Dev Genes Evol (2004) 214: 466–472
DOI 10.1007/s00427-004-0426-0

SHORT COMMUNICATION

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Analysis of *EphA4* in the lesser spotted catshark identifies a primitive gnathostome expression pattern and reveals co-option during evolution of shark-specific morphology

Received: 14 May 2004 / Accepted: 30 June 2004 / Published online: 6 August 2004

Abstract The Eph family is the largest known group of structurally related receptor tyrosine kinases (RTKs). Each Eph receptor has a specific Ephrin ligand, and these function to define spatial boundaries during development. Analyses of *EphA4* in mouse, chick, frog and zebrafish embryos have implicated this gene in a number of developmental processes, including maintenance of segmental boundaries, axon guidance, limb development, neural crest migration and patterning of the ear. In order to determine which components of *EphA4* function may be primitive for gnathostomes, we cloned *EphA4* from the lesser spotted catshark (*Scyliorhinus canicula*) and examined its expression pattern during shark embryonic development. Consistent with the patterns reported for bony fish and tetrapods, we observed segmental expression of *EphA4* in the developing hindbrain and later in the pharyngeal arches of shark embryos. *EphA4* was also detected during sensory organogenesis, in the developing ear, eye, nasal pits and lateral line. A dynamic pattern of *EphA4* expression occurs during shark fin development, suggesting an early role in outgrowth and patterning of the fin buds and a later role in tissue differentiation. We also observed several novel domains of *EphA4* expression that have not been reported in other vertebrates, including external gill buds, dermal denticles, median fins and claspers. While some of these domains may reflect co-option of *EphA4* expression to novel sites for development of shark-specific characters, others are more likely to be ancestral patterns of expression that were lost in other vertebrate lineages.

Keywords *EphA4* · Shark embryo · Hindbrain · Fin development · Lateral line

Edited by R. P. Elinson

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Materials and methods

PCR cloning and sequence analysis

Degenerate primers were designed from highly conserved motifs of the tyrosine kinase catalytic domain (sense sequence MIITEYM; antisense sequence SDVWSFG) and used in standard RT-PCR reactions to amplify fragments using RNA from a stage 28 *Scyliorhinus canicula* (Ballard et al. 1993). A 25-μl PCR reaction was performed using 100 pmol/μl sense and antisense primers and an annealing temperature of 51°C. The amplified fragments were cloned into a PGEM-T Easy Vector (Promega) and sequenced in both directions. The identity of the cloned fragment was determined by BLAST (NCBI) searches and protein alignments with related vertebrate sequences using Clustal X. Maximum likelihood phylogenetic trees were constructed using Tree-Puzzle software. The sequence has been submitted to GenBank (accession number AY667434).

Whole-mount in situ hybridization

The expression pattern of *EphA4* in *S. canicula* was detected by whole-mount in situ hybridization, from stage 18 to stage 33 (Ballard et al. 1993), using the method of Nieto et al. (1996) with the following modifications. The proteinase K step was replaced at early stages (< st. 29) by treatment with 10% TritonX-100 in dimethyl sulfoxide and methanol (1:1). Levamisol (2 mM) was added to both KTBT and NTMT washes. The pre-hybridization and hybridization was performed at 70°C. For the color

reaction, 10% dimethyl formamide (DMF) was added to maintain NBT and BCIP in solution.

Cryosections of whole-mount embryos

Following whole-mount in situ hybridization, embryos were equilibrated in 30% sucrose in phosphate buffered saline (PBS) and embedded in 20% gelatine at 50°C overnight. The specimens were frozen in liquid nitrogen and mounted in Tissue-Tek O.C.T. for cryosectioning. Sections of 35 μm were cut and mounted onto gelatinized slides.

Whole-mount immunochemistry

Embryos were washed in PBS with 1% triton (PBT-1) for 3 h, incubated in 0.25% trypsin for 2–5 min and immersed in pre-cooled acetone. After being rinsed in PBT-1, the specimens were placed in 10% goat serum (GS), 1% dimethyl sulfoxide and 5% H_2O_2 in PBT-1, overnight. The 3A10 and the HNK1 antibodies were used at concentrations of 1:500 and 1:70, respectively, in PBT-1 containing 10% GS. Peroxidase-conjugated secondary antibodies were used at a concentration of 1:500 in PBT-1 containing 1% GS. Embryos were then washed in 1% GS in PBT-1 followed by PBS before being incubated in 0.5 mg/ml diaminobenzidine (DAB). Reaction was developed by transferring embryos to fresh DAB activated with 0.003% H_2O_2.

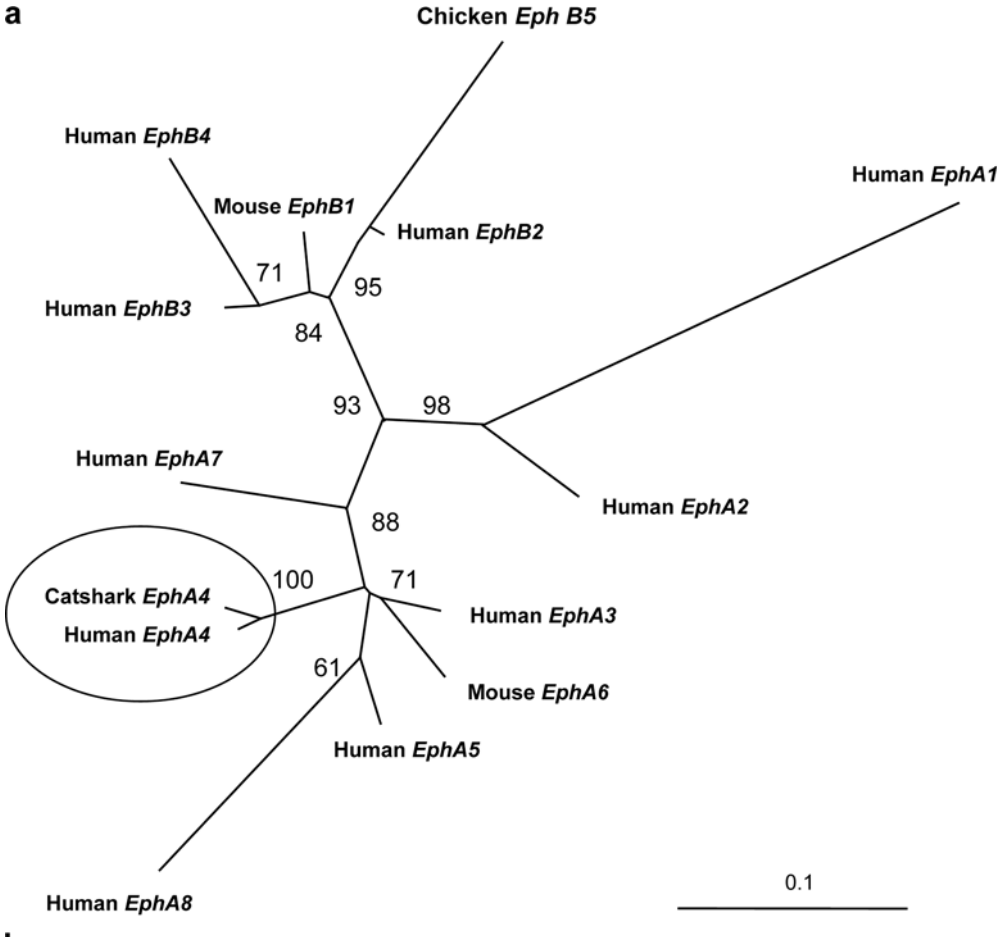

Fig. 1a, b Amino acid sequence analyses of *Scyliorhinus canicula EphA4*. **a** Maximum likelihood radial phylogram comparing the deduced amino acid sequence of the catshark *EphA4* fragment with representatives of the tetrapod Eph family. The degree of similarity is indicated by *branch length*, and support values are indicated for each branch. Note orthology of catshark *EphA4* and human *EphA4* (*circled*). **b** Alignment of catshark and tetrapod *EphA4* amino acid sequences used to generate tree shown in **a**. Species names are indicated in the *left column*; *Sc EphA4 S. canicula*, accession number AY667434; *Hs EphA4 Homo sapiens*, accession number NP004429; *Mm EphA4 Mus musculus*, accession number AAH04782; *Gg EphA4 Gallus gallus*, accession number CAA79509. *Single dots* represent residues identical to catshark *EphA4*. The conserved tyrosine motif is *shadowed*, corresponding to the characteristic autophosphorylation site in tyrosine kinase receptors

Scanning electron microscopy

Specimens were fixed in 1% glutaraldehyde in PBS. After post-fixation in 1% osmium tetroxide, specimens were dehydrated in a graded ethanol series and transferred to acetone. Specimens were then critical-point dried, mounted onto carbon discs and sputter-coated with gold particles.

Results and discussion

Cloning and sequencing of *EphA4* in *S. canicula*

A 348-bp fragment of the catshark (*S. canicula*) was amplified by degenerate RT-PCR. A BLAST search indicated that the deduced amino acid sequence was most closely related to *EphA4* from tetrapods (97% identity with *Xenopus*, chicken, mouse and human

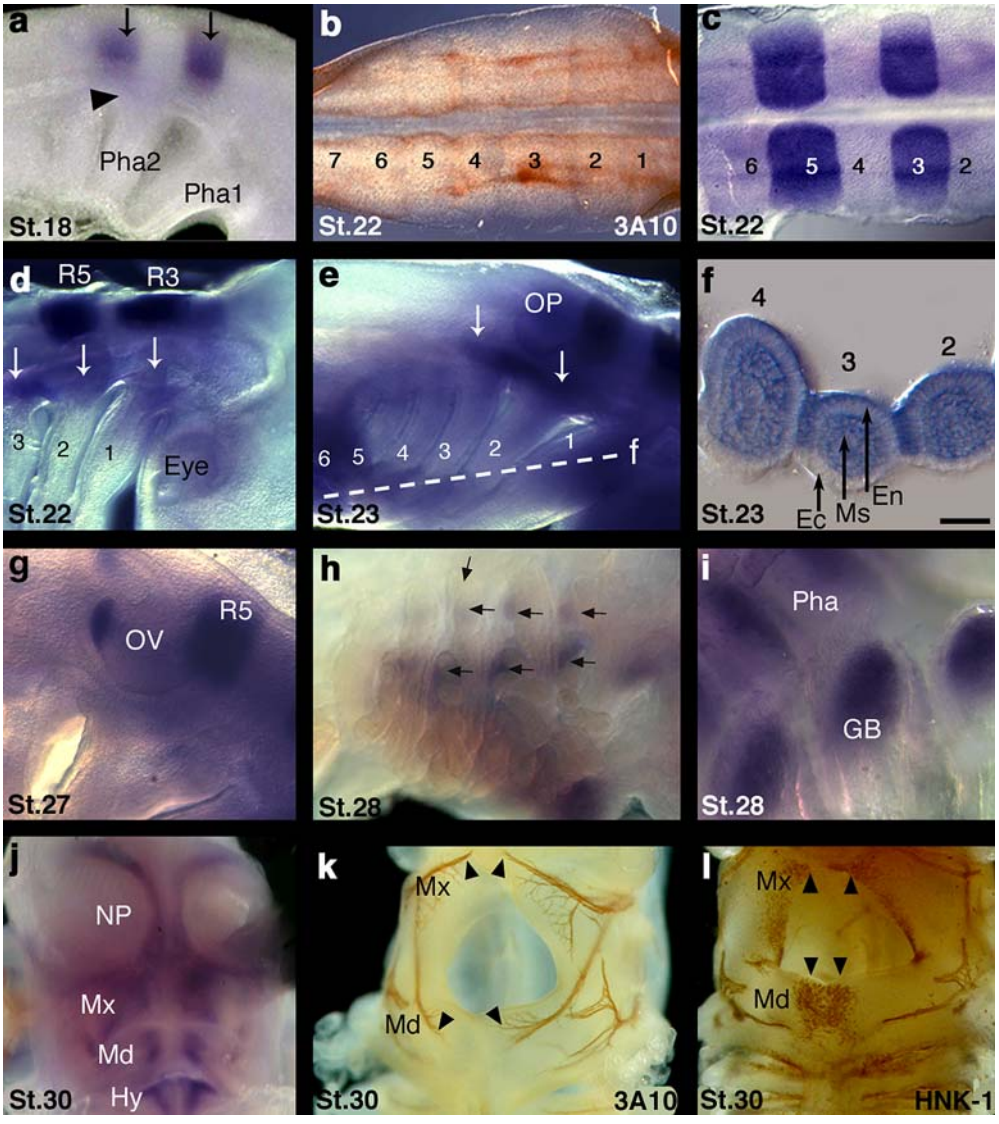

Fig. 2a–l *EphA4* expression during shark brain and craniofacial development. Stage of development is indicated in *lower left corners*. Anterior is to *right* in **a–i**, and to *top* in **j–l**. **a** Lateral view of segmental *EphA4* expression in the rhombencephalon (*arrows*). *Arrowhead* marks expression at dorsal edge of second pharyngeal arch (*Pha*). **b** Immunolocalization with 3A10 antibody highlighting rhombomere organization within the hindbrain. Hindbrain is flat-mounted and the right half of each rhombomere is *numbered*. **c** *EphA4* expression in rhombomeres 3 and 5 (compare with **b**). **d** *EphA4* expression in rhombomeres (*R*) 3 and 5, eye, and dorsal aspect of the anterior pharyngeal arches (*arrows*). **e** *EphA4* expression extending into the pharyngeal arches. *Arrows* point to the dorsal expression in pharyngeal arches 2 and 3. Note *EphA4* expression in the otic placode (*OP*). *Broken line* indicates plane of section shown in **f**. **f** Coronal section through pharyngeal arches 2–4 showing *EphA4* expression in the pharyngeal arch mesenchyme (*Ms*) and endoderm (*En*). Note absence of signal in overlying ectoderm (*Ec*). *Scale bar* 200 μm. **g** *EphA4* expression in the posterior region of the otic vesicle (*OV*). **h** *EphA4* expression in the internal walls of the pharyngeal arches (*arrows*). **i** Detail of the *EphA4* expression in the gill buds (*GB*) and internal walls of the pharyngeal arches (*Pha*). **j** *EphA4* expression surrounding the nasal pits (*NP*) and at the ventral tips of the maxillary (*Mx*), mandibular (*Md*) and hyoid arches (*Hy*). **k** Immunolocalization with 3A10 antibody shows the innervation of the jaws (*arrowheads* mark growth cones). **l** Immunolocalization of HNK1 in the shark face. Note expression at the ventral tips of the fusing jaws (*arrowheads*)

EphA4). Maximum likelihood phylogenetic analysis confirmed the orthology of the catshark fragment and human *EphA4* (Fig. 1a). Sequence comparisons revealed only three substitutions in the shark *EphA4* over 116 amino acids of the catalytic domain (Fig. 1b). We also detected a conserved tyrosine motif that corresponds to an autophosphorylation site (Fig. 1b), which has been shown to be involved in the recruitment of downstream cytoplasmic signaling molecules following ligand binding (Bovenkamp et al. 1997).

EphA4 expression during hindbrain segmentation and pharyngeal arch development

Development of the central nervous system in vertebrates involves the anteroposterior subdivision of the embryonic hindbrain into a series of segmental bulges known as rhombomeres (*R*). During catshark development we detected *EphA4* expression in two stripes within the shark rhombencephalon at stage 18 (Fig. 2a). Immunolocalization of the neurofilament protein 3A10 confirmed the identity of the rhombomeres expressing *EphA4* as r3 and r5 (compare Fig. 2b, c). By stage 22, lower levels of *EphA4* expression were also detected in r2, r4 and r6 (Fig. 2c), similar to the expression pattern reported for the tetrapod hindbrain (Hirano et al. 1998). In vertebrates, *EphA4* is involved in the segmental restriction of cells within the embryonic hindbrain (Xu et al. 1999). The observation of strong *EphA4* expression in shark rhombomeres 3 and 5 suggests that the role of *EphA4* signaling in hindbrain segmentation evolved prior to the gnathostome radiation. During shark hindbrain development, we also observed a domain of *EphA4* expression approaching the second pharyngeal arch, ventral to rhombomere 5, as early as stage 18 (Fig. 2a). *EphA4* transcripts were then detected in streams of expression that extended ventrally from the rhombomeres into the dorsal aspect of the three most anterior pharyngeal arches at stage 22 (Fig. 2d). The *EphA4* expression domain continued to spread ventrally throughout the pharyngeal arches, with the strongest expression remaining at the dorsal edge of pharyngeal arches 2 and 3 (Fig. 2e). Histological analysis revealed that *EphA4* transcripts were present in pharyngeal arch mesenchyme and endoderm, but could not be detected in overlying ectoderm (Fig. 2f). Interestingly, at later stages of development (st. 28), we observed narrow domains of *EphA4* expression adjacent to the gill clefts and in the emerging gill buds (Fig. 2h, i). Eph signaling is involved in the control of axonal pathfinding and neural crest migration from the hindbrain into the pharyngeal arches (Robinson et al. 1997; Küry et al. 2000). Our observations suggest that *EphA4* has a conserved role during the development of these tissues in sharks, and raise the possibility of a novel role for Eph signaling in gill development.

EphA4 expression during ear development

Recent work has implicated Eph-Ephrin signaling in vertebrate inner ear development (Pickles et al. 2002). We detected *EphA4* expression in the otic placode from the earliest stages of ear development in the catshark (Fig. 2e). Expression persisted during ear development, and became restricted to the most posterior half of the invaginating otic vesicles by stage 27 (Fig. 2g). In zebrafish, fate maps show that the posterolateral component of the otic vesicle, which also expresses *bmp2*, *bmp4*, and *msxC*, contributes to the posterior cristae, where the sensory receptors reside (Mowbray et al. 2001). Thus, *EphA4* localizes to sites of cristae development in the developing ears of both fishes and tetrapods. Taken together, these data suggest a conserved mechanism of inner ear formation in vertebrates.

EphA4 expression during craniofacial development

During shark craniofacial development, *EphA4* expression was detected surrounding the optic and nasal placodes as early as stage 22, and expression persisted around the developing eyes and nasal pits (Fig. 2d, j). Within the pharyngeal arches, *EphA4* became restricted to the most ventral or distal tips of the maxilary, mandibular and hyoid arches, where they fuse to form the ventral midline of the face (Fig. 2j). Interestingly, the growth cones of the trigeminal nerve branches appeared to migrate towards the *EphA4* expression domains (compare Fig. 2j, k), while cells positive for HNK1, a neural crest marker, clustered within the *EphA4* domains at the points of midline fusion (Fig. 2l). In chick embryos, the expression of Ephs and Ephrins at the distal tips of the mandibular arches may repel nerve growth to prevent right and left nerves from crossing the facial midline (Küry et al. 2000). The similarities between the shark and tetrapod patterns of *EphA4* expression are consistent with the conserved regulation of early craniofacial morphogenesis across gnathostomes.

EphA4 expression in paired fins

During early stages of paired fin development, prior to the differentiation of the apical ectodermal ridge (AER), *EphA4* was transcribed along the entire proximodistal axis of the fin bud mesenchyme (Fig. 3a, b). By stage 25, *EphA4* expression became restricted to the distal mesenchyme beneath the AER (Fig. 3c, d). Expression decreased as the pectoral fins developed but was later upregulated in the sites of radial formation during skeletogenesis (Fig. 3e, f). Interestingly, the distal boundary of *EphA4* expression in the fin colocalizes with the boundary between endoskeletal elements (radials) and prospective dermal rays, which form in the apical ectodermal fold (AEF; Fig. 3e, f). Kimmel et al. (2001) noted that pharyngeal cartilage joints develop at the boundary of *EphA3*

expression, and suggested that zones of non-chondrification may be specified at the boundaries of Eph expression domains in the face. This hypothesis may bear on the question of how the distal limit of endoskeletal development is specified in fins.

The *EphA4* expression in the pelvic fins resembled the pattern observed in the pectoral fins (data not shown). A departure from the tetrapod pattern was observed during the differentiation of the shark intromittent organs (claspers) from the posterior margins of the pelvic fins. *EphA4* transcription persisted in the most distal regions of the developing claspers, immediately beneath the apical ectodermal fold, after the gene was downregulated in the rest of the pelvic fins (compare Fig. 3g, h). An early and late phase of *EphA4* expression was also described during the development of tetrapod paired limbs (Patel et al. 1996). *EphA4* is expressed early the distal mesenchyme of the limb, under the AER, where it is thought to be

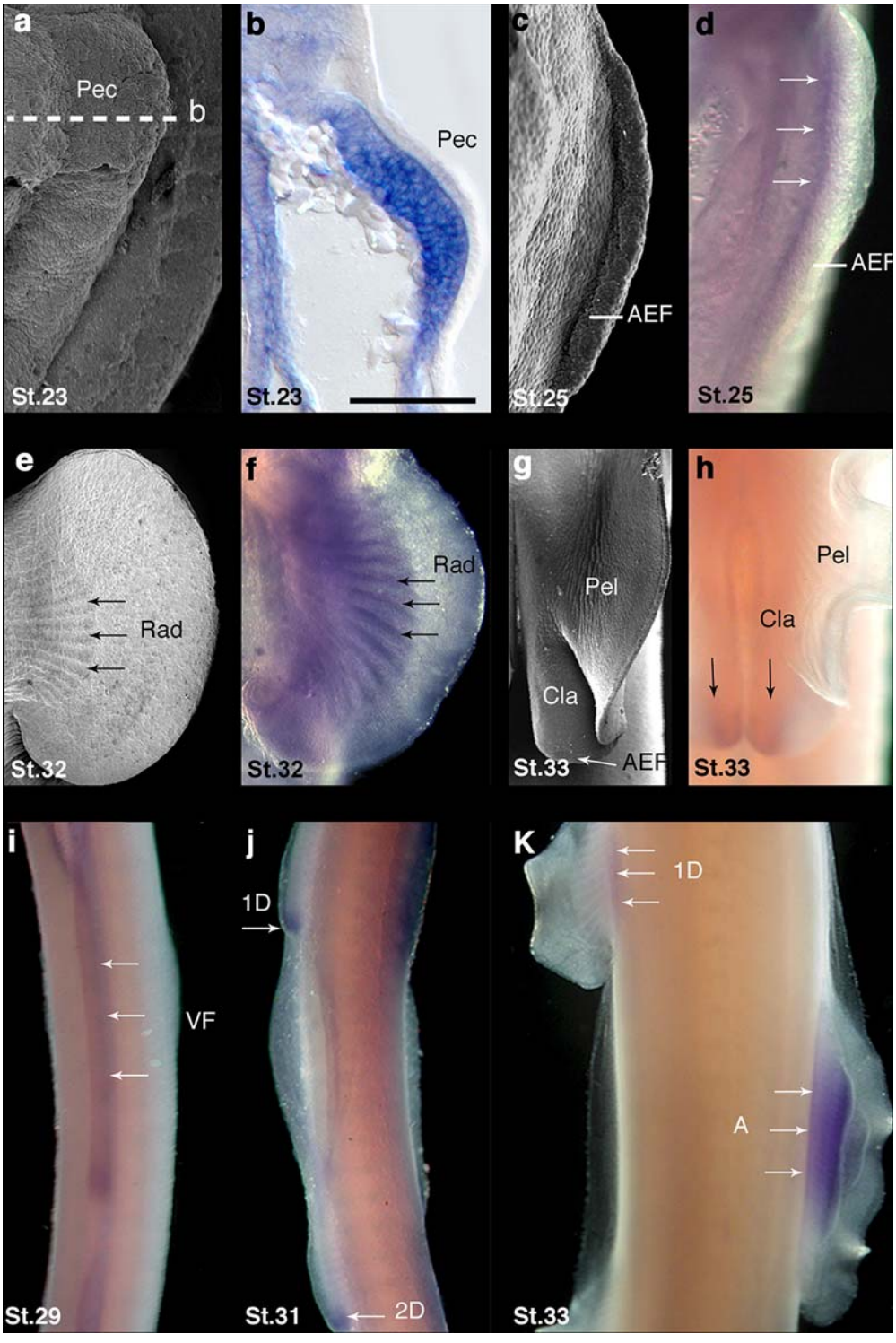

Fig. 3a–k *EphA4* expression during catshark fin development. Stage of development is indicated in *lower left corners*. Anterior is to the *top* in **a, c–k** and dorsal is to the top in **b**. **a** Scanning electron micrograph of a pectoral fin bud (*Pec*) prior to the development of the apical ectodermal ridge (AER). *Dashed line* indicates plane of section shown in **b**. **b** Histological section showing *EphA4* expression along the entire proximodistal axis of the pectoral fin bud mesenchyme at the same stage as **a**. *Scale bar* 200 μm. **c** SEM of pectoral fins after the development of the apical ectodermal fold (*AEF*). **d** *EphA4* expression in the distal mesenchyme under the AEF (*arrows*; compare with **c**). **e** SEM of the pectoral fin during the development of the radials (*Rad*). **f** *EphA4* expression in the radials of the pectoral fin skeleton (compare with **e**). **g** SEM of the claspers (*Cla*) developing from the pelvic fins (*Pel*). **h** Ventral view of pelvic fins showing *EphA4* expression at the distal tip of the prospective claspers (*Cla*). **i** Ventral view of trunk showing *EphA4* expression in the ventral median finfold (*VF*). **j** Lateral view of trunk showing *EphA4* expression at the distal posterior margin of the first and second dorsal fins (*1d*, *2d*). **k** Lateral view of trunk showing *EphA4* expression in the radials of the first dorsal (*1d*) and anal (*a*) fin skeletons

involved in coordination of polarizing and outgrowth signals. Later, during skeletogenesis, it is up-regulated in tendons (Patel et al. 1996), motor axons (Helmbacher et al. 2000) and muscle (Swartz et al. 2001). Our data highlight a conserved regulation of *EphA4* expression in fin and limb development, however the pattern detected during the outgrowth of the claspers points to a modulation of *EphA4* regulation in the evolution of shark-specific morphology.

EphA4 expression in median fins

The lesser spotted catshark develops a median finfold along the dorsal and ventral midline, which later subdivides to form the unpaired median fins (two dorsals, an anal and a caudal fin). We observed a stripe of *EphA4* expression along the dorsal and ventral midlines during early development of the median finfold (Fig. 3i). As the individual median fins develop from the primary finfold, expression of *EphA4* becomes restricted to the posterior mesenchyme of the dorsal fin buds (Fig. 3j). During skeletogenesis, *EphA4* is re-expressed proximally in the developing radials (Fig. 3k). The dynamics of *EphA4* expression in the median fins closely mirror that of the paired fins, with both exhibiting early and late phases of expression. The early phase is somewhat different in median and paired fin buds, as transcripts were detected along the entire anteroposterior axis in the paired fins, but only posteriorly in the median fins. Given that paired fin outgrowth is directed distally, whereas median fin outgrowth is directed posteriorly, our findings suggest that early transcription of *EphA4* within the fin bud may relate to the directionality of outgrowth.

EphA4 expression in the lateral line

The lateral line is an important sensory system of mechanoreceptive neuromasts and nerve tracts along the flank of amphibians and fishes. During embryogenesis, the lateral line develops along the trunk in an anterior to posterior direction, depositing neuromasts in its wake (Coombs et al. 1989). Lateral line neuromasts are reported to have a dual embryonic origin, consisting of neural crest and placodal cells (Collazo et al. 1994). Expression of *Ephs* or *Ephrins* has not previously been examined during lateral line development in any vertebrate. We find that *EphA4* is expressed during catshark lateral line development, marking the entire lateral line of the trunk until shortly before hatching (Fig. 4a). Given that Eph signaling is involved in axonal pathfinding and neural crest migration (Robinson et al. 1997; Küry et al. 2000), our finding suggests that *EphA4* may be involved in the coordination of lateral line organogenesis. The lateral line and inner ear transduce extrinsic signals using mechanosensory hair cells and, as such, these organ systems share structural and functional properties. Recent work has identified several molecules that play similar roles in the development of both inner ear and lateral line (Mowbray et al. 2001). Our finding that *EphA4* is expressed during development of these two structures reinforces the idea that they share a common developmental program.

EphA4 expression in dermal denticles and teeth

An embryonic feature of several shark species is the presence of two dorsal lateral rows of dermal denticles along the trunk (Ford 1921). We observed a polarized, chevron-shaped expression domain of *EphA4* along the leading edge of each of these dermal denticles (Fig. 4a–c). This pattern of *EphA4* in the dermal denticles is reminiscent of *EphA4* expression in developing feathers and scales, both of which bud from dermal epithelium (Ellis et al. 1995; Patel et al. 1996). Interestingly, we also observed expression of *EphA4* in the oral epithelium, in the region of the prospective tooth rows (Fig. 4d). Taken together with the observation that Eph receptors are

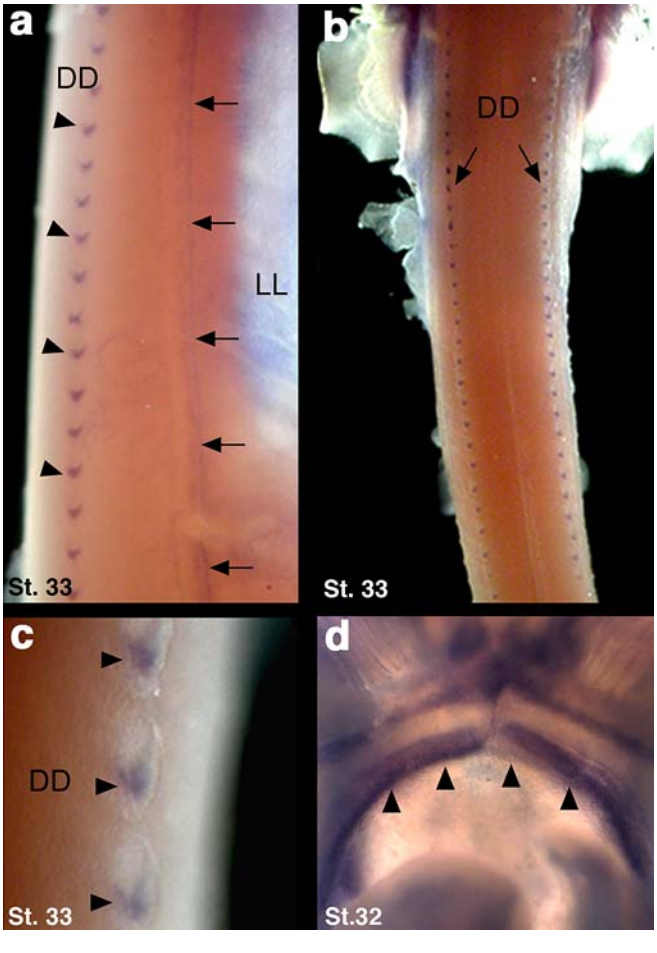

Fig. 4a–d *EphA4* expression in the shark lateral line, dorsal dermal denticles and oral epithelium. Stage of development is indicated in *lower left corners*. Anterior is to the *top*. **a** Lateral view of *EphA4* expression in the lateral line (*LL*; indicated with *arrows*) and dorsal dermal denticles (*DD*; *arrowheads*). **b** Dorsal view of *EphA4* expression in the two rows of embryonic dorsal dermal denticles. **c** Detail of the chevron-shaped *EphA4* expression domains in the dorsal dermal denticles. **d** *EphA4* expression in the oral epithelium in the prospective tooth rows (*arrowheads*).

expressed during tetrapod tooth development (Ellis et al. 1995), our findings indicate that a similar molecular mechanism regulates the development of primitive odontoids, teeth and dermal denticles in shark embryos.

Acknowledgements We thank Anthony Graham for providing the HNK1 antibody, and the Electron Microscopy Core Laboratory, Biotechnology Program, University of Florida for use of their facilities. R.F. is a Ph.D. student of the GABBA Graduate Program of Oporto University (Portugal) and was supported by a fellowship from FCT, Praxis XXI.

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