarily diverse gnathostomes.

*A conserved dental gene set controls development of the elasmobranch dentition*

Expression patterns of *pitx1*, *pitx2*, *shh*, *MK*, *bmp4*, *β-cat* and *lef1* throughout early development of the skate dentition are indicative of a conserved mechanism for tooth

development throughout diverse elasmobranch groups, and gnathostomes more generally. These data suggest that elasmobranchs utilise the same core dental gene set for the initial development and subsequent regeneration of their teeth over time. Some variations in gene expression patterns were noted, for example *pitx2* expression is epithelial in tooth development of most gnathostomes<sup>45,48</sup>, although in the shark and skate it is both epithelial and mesenchymal<sup>4</sup> (Fig. 7D-F). This may be an elasmobranch specific expression pattern, which has subsequently shifted in comparatively derived vertebrates. The relatively rapid and continuous nature of elasmobranch tooth regeneration may explain some shifts in the deployment of this conserved dental gene set.

*An enamel knot-like signalling centre may regulate diversity of the elasmobranch dentition*

Cusp development in elasmobranchs is highly variable. Within sharks, teeth lacking cusps are limited to a small number of genera, wherein all (e.g. *Mustelus*) or some (e.g. *Heterodontus*) of the teeth lack true cusps. Within batoids, a lack of cusps is widespread. Taxa lacking cusps or showing reduced cusps are present within all clades. Whether this diversity in tooth form is related to the capacity for continuous tooth regeneration in these groups remains unclear. It has been shown that morphology can shift from embryonic to adult form, with new phenotypes emerging over multiple rounds of tooth regeneration<sup>3</sup>. However, we are yet to understand the evolutionary triggers for such variation in cusp diversity. Within the Rajidae, some genera (e.g. *Leucoraja*) exhibit cusped teeth in either sex, although in many genera (e.g. *Raja*) cusped teeth are specific to mature males. The response of perpetual development and tooth regeneration to elevated levels of breeding-associated hormones remains unknown, although synchronization between changes in tooth morphology and breeding cycles has been recorded<sup>62</sup>. Varied tooth morphology is common in rays (Batoidea), for example the teeth of

plankton feeding rays (e.g. *Manta*, *Mobula* species) have simple cusps (and little else) despite their ancestors having flat teeth<sup>63</sup>. Whilst the earliest fossil batoid has cusped teeth, all other batoids from their first 70 million years of evolution had little or nothing in the way of a true cusp<sup>64</sup>.

The ray dentition typically consists of flattened interlocking teeth, together forming a crushing pad, whereas shark teeth are typically pointed, multi-cuspid units (Fig. 1)<sup>3,65</sup>. Gnathostome tooth cusp morphology is proposed to result from initial activation of the primary EK; a cell cluster expressing genes in a coordinated activation and inhibition pattern to direct surrounding cellular activity<sup>19,66</sup>. Evidence for the conservation of an EK-like signalling centre in elasmobranch dentitions remains inconclusive<sup>52</sup>, although in the shark, expression patterns of several markers including *shh*, *MK*, *fgf3*, *fgf10* and *lef1*, provide support for such a module<sup>4</sup>. Cell proliferation throughout the dental epithelium of first and second-generation skate teeth is interrupted by a transient region of non-proliferating cells (Figure 6A and C) and a small subset of inner dental epithelial cells that express *fgf* genes in a restricted pattern (Figure 9). This is in contrast to shark tooth development, which shows a marked reduction in cell proliferation in the medial epithelial tip of successive teeth (Rasch et al. 2016; Fig. 5J-L), linked with the EK-like structure expressing a collection of conserved genes (Figure 10). Perhaps a more restricted and more transient EK-like structure is necessary for the Rajid enameloid cusplet that is capable of further directing the complete development of the flattened cusp. Our developmental data indicate that shifts in the expression of a core dental gene set may explain the dramatic phenotypic diversity observed throughout elasmobranch tooth morphology.

In known ray clades (batoidea), as a character of their more flattened tooth morphology, shark-like pointed, multi-cuspid units are relatively rare. Given the extensive conservation of EK-related signalling throughout the dental development of diverse gnathostomes, this

variation in tooth morphology may be the result of modified gene expression patterns within such a signalling module. We propose that comparatively expanded gene expression and cell proliferation domains in the successive teeth of the skate may represent a shift in the influence of the EK-like signalling centre, compared to the shark (Fig. 5, 6)<sup>4</sup>, thereby contributing to variation in tooth cusp morphology (Figure 10). In the skate, the lack of an extensive reduction in PCNA immunoreactivity in the medial epithelial tip of the anlagen may be linked to the flattened molariform-like tooth morphology (Fig. 5A-C). Conversely, the concentrated PCNA reduction in the shark may contribute to the sharp final cusp morphology (Fig. 5L). This highlights the potential of the EK signalling centre as a broad driving force in the evolution of diverse gnathostome tooth morphologies.

#### *The link between elasmobranch tooth and taste bud development and regeneration*

Despite having a diverse range of tooth morphologies, the elasmobranch dentition exhibits conserved mechanisms of development and continuous regeneration<sup>3,4,65</sup>. The development of a permanent and continuous DL is essential for this process<sup>26,39,55,67</sup>. In the shark, expression of *sox2* is conserved within the odonto-gustatory band (OGB); the epithelial sheet competent to make the first generation of teeth on either jaw<sup>13</sup>. The OGB contains epithelial precursor cells from which both teeth and taste buds emerge in close proximity, on the jaw margins.

In the skate, this link between dental and oral epithelia is also apparent. The skate possesses an OGB comparable to that of the shark, marked in early stages of dental development by sustained expression of tooth-specific markers including *sox2*, *pitx1/2*, *shh*, *MK*, *bmp4* and  $\beta$ -*cat* (Figs. 4, 5, 6, 7). Expression of these genes within the skate tooth and prospective taste bud territories highlights the importance of the OGB for the development and regeneration of teeth, and the link between development of regenerative teeth and taste buds<sup>68</sup>.

### *Conclusion*

Overall, we show that despite their divergence in form<sup>3,65</sup>, the development and regeneration of the elasmobranch dentition is broadly conserved. We suggest *sox2* is important in initiating and maintaining the dental lamina and the continuous regeneration of teeth among elasmobranchs, alongside a conserved set of dental genes employed widely throughout tooth development of diverse gnathostomes<sup>4</sup>. Finally, alterations to the expression of genes comprising an EK-like signalling centre may explain the morphological diversity of teeth both within elasmobranchs and other gnathostomes.

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### Author contributions

LJR and GJF designed the study. LJR, RCL, CU, WAD, APT and GJF performed the data analyses. LJR, RLC, CU, WAD, APT and GJF wrote the manuscript. All authors edited and approved the final manuscript.

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## Figure legends

**Figure 1: Morphological diversity of the elasmobranch dentition.** MicroCT of the adult skate jaws (*R. clavata*) reveals a characteristic ray dentition (A), with interlocking rows of teeth forming a continuous crushing surface. The shark (*S. canicula*) upper jaw dentition (B) comprises a number of replacement tooth families separated into distinct files; at least four replacement teeth visible per file, forming an arc spanning both jaw parasymphyses, separated by a central toothless region. Individual teeth form pointed, multi-cuspid units, with functional rows becoming vertical and terminating at the jaw margin. Alizarin red staining

reveals the flattened morphology of mineralised skate teeth (C), (D) close-up of the *R. clavata* tooth with the small mineralised (enameloid) cusplet at the posterior side of the rounded cusp (arrowhead); and the pointed, multi-cuspid morphology of blade-like shark teeth (E-F). Shark teeth develop as a staggered series, and dermal denticles are visible across the ventral surface of the rostrum (E-F). Thin paraffin sections stained with DAPI in skates and sharks (Stage 31-32), reveal replacement teeth initiating from the successional lamina (SL), continuous with the dental lamina (DL); the epithelial-mesenchymal compartment (EP/MES) within which teeth develop (G-J). The maxillary valve (MV) is visible in the shark (I). Ray and shark silhouettes were obtained from [www.phylopic.org](http://www.phylopic.org). Scale bar lengths: A = 10 mm; B, E unscaled; C = 1 mm; D, F, H, J = 100  $\mu$ m; G, I = 200  $\mu$ m.

**Figure 2: MicroCT imaging of the developing Little Skate jaw.** MicroCT scanning of the little skate (*L. erinacea*; stage 33<sup>30</sup>) reveals rows of taste buds (A; green colour) lingual and oral to the first generation of developing teeth (blue colour; B). B, CT segmentation sections remove surface tissue to expose the first generation of teeth (blue). Virtual sections reveal mineralisation of these first-generation teeth (C, D). (C, D) unerupted first generation tooth just under the oral epithelial surface (arrowhead; blue), teeth contained within the epithelial dental lamina (magenta colour).

**Figure 3: *sox2* expression during early dental development in the little skate.**

Wholemout *ISH* was undertaken to track *sox2* expression throughout early dental development in the skate (*L. erinacea*). Ampullae of Lorenzini (AL) are visible prior to dental development, cover the ventral surface of the skate head (A-B). Two rows of distinct ampullae we observed beneath the lower jaw (B). At Stage 29, the odontogenic band (OB) is

visible in the upper jaw (C-D). Within the OB stronger units of *sox2* expression are now visible as the epithelial thickenings, which give rise to individual teeth (Stage 28-29) (panel E;T), begin to form. First (T1) and second (T2)-generation are visible (F). The ray silhouette was obtained from [www.phylopic.org](http://www.phylopic.org). Scale bar lengths: A, C, E = 1000  $\mu\text{m}$ ; B, D, F = 300  $\mu\text{m}$ .

**Figure 4: *sox2* defines dental competence in the skate and shark.** Section ISH (AP, pseudo-coloured pink, DAPI counterstain) and IHC (DAB, methyl green counterstain) revealed localised epithelial (Ep) *sox2* expression in dorso-ventrally opposing epithelial regions of the upper and lower skate (*R. clavata*) jaws, prior to the formation of the DL (A-F) (Stage 28-29). Expression was not observed in the mesenchyme (Mes). Ampullae of Lorenzini (AL) also expressed *sox2* (A). ISH for *sox2* in the shark (*S. stellaris*) jaw revealed a similar pattern of localised expression (G-I), implying a conserved role of *sox2* in the initiation of elasmobranch dental development. Ray and shark silhouettes were obtained from [www.phylopic.org](http://www.phylopic.org). Scale bar lengths: A, D, G = 1 mm; B – C, E – F, H – I = 100  $\mu\text{m}$ .

**Figure 5: PCNA and *sox2* expression during early tooth development of the skate and shark.** IHC for PCNA (DAB, methyl green counterstain) demarcates the infolding dental lamina (A-C) (Stage 31-32) and associated cartilages (CA) in the upper and lower jaws of the skate (*R. clavata*). The PCNA+ dental epithelium is continuous with vestigial first and second-generation teeth (T1, T2) (B-C). *sox2* expression from ISH (AP, pseudo-coloured Magenta, DAPI counterstain, grey) and IHC (pseudo-coloured green, DAPI counterstain, grey) patterns further define epithelial regions associated with the dental lamina (D-I; for comparison H is from Martin et al. 2016). In either jaw, two sets of early-stage teeth are



visible (T1, T2). In the upper jaw (E), *sox2* expression terminates lingually to T2. Further clustering of Sox2<sup>+</sup> cells at the oral dental junction (F, arrowhead) may be a stem cell niche, required to maintain continuous tooth regeneration as observed in the shark. The shark jaw (*S. canicula*) is morphologically distinct from the skate, as it has a maxillary valve (MV) (J). Along the ventral surface of the MV, PCNA immunoreactivity marks cell proliferation in the outer oral epithelium and developing taste buds (J). Similar to the skate, cell proliferation is largely uniform throughout the DL (K). However, in developing shark teeth, we observed a distinct PCNA-negative medial region of the tooth epithelium (L, arrowhead). In mammalian teeth, this non-proliferative cell cluster is termed the enamel-knot (EK)<sup>20</sup>. An EK-like signalling centre may be present during shark tooth development (J-L; for comparison K and L from Rasch *et al.* 2016) but was not observed by DAB-PCNA immunoreactivity during skate tooth development (A-C); we do however see a transient reduction of PCNA at the posterior cusplet region with immunofluorescence (Figure 6C). Ray and shark silhouettes were obtained from [www.phylopic.org](http://www.phylopic.org). Scale bar lengths: A, D, G, J=200  $\mu\text{m}$ ; B – C, E – F, H – I, K – L=100  $\mu\text{m}$ .

**Figure 6: Immunofluorescence of Sox2 and PCNA during tooth and dental lamina development and later-stage tooth regeneration in the little skate (*L. erinacea*).**

Immunofluorescence of Sox2 (magenta) was observed in the sub-surface epithelial layer (red arrowheads) linking presumptive taste fields with the progressive development of the first two generations of flattened teeth within the invaginating dental lamina (stage 32<sup>29,31</sup>) (A-B). (C) A slight reduction in PCNA (green) expression was observed at the polarized apical region of the inner dental epithelia (white arrow), equivalent to the shark enameloid knot<sup>4</sup>. At the hatching stage (stage 34<sup>29,31</sup>), developing tooth generations have a more flattened appearance, with a slightly pointed enameloid cusplet polarised to the posterior aspect of the

tooth (in sagittal section) (D-F). Sox2 continues to be expressed but is restricted to the successional lamina associated with the emergence of new tooth generations (white arrowheads) and the sub-surface pocket at the dental/oral junction linking the dental lamina with the taste field (red arrowheads). PCNA labels generic proliferative cells of the jaws and dentition, with the dental lamina and the developing teeth showing higher levels of proliferation. DAPI staining is shown in blue. Scale bars are 60  $\mu\text{m}$  in length. MC=Meckel's cartilage.

**Figure 7: A conserved dental gene set governs skate tooth development.** ISH (AP, pseudo-coloured pink, DAPI counterstain) during first and second-generation tooth (T1, T2) development was undertaken in the upper and lower jaws of the thornback skate (*R. clavata*) (Stage 31-32). *pitx1* is expressed within a restricted region of inner oral epithelium in either jaw, continuous with the dental epithelium and SL (A-C, arrowheads). Expression is absent within the anlagen and surrounding mesenchymal tissue. Expression of *pitx2* was similar, although additional expression was observed in the dental mesenchyme underlying developing teeth (T1, T2) (D-F). *shh* expression was observed in the medial dental epithelium of the tooth in the lower jaw (G, I), and within a restricted region of the inner oral epithelium (H). *MK* expression localises to specific regions of the DL in either jaw, including the medial tooth epithelium, dental epithelium and SL (J-L, arrowheads). Expression of *MK* within the anlagen corresponds to *shh* expression, potentially demarking an EK-like signalling centre. *bmp4* is expressed within the epithelium and mesenchyme in either jaw (M-O). Expression was noted in the inner oral mesenchyme underlying developing teeth (T1, T2). Overall, these results suggest that a conserved dental gene set underlie development of the skate dentition. Scale bar lengths: A, D, G, J, M = 200  $\mu\text{m}$ ; B – C, E – F, H – I, K – L, N – O = 100  $\mu\text{m}$ .

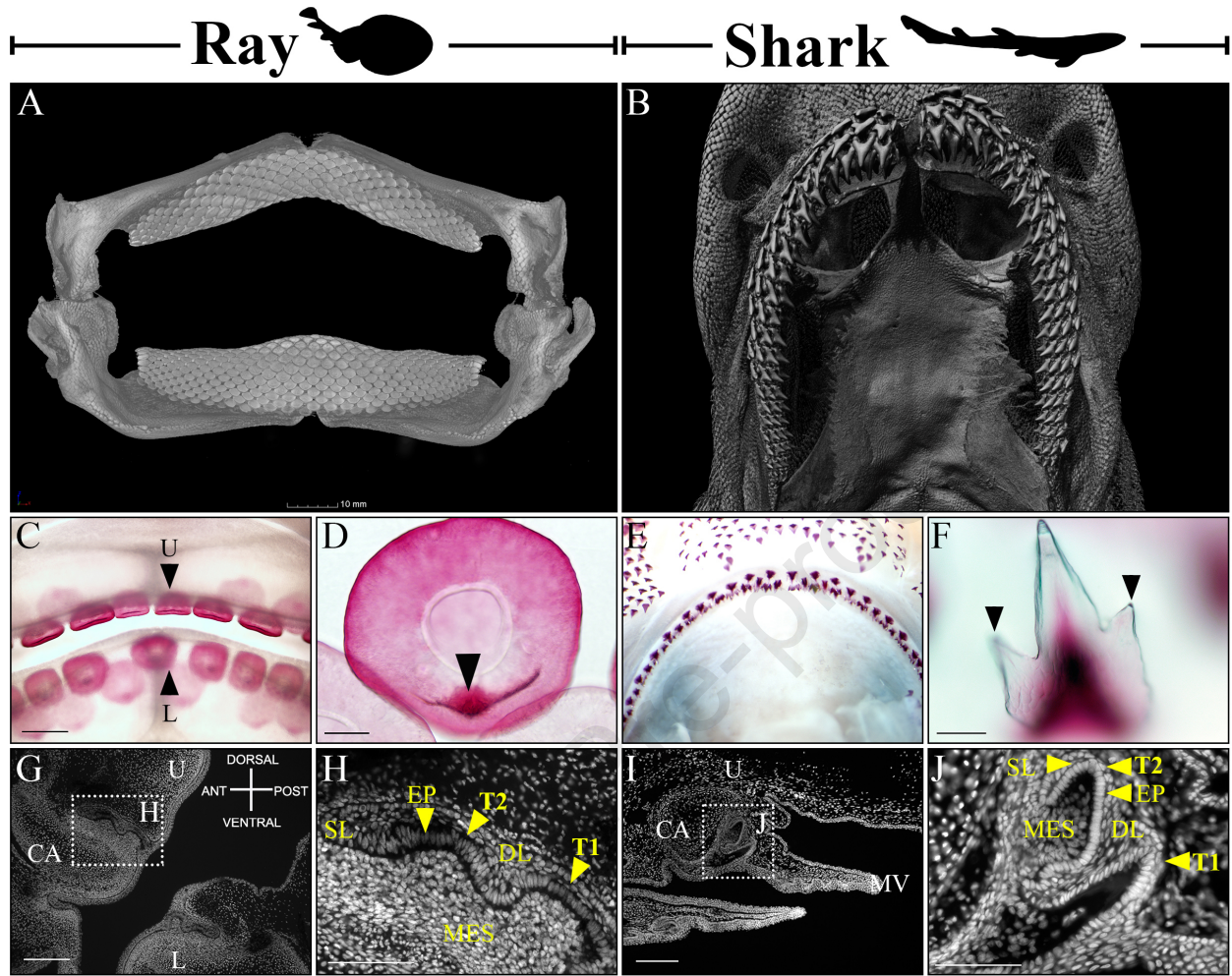
**Figure 8: A conserved dental gene set governs skate tooth development (continued).** The canonical Wnt pathway-associated markers, *β-cat* and *lef1*, were strongly expressed in the oral epithelium of either jaw of the thornback skate (*R. clavata*) (A-F) (Stage 31-32). Localised expression was observed in the epithelium of developing first and second-generation teeth (T1, T2). *β-cat* was observed in the inner oral epithelium, continuous with the DL and SL (A-C), whereas *lef1* expression was primarily restricted to developing teeth (D-F). Coronal sections of the developing little skate (*L. erinacea*) jaw were also used for ISH and IHC (G-K) (Stage 32-33). *pitx1* was expressed throughout the dental epithelium but absent from the inner dental epithelium and papillary dental mesenchyme (G, H). PCNA immunoreactivity revealed a highly proliferative dental epithelium (I). ISH for *sox2* revealed expression in either jaw, with stronger expression towards the SL (J-K; for comparison K is from Martin *et al.* 2016). This corresponded to Sox2 IHC (L). Overall, these expression patterns bear notable similarity to those of the shark, suggesting conservation of elasmobranch dental development<sup>4,13</sup>. Scale bar lengths: A, D = 200 μm; B – C, E – F, H – I, K – L = 100 μm; G, J = 1 mm.

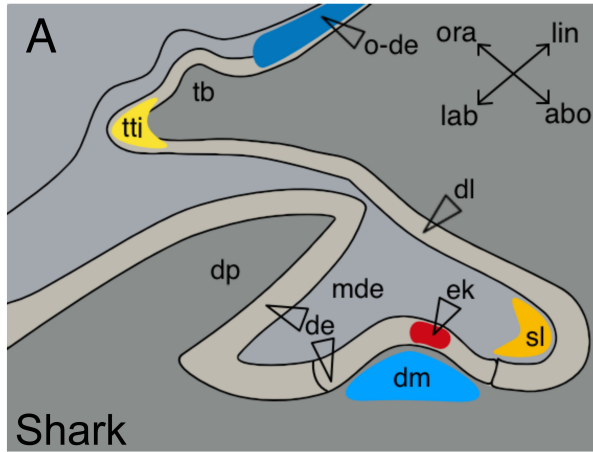
**Figure 9: Restricted *Fgf* gene expression highlights a potential EK-like structure that corresponds to the posterior enameloid cusplet in the little skate (*L. erinacea*).** (A)

Trichrome stained histological section (St. 34) showing the early stages of new tooth formation within the successional lamina (arrowhead; equivalent position of possible EK-like structure shown by *fgf* gene expression in B-D). The predecessor tooth shows the developing enameloid cusplet on the posterior aspect of the flattened cusp (arrow). (B) *fgf4* expression (Stage 33) localised to the restricted presumptive EK-like cells of the inner dental epithelium (IDE; arrowhead). Expression also observed in the mesenchymal dental papilla. (C) *fgf3* expression is similarly expressed in the EK-like cells within the IDE of the first-generation

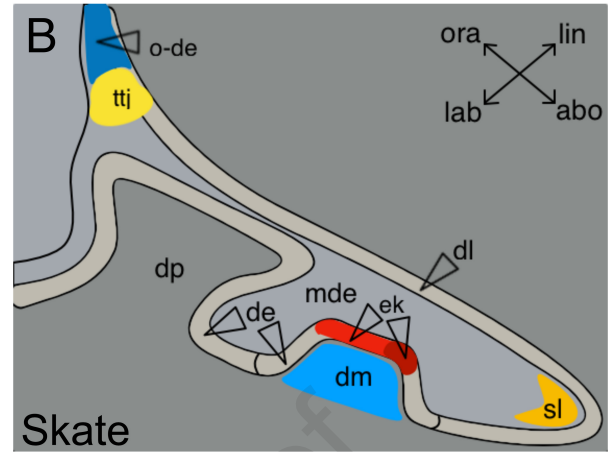
tooth (arrowhead; St. 32). (D) *fgf10* shows a more restricted EK-like pattern, again associated with the same posteriorly located cell cluster (arrowhead). Scale bar in A and B=50 $\mu$ m. B-D same magnification.

**Figure 10. Schematic diagram comparing the early generations of the shark and skate dentition.** (A, B) scheme based on sagittal thin sections (stage 31-32) through the dental lamina (dl) of the shark (A; based on *S. canicula*) and skate (B; based on *L. erinacea*) showing the developing first two tooth generations, the progenitor niches (ttj and sl, yellow), and the EK-like signalling centres (red). The dental lamina in skates (B) further extends into the underlying mesenchyme permitting development of new flattened teeth. Expanded IDE (EK; light red) expresses genes known to be conserved in EK-like structures (i.e. *shh* and *lef1*), however, a more restricted cell cluster (dark red) could reveal the true EK-like signalling centre in the skate expressing *fgf* genes, associated with development of a small enameloid cusplet (Figure 9). Key shows the known gene co-expression clusters. de, dental epithelium; dm, dental mesenchyme; ek, enamel knot-like structure; mde, middle dental epithelium; o-de, oral epithelium; sl, successional lamina; ttj, tooth-taste junction; tb, taste bud; dp, dental papilla; Orientation axis: ora, oral; abo, aboral; lab, labial; lin, lingual.

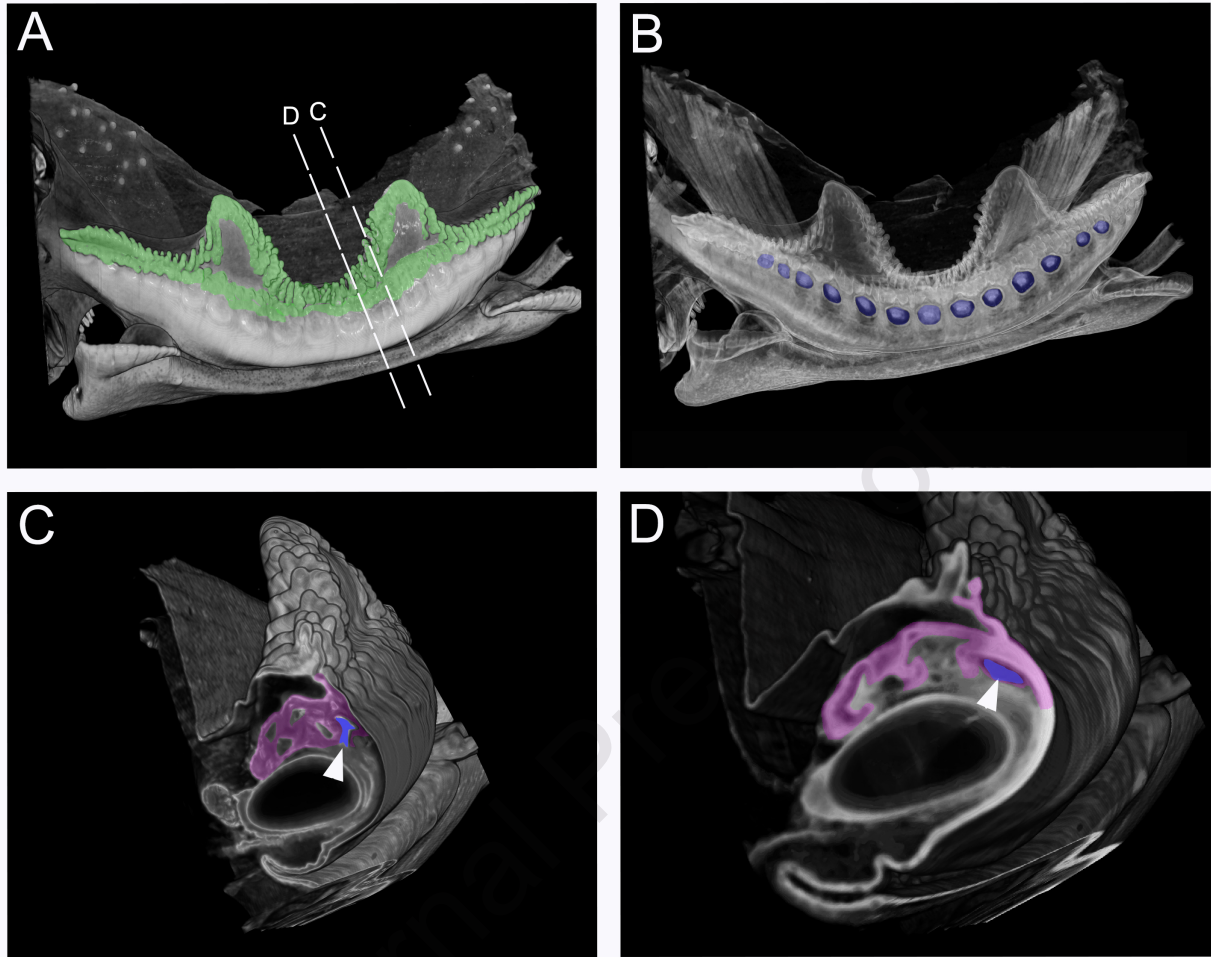


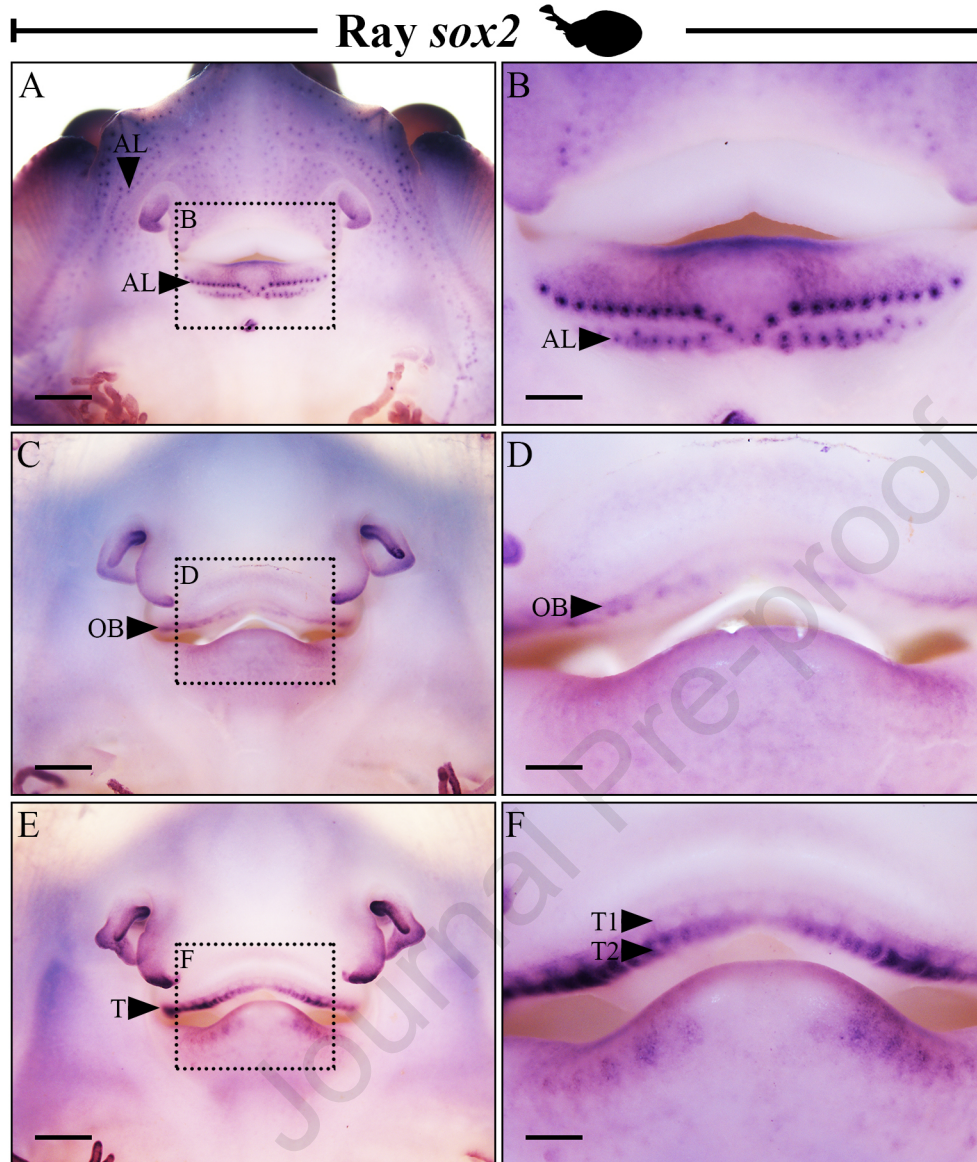


de	<i>β-cat; lef1; pitx1; pitx2; ptc2; bmp4</i>
dm	<i>pitx2; bmp4; β-cat; lef1; ptc2; fgf3; bmp4; MK</i>
ek	<i>shh; ptc2; MK; fgf3; fgf10; bmp4; lef1</i>
mde	<i>pitx1; pitx2</i>
o-de	<i>sox2; pitx1; shh; MK; bmp4; β-cat</i>
sl	<i>sox2; pitx1; pitx2; MK; bmp4; β-cat</i>
ttj	<i>sox2; pitx1; pitx2; bmp4; β-cat</i>

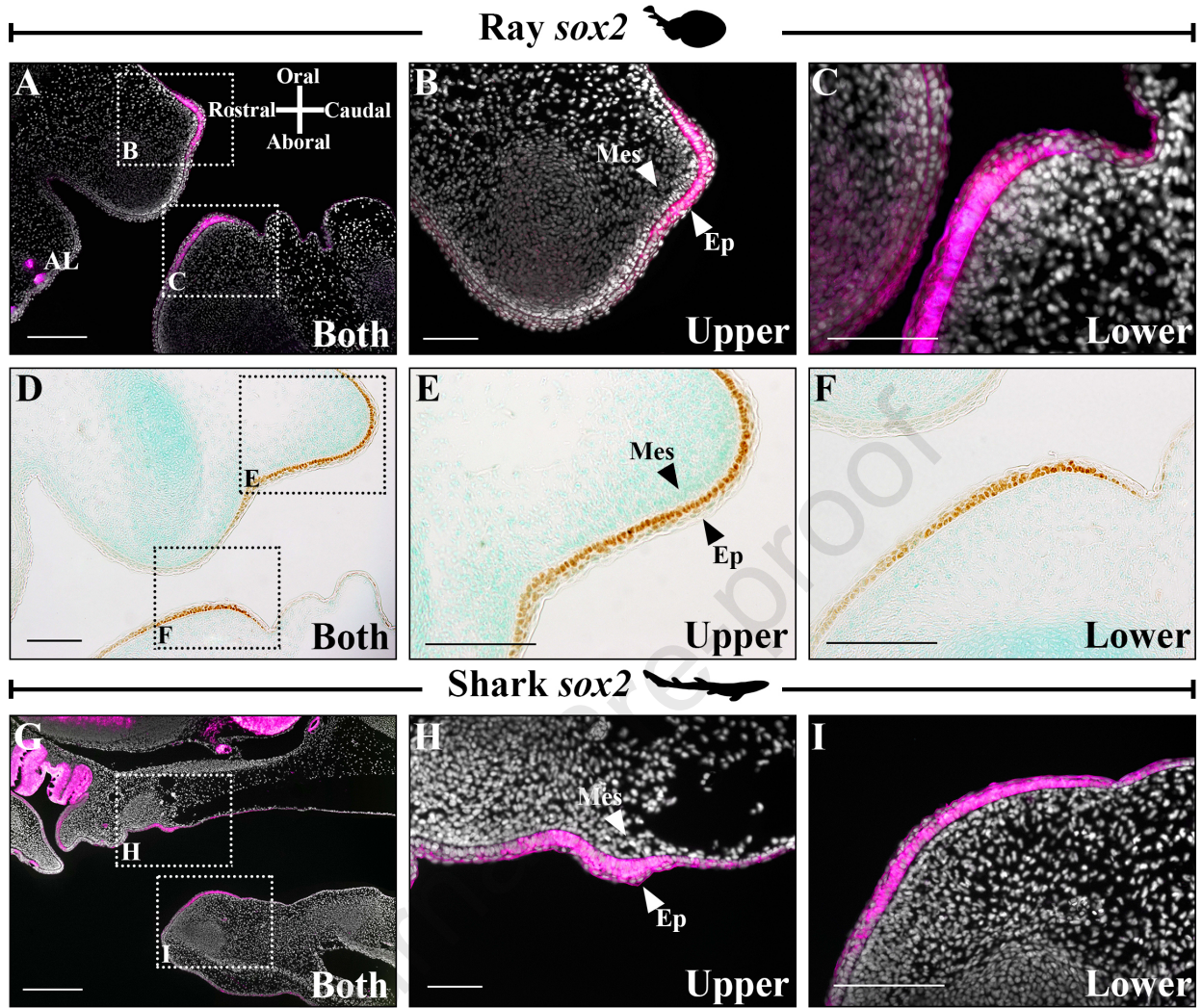


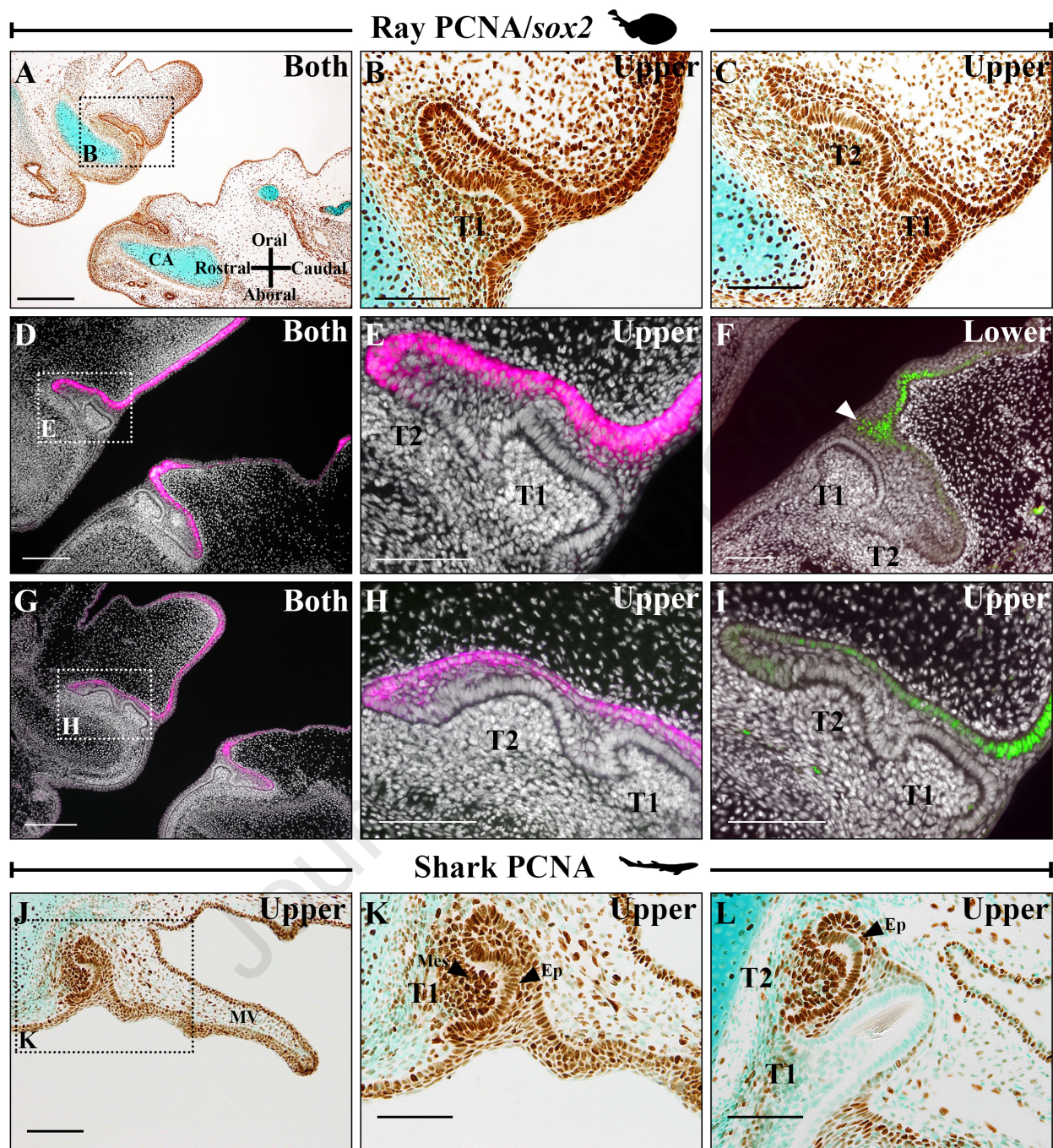
de	<i>β-cat; lef1</i>
dm	<i>pitx2; bmp4; β-cat; lef1</i>
ek	<i>fgf3; fgf4; fgf10; MK; shh; lef1</i>
mde	<i>pitx1; pitx2</i>
o-de	<i>sox2; pitx1; shh; MK; bmp4; β-cat</i>
sl	<i>sox2; pitx1; pitx2; MK; bmp4; β-cat</i>
ttj	<i>sox2; pitx1; pitx2; bmp4</i>

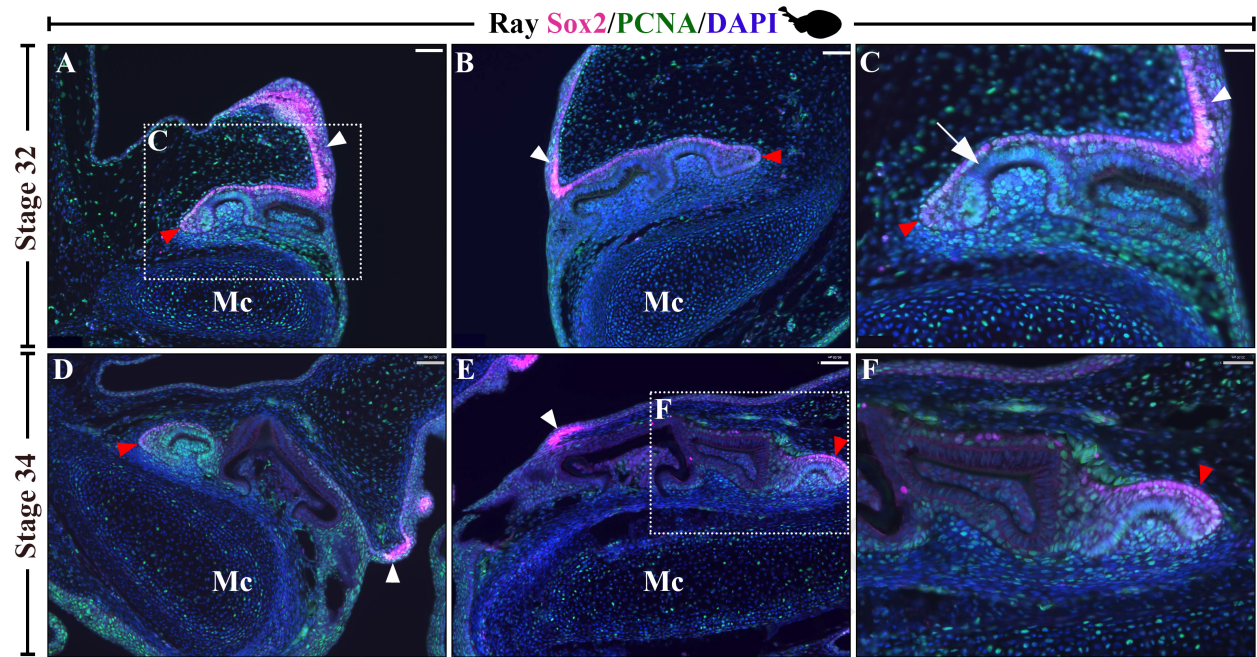


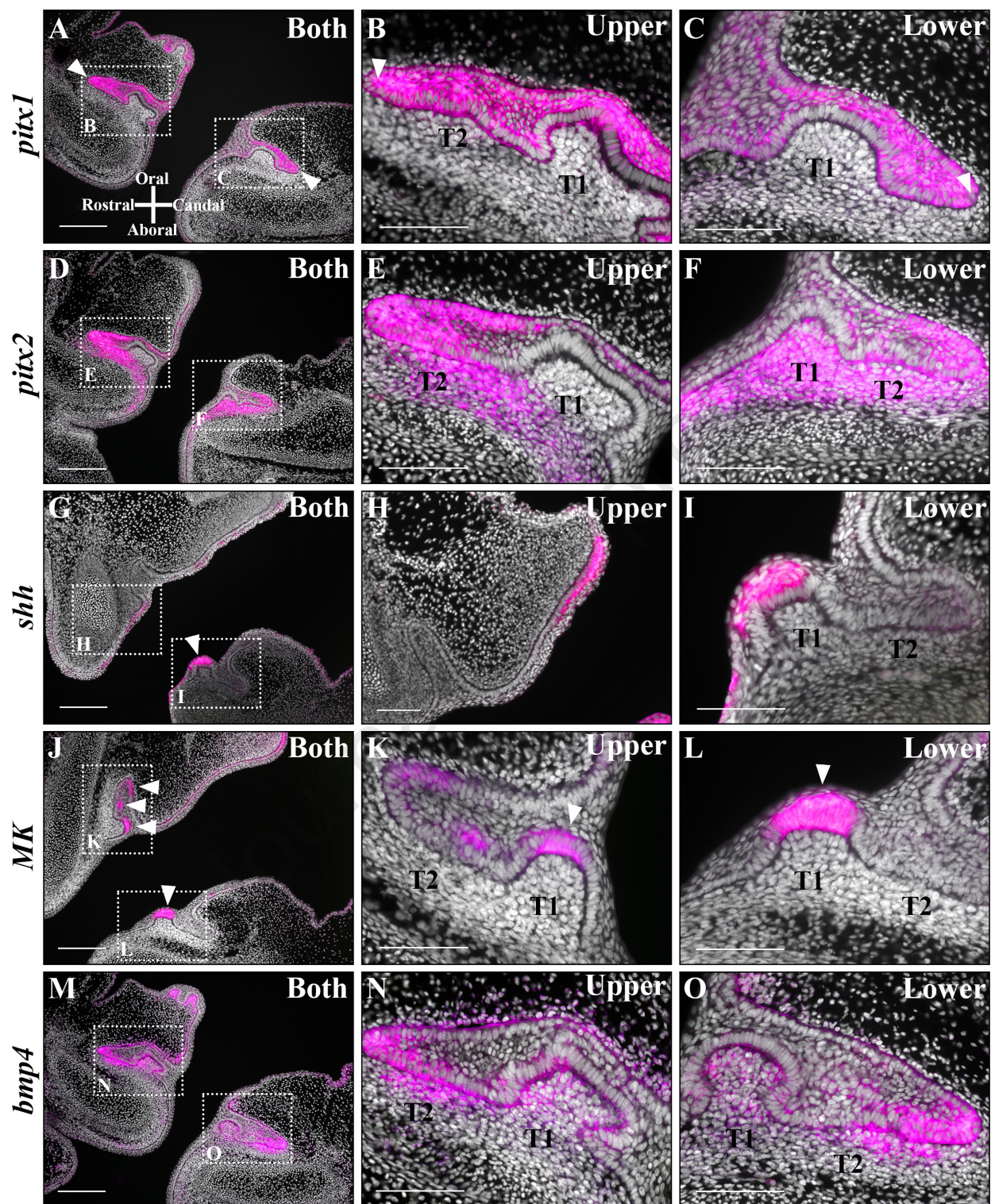


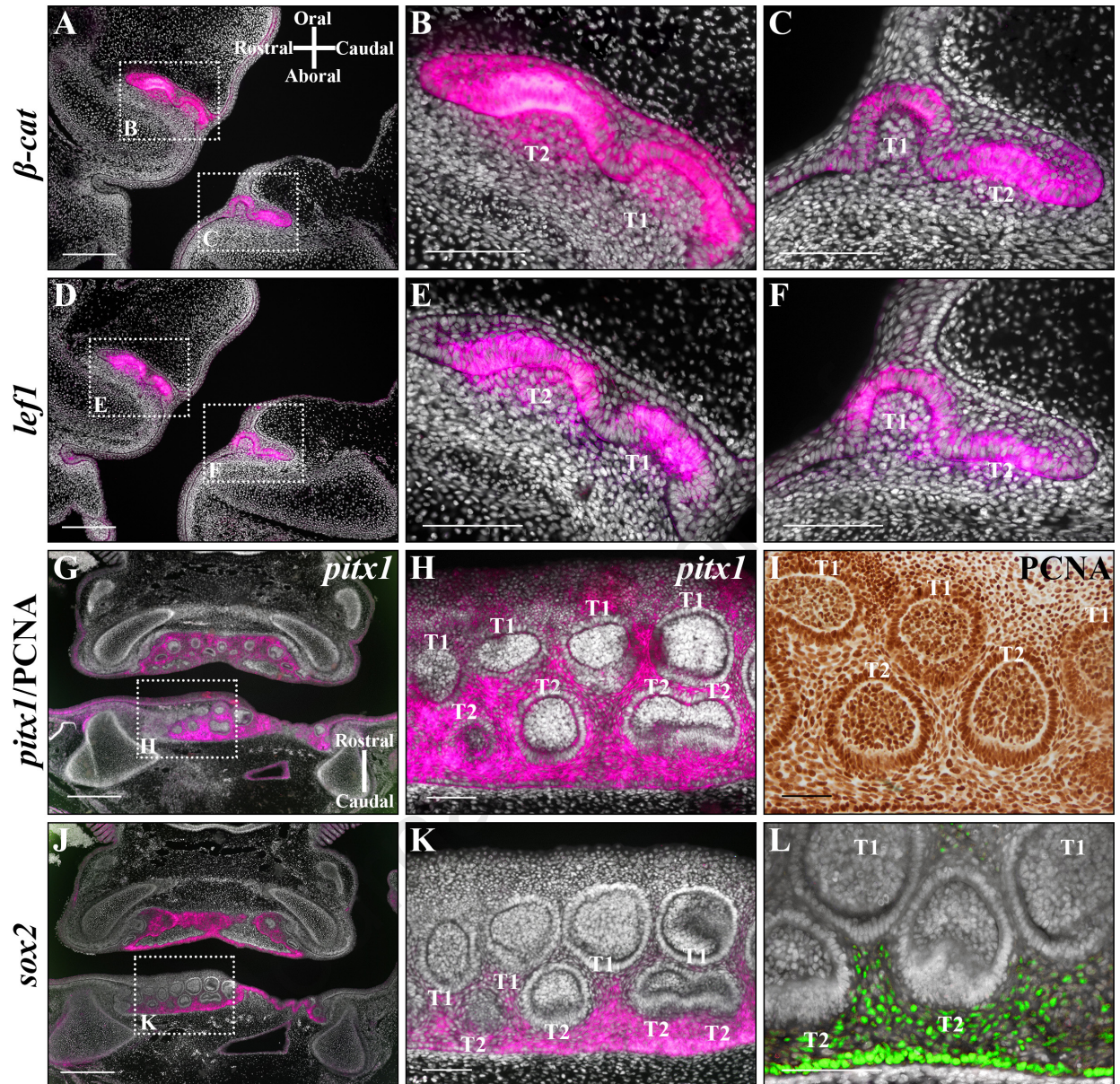


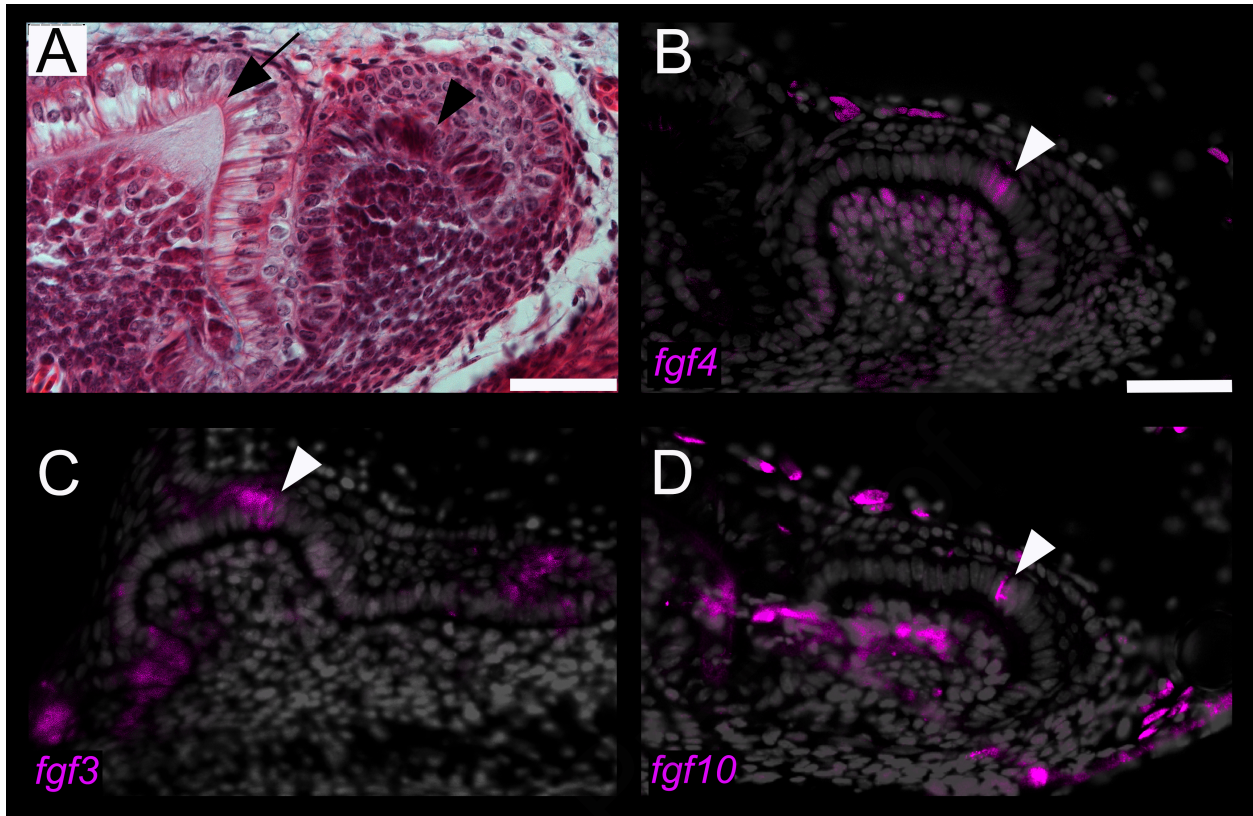












## **Highlights**

### **Rasch et al. Development and regeneration of the crushing dentition in skates (Rajidae)**

Crushing teeth in rays show highly conserved development to other vertebrate teeth

Like sharks, skates have a permanent dental lamina for continuous dental regeneration

Flattened skate tooth cusps develop with reduced yet conserved enamel knot signaling

Diversity of elasmobranchs tooth type is linked to shifts in enamel knot signaling