Development 133, 3127-3137 (2006) doi:10.1242/dev.02459

Developmental genetic mechanisms of evolutionary tooth loss in cypriniform fishes

David W. Stock*, William R. Jackman and Josh Trapani†

The fossil record indicates that cypriniform fishes, a group including the zebrafish, lost oral teeth over 50 million years ago. Despite subsequent diversification of feeding modes, no cypriniform has regained oral teeth, suggesting the zebrafish as a model for studying the developmental genetic basis of evolutionary constraint. To investigate the mechanism of cypriniform tooth loss, we compared the oral expression of seven genes whose mammalian orthologs are involved in tooth initiation in the zebrafish and the Mexican tetra, *Astyanax mexicanus*, a related species retaining oral teeth. The most significant difference we found was an absence in zebrafish oral epithelium of expression of *dlx2a* and *dlx2b*, transcription factors that are expressed in early *Astyanax* odontogenic epithelium. Analysis of orthologous genes in the Japanese medaka (*Oryzias latipes*) and a catfish (*Synodontis multipunctatus*) suggests that expression was lost in cypriniforms, rather than gained in *Astyanax*. Treatment of *Astyanax* with an inhibitor of Fibroblast growth factor (Fgf) signaling produced a partial phenocopy of the zebrafish oral region, in that oral teeth, and expression of *dlx2a* and *dlx2b*, were lost, whereas *shh* and *pitx2*, genes whose expression is present in zebrafish oral epithelium, were unaffected. We hypothesize that a loss of Fgf signaling to oral epithelium was associated with cypriniform tooth loss.

KEY WORDS: Zebrafish, *Astyanax*, Medaka, Catfish, Dlx, Fgf, Constraint

INTRODUCTION

The ancestral bony fish dentition comprised numerous teeth lining the oral and pharyngeal cavities (Huysseune and Sire, 1998; Stock, 2001). Reduction of dentition from this state has occurred repeatedly, whereas the gain of teeth has been less common (Huysseune and Sire, 1998; Stock, 2001; Sire, 2001). Two potential explanations for this macroevolutionary trend are that (1) reduced dentitions are adaptively superior to more complete ones and (2) mechanisms of tooth loss constrain the ability to regain teeth. A potential example of tooth loss constraining dental evolution is the loss of oral teeth in cypriniform fishes, which include the zebrafish. The cypriniform fossil record (Cavender, 1991) suggests that oral teeth were lost at least 50 million years ago. Since then, the group has diversified into over 3000 extant species in five families (Nelson, 1994), and included within this diversity are feeding modes, such as predation on fishes, for which oral teeth are likely adaptive (Sibbing, 1991; Portz and Tyus, 2004) (D.W.S., unpublished). This raises the possibility that no cypriniform has regained oral teeth because of developmental genetic constraints on the ability to do so, which would be further supported by finding a complex series of genetic modifications associated with tooth loss.

We investigated the developmental genetic mechanism of cypriniform tooth loss by comparing oral development in the zebrafish and a member of the closest lineage that retains oral teeth – the characiform *Astyanax mexicanus*, or Mexican tetra (Fink and Fink, 1996; Jeffery, 2001). Cypriniforms and characiforms are members of the Superorder Ostariophysi (Nelson, 1994); we extended some comparisons to an additional ostariophysan (the siluriform cuckoo catfish, *Synodontis multipunctatus*) and to an outgroup (the Japanese medaka, *Oryzias latipes*, a member of the Superorder Acanthopterygii), both of which possess oral teeth.

Tooth development has been studied most extensively in the mouse (Jernvall and Thesleff, 2000; Tucker and Sharpe, 2004; Zhang et al., 2005), in which the earliest sign of initiation is thickening of the oral epithelium to form the dental lamina. This epithelium then invaginates into the underlying mesenchyme to form a bud. Mesenchyme condenses around the bud and folding of the epithelium occurs, prefiguring the crown shape of the tooth. Tooth initiation and morphogenesis are similar in larval teleost fishes, although mesenchymal condensation has been more difficult to document, perhaps because of the small number of cells involved (Huysseune et al., 1998; Sire et al., 2002).

Because no morphological evidence of oral tooth development has been observed in zebrafish (Huysseune et al., 1998), we focused our comparisons of gene expression and function on those involved in the earliest stages of mammalian tooth development. Even before dental lamina formation in the mouse, signaling from odontogenic epithelium through the Fibroblast growth factor (Fgf) pathway induces the expression of multiple transcription factors in the underlying mesenchyme (Neubüser et al., 1997; Trumpp et al., 1999). Although pathways regulating dental lamina formation are less well understood (Jernvall and Thesleff, 2000; Tucker and Sharpe, 2004), several genes have been shown to mark this structure, including *Shh* (Dassule and McMahon, 1998; Hardcastle et al., 1998), *Dlx2* (Thomas et al., 2000; Zhao et al., 2000) and *Pitx2* (Mucchielli et al., 1997; Keränen et al., 1999).

We examined expression in the zebrafish and *Astyanax* of the Fgf pathway ligand Fgf8, its putative downstream targets Pax9 and Lhx6, and the dental lamina markers Shh, Pitx2, Dlx2a and Dlx2b. Fgf signaling from oral epithelium to mesenchyme appears conserved in both species, although this pathway may not be involved in tooth development, as it is in the mouse. Dental lamina markers are expressed in the odontogenic epithelium of *Astyanax* in a pattern similar to that of the mouse. However, whereas *pitx2* and *shh* are expressed in zebrafish oral epithelium, *dlx2a* and *dlx2b* are not.

Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309-0334, USA.

*Author for correspondence (e-mail: David.Stock@Colorado.edu)
†Present address: Department of Paleobiology, National Museum of Natural History, Smithsonian Institution, NHB, MRC 121, Washington, DC 20013-7012, USA

Accepted 25 May 2006

Examination of Dlx2 ortholog expression in additional fish species suggests that oral epithelial expression was lost in the zebrafish lineage rather than gained in *Astyanax*. Inhibition of Fgf signaling in *Astyanax* produced a partial phenocopy of the gene expression characteristics of zebrafish oral epithelium. We hypothesize that multiple Fgf signaling events occur in the jaw of toothed fishes, and that alteration of one of these led to the loss of cypriniform oral teeth.

MATERIALS AND METHODS

Animals

Zebrafish (*Danio rerio*) were from a commercial supplier (Fish2U.com). *Astyanax mexicanus* occurs in both eyed and blind cave forms (Jeffery, 2001). cDNAs were cloned from an eyed population collected in Texas (Dowling et al., 2002). In situ hybridization and histology were performed with blind larvae from a commercial population originating from La Cueva Chica, while imaging of live larvae and inhibition of Fgf signaling employed a population from La Cueva de El Pachón (Jeffery and Martasian, 1998). No features examined differed significantly among these populations. Japanese medaka were of the Cab strain (Wittbrodt et al., 2002). *Synodontis* embryos and larvae were from a colony maintained by Alexander Cruz (University of Colorado). Embryos and larvae of all species were staged by hours post-fertilization (hpf). Zebrafish were raised at 28.5°C, *Astyanax* at 25°C and medaka at 26°C; rearing temperatures of *Synodontis* were not closely controlled. For zebrafish larvae examined in whole mount, pigmentation was inhibited with 0.003% 1-phenyl-2-thiourea.

Cloning and sequence analysis

RNA was isolated from larvae (or adult ovary) of *Astyanax*, medaka and *Synodontis* using the Ultraspec System (Biotecx). Reverse transcriptase-mediated PCR was carried out with the following degenerate primer pairs (added restriction sites are underlined):
dlx2a, GCCGGGATCCATGACNGGNGTNTTYGAYAG, GCCGGAATTCCAADATNGTNCCNGCRCTNAC;
dlx2b, GCCGGGATCCATNGTNAAYGGNAARCCNAA, GCCGGAATTCTGRAACCADATYTTNACYTG;
fgf8, GCCGGGATCCACNAGYGGNAARCAYGTNCA, GCCGGAATTCGGNARNCKYTTCATRAARTG;
lhx6, GCCGGGATCCGCNTGYTTYGCNTGYTTYTC, GCCGGAATTCARTTYTGRAACCANACYTG;
pax9, GCCGGGATCCGCNTTYGGNGARGTNAAYCA, GCCGGAATTCACNGARTGNGANGANGGCCA;
pitx2, GCCGGGATCCARMGNACNCAYTTYACNAG, GCCGGAATTCTTRCANGTRTCNCKRTANAC.

PCR products were cloned into pCR4-TOPO (Invitrogen) and subjected to automated sequencing. The SMART RACE Kit (BD Clontech) was used to generate complete cDNA sequences for *Astyanax dlx2a*, *dlx2b*, *fgf8*, *pax9* and *pitx2*, medaka *dlx2a*, and *Synodontis dlx2a* and *dlx2b*. A partial cDNA sequence for *Astyanax lhx6* was obtained similarly. All sequence positions reported (GenBank Accession numbers DQ822509-DQ822517) were determined for at least five independent clones, representing both strands.

The sequences were translated and potential orthologs identified by BLAST searches of GenBank and the zebrafish and Fugu genome databases (http://www.ensmbl.org). Alignments of amino acid sequences were performed with the Clustal X program (Thompson et al., 1997), and phylogenetic analyses were carried out with the neighbor-joining algorithm of MEGA version 2.0 (Kumar et al., 2001).

In situ hybridization

Whole-mount in situ hybridization and sectioning followed the protocol of Jackman et al. (Jackman et al., 2004), with proteinase K pre-treatment at 2.5-25 μg/ml. Each gene was examined in zebrafish fixed at 4-hour intervals between 28-60 hpf and at 12-hour intervals thereafter through 120 hpf. All *Astyanax* genes were examined in specimens aged 30, 36, 42, 48, 60 and 72 hpf. Digoxigenin-labelled antisense riboprobes were synthesized from cloned cDNA fragments as follows: *Astyanax dlx2a* (nucleotides 190-1019 of GenBank DQ822509), *dlx2b* (13-587 of DQ822510), *fgf8* (898-2103 of DQ822511), *lhx6* (56-897 of DQ822512), *pax9* (189-775 of DQ822513), *pitx2* (427-958 of DQ822514) and *shh* (194-1187 of AY661433); zebrafish *dlx2b* (Ellies et al., 1997), *dlx2a*, *fgf8*, *lhx6*, *pax9*, *pitx2* (Jackman et al., 2004) and *shh* (918-1573 of NM_131063); *O. latipes dlx2a* (158-987 of DQ822515); and *Synodontis dlx2a* (199-1028 of DQ822516) and *dlx2b* (264-955 of DQ822517).

Morphological analysis and histology

Astyanax oral development was examined using differential interference contrast (DIC) microscopy in anaesthetised specimens embedded in 0.5% agarose. *Astyanax* and zebrafish were cleared and stained with Alcian green (Jackman et al., 2004), or Alcian blue and Alizarin red (Hanken and Wassersug, 1981), to visualize teeth. In addition, 2 μm serial sections of glycol-methacrylate-embedded zebrafish and *Astyanax* larvae were cut with glass knives and stained with 0.1% Toluidine blue.

SU5402 treatment

SU5402 (Mohammadi et al., 1997) was used to inhibit signaling through Fgf receptors in the zebrafish and *Astyanax*. Zebrafish were dechorionated and treated with 25 μM SU5402 in 0.5% DMSO from 24 or 32 hpf through 56 hpf, followed by fixation and in situ hybridization (Jackman et al., 2004). A range of SU5402 concentrations was examined in *Astyanax* to find one affecting teeth while minimizing other phenotypic abnormalities; the concentration used in the data presented was 10 μM. Larvae were raised in SU5402 beginning at 30 or 36 hours. Specimens for in situ hybridization were fixed at 84 hpf and additional embryos were allowed to develop to 108 hpf for skeletal staining. Control embryos of both species were raised in 0.5% DMSO. Any larvae showing gross morphological abnormalities or severe developmental retardation were excluded from consideration.

RESULTS

Oral teeth are present in *Astyanax* larvae, but absent from all stages of the zebrafish

As reported by Valdéz-Moreno and Contreras-Baldera (Valdéz-Moreno and Contreras-Baldera, 2003), adult *A. mexicanus* possess teeth attached to premaxillary, maxillary and dentary bones (Fig. 1A). Trapani et al. (Trapani et al., 2005) identified the first teeth to appear in larvae as one attached to each premaxillary bone and one on each side of the dentary bone midline (Fig. 1B,C). Germs of these could not be identified reliably in histological sections at 60 hpf, whereas most 72 hpf specimens exhibited bell-shaped germs (Fig. 1D-F), corresponding to ongoing morphogenesis or cytodifferentiation stages in zebrafish pharyngeal tooth development (Van der heyden and Huysseune, 2000).

The absence of oral teeth in adult cypriniforms generally (Nelson, 1994) and in zebrafish specifically (Kimmel et al., 1995; Huysseune et al., 1998) is well known (Fig. 1G). Similarly, extensive skeletal staining of zebrafish larvae (Schilling, 2002) failed to reveal mineralized oral teeth (Fig. 1H). To search for arrested oral tooth germs in zebrafish larvae, we produced serial histological sections of several individuals each of 60, 72, 84, 96, 108 and 120 hpf. No evidence for mandibular tooth germs was found in any specimen (Fig. 1I-L).

Gene orthology

It has been suggested that genome duplication occurred in ray-finned fishes before the divergence of the Ostariophysi (zebrafish, *Astyanax* and *Synodontis*) and the Acanthopterygii (medaka and Fugu) (Meyer and Van de Peer, 2005). Of the genes we examined, two zebrafish orthologs are known for tetrapod Shh (Zardoya et al., 1996) and Dlx2 (Stock et al., 1996). Yamamoto et al. (Yamamoto et al., 2004) established the orthology of *Astyanax shh*, while phylogenetic analysis (Fig. 2A) identified Dlx2a and Dlx2b orthologs in *Astyanax* and *Synodontis*, and *dlx2a* in medaka. Despite extensive sequencing of Dlx homeodomains, we found no evidence for medaka *dlx2b*. This gene may be absent from acanthopterygians, as BLAST searches of the Fugu genome with the zebrafish homeodomain failed to detect it.

A single zebrafish ortholog is known for tetrapod Fgf8, Pax9, Lhx6 and Pitx2. Phylogenetic analyses of Fgf8 and Pax9 (Fig. 2B,C) identify the *Astyanax* genes as orthologs rather than recently formed paralogs of their zebrafish counterparts because of concordance between gene and species relationships. Although phylogenetic analysis of Lhx6 and Lhx8 sequences has identified the *Astyanax* gene cloned as *lhx6* (Fig. 2D), we cannot rule out the possibility that it is a recently formed paralog of zebrafish *lhx6* because of lack of other teleost sequences.

Phylogenetic analyses did not unambiguously identify the orthology of the *Astyanax* Pitx gene cloned. Three Pitx genes are known in vertebrates (Gage et al., 1999) and phylogenetic analyses (not shown) clustered the *Astyanax* gene with, but outside of, Pitx2 genes from other vertebrates. Pitx2 is alternatively spliced (Arakawa et al., 1998; Essner et al., 2000), with the cDNA we characterized corresponding to Pitx2a. We searched the zebrafish genome with each *Astyanax* exon separately, but did not find any genes other than *pitx1*, *pitx2* and *pitx3*. Alignment of Pitx genes revealed no similarity between exon 2 of Pitx2 and any region of Pitx1 or Pitx3. By contrast, exon 2 exhibited a single amino acid difference (out of fifteen) between the *Astyanax* gene and zebrafish *pitx2* (Fig. 2E). We conclude that the *Astyanax* gene is orthologous to zebrafish *pitx2*, but has undergone substantial sequence divergence.

Dlx2 orthologs mark *Astyanax* odontogenic epithelium

Jackman et al. (Jackman et al., 2004) identified the homeodomain transcription factor *dlx2b* as a marker of zebrafish pharyngeal tooth epithelium. We examined *dlx2b* expression in *Astyanax* to determine if it also marked oral tooth epithelium. At 72 hpf, staining is present in localized domains on either side of the midline in upper and lower jaws (Fig. 3H). Sectioning revealed that each corresponds to a tooth germ, based on epithelial morphology and mineralization (Fig. 3I,M,N). Expression is strongest in the epithelial layer, but prolonged staining revealed expression in tooth germ mesenchyme (not shown).

To determine whether *dlx2b* marks earlier odontogenic epithelium, we traced the tooth germ expression domains to earlier stages. In the specimens examined, the oral plate was intact at 42 hours and ruptured by 48 hours (Fig. 3A,B). Between 48 and 72 hours, the mouth opening enlarges substantially and moves from a midventral to a terminal location (Fig. 3B-D), similar to that of the zebrafish (Kimmel et al., 1995). We detected epithelial expression domains of *dlx2b* reminiscent of tooth germs as early as 36 hpf (Fig. 3E-G,J-L). These move medially between 42 and 60 hpf (Fig. 3E-G), and are likely to correspond to odontogenic epithelium.

In addition to expression in odontogenic epithelium and mesenchyme, mouse *Dlx2* is expressed in mandibular arch mesenchyme outside tooth germs (Qiu et al., 1997; Thomas et al., 2000; Zhao et al., 2000). This latter expression is lacking for *Astyanax dlx2b*, but present for *dlx2a* (lateral to tooth germs). At 72 hpf, *dlx2a* was expressed in the epithelium and mesenchyme of all four tooth germs (Fig. 3R,S). Tooth germ expression appeared at 60 hpf (Fig. 3Q), while lateral (non-dental) mesenchyme expression could be detected at 30 hours, the earliest stage examined (Fig. 3O,P).

Loss of epithelial Dlx2 ortholog expression is associated with cypriniform tooth loss

Initial characterization of zebrafish *dlx2a* (Akimenko et al., 1994) and *dlx2b* (Ellies et al., 1997) identified mandibular arch expression of the former but not the latter. We closely examined the expression

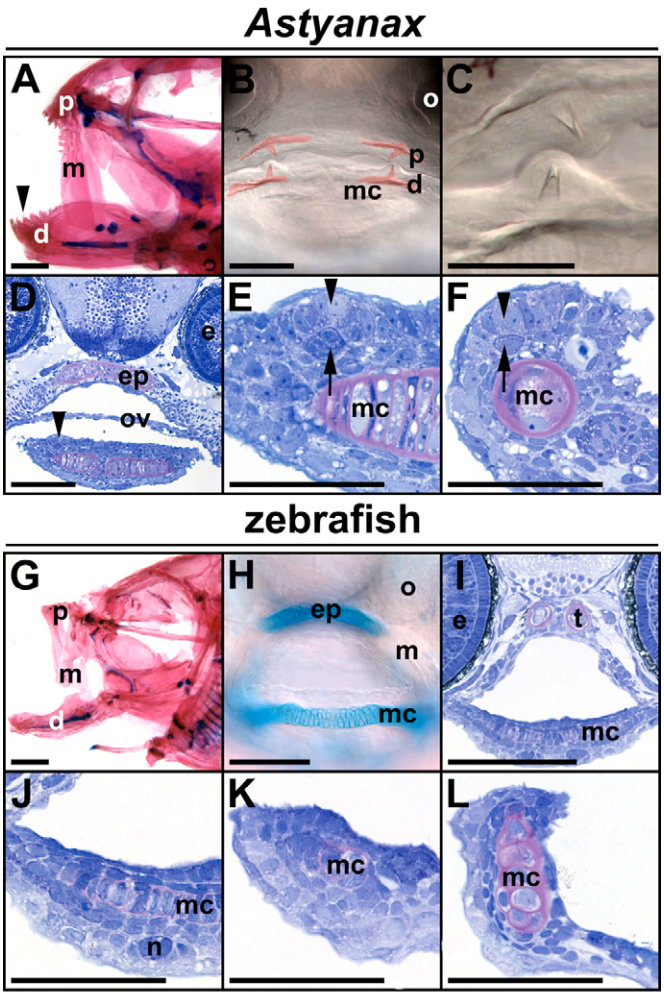

Fig. 1. Oral morphology in *Astyanax* and zebrafish. (**A**) Teeth are present on premaxillary (p), maxillary (m) and dentary bones (d, arrowhead) of adult *Astyanax* (lateral view, cleared and stained with Alcian blue and Alizarin red). (**B**,**C**) A single tooth is present on each premaxillary and dentary bone in 120 hpf *Astyanax* (frontal views; teeth and bones digitally colored red in B). (**D-F**) Bell-shaped tooth germs in 72 hpf *Astyanax* lower jaw. Dental epithelium indicated by arrowhead, darkly-stained dental mesenchyme by arrow. Transverse sections in D,E; sagittal in F. (**G**) Toothless oral cavity of adult zebrafish. (**H**) Toothless oral cavity of 124 hpf zebrafish larva cleared and stained with Alcian green. (**I-L**) No tooth germs are visible in sectioned, Toluidine blue-stained zebrafish larvae. (I,J) Identical transverse sections of a 72 hpf specimen. (K,L) Sagittal views of the lower jaw of 72 hpf and 120 hpf specimens, respectively. d, dentary; e, eye; ep, ethmoid plate; m, maxillary; mc, Meckel's cartilage; n, neuromast; o, olfactory organ; ov, oral valve; p, premaxillary; t, trabecula. Scale bars: 1 mm in A,G; 100 μm in B,D,H,I; 50 μm in C,E,F,J-L.

of both genes in zebrafish aged 28-120 hpf. *dlx2b* was not expressed in mandibular arch epithelium or mesenchyme at any stage (Fig. 4A-C), whereas mandibular arch expression of *dlx2a* was limited to lateral mesenchyme, corresponding to the non-dental expression of its *Astyanax* ortholog (Fig. 4D-F).

We next investigated whether Dlx2 ortholog expression was gained in the lineage leading to *Astyanax* or lost from that of zebrafish. We isolated *dlx2a* and *dlx2b* orthologs (Fig. 2A) from the catfish *Synodontis*, an ostariophysan more closely related to *Astyanax* than is the zebrafish (Saitoh et al., 2003). *Synodontis*

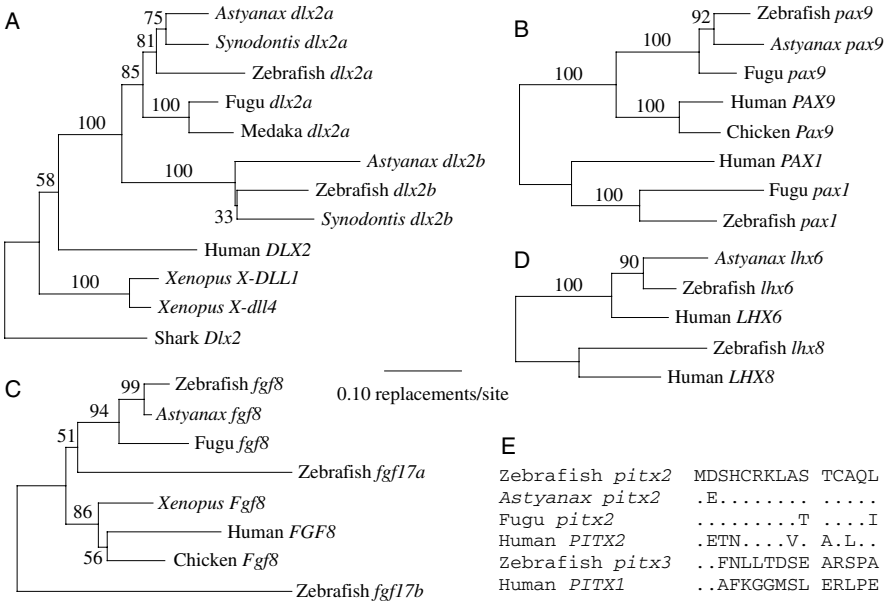

Fig. 2. Gene orthology. Numbers indicate percentage of 1000 bootstrap replications in which the branch was found. (**A**) Phylogeny of Dlx2 genes rooted with a shark sequence. Monophyletic clades containing Dlx2a genes and Dlx2b genes were found in 85% and 100% of bootstrap replicates, respectively. (**B**) Phylogeny of Pax9 genes (rooted with Pax1 genes) matches that of the species with high bootstrap support (92-100%). (**C**) Phylogeny of Fgf8 and Fgf17 genes rooted in the midpoint of the longest path between terminal taxa. Ninety-nine percent of the bootstrap trees support orthology of zebrafish and *Astyanax fgf8*. (**D**) Phylogeny of Lhx6 and Lhx8 genes with midpoint rooting. Ninety percent of the bootstrap trees support orthology of zebrafish and *Astyanax lhx6*. (**E**) Alignment of exon 2 of Pitx2, Pitx3 and Pitx1. Periods indicate matches to the uppermost sequence. The *Astyanax* gene has a single mismatch with zebrafish *pitx2*, but no recognizable homology to Pitx1 and Pitx3 genes.

dlx2a and *dlx2b* expression closely matched that in *Astyanax*, with both being present in odontogenic epithelium but only *dlx2a* present laterally in jaw mesenchyme (Fig. 4G-L). We were only able to isolate *dlx2a* (Fig. 2A) from the medaka, an outgroup of the Ostariophysi. As in the other teleosts with oral teeth, medaka *dlx2a* is expressed both laterally in jaw mesenchyme and in oral epithelium (Fig. 4M-O). These data suggest a loss of epithelial Dlx2 ortholog expression in association with cypriniform oral tooth loss.

pitx2 and *shh* are expressed in zebrafish oral epithelium, but provide no indication of tooth initiation

The homeodomain transcription factor *Pitx2* and the secreted ligand *Shh* are expressed in the early dental epithelium of the mouse (Mucchielli et al., 1997; Dassule and McMahon, 1998; Keränen et al., 1999). *Pitx2* is expressed broadly in stomodeal epithelium well before the appearance of tooth germs, becoming progressively restricted to odontogenic epithelium. *Astyanax pitx2* is expressed

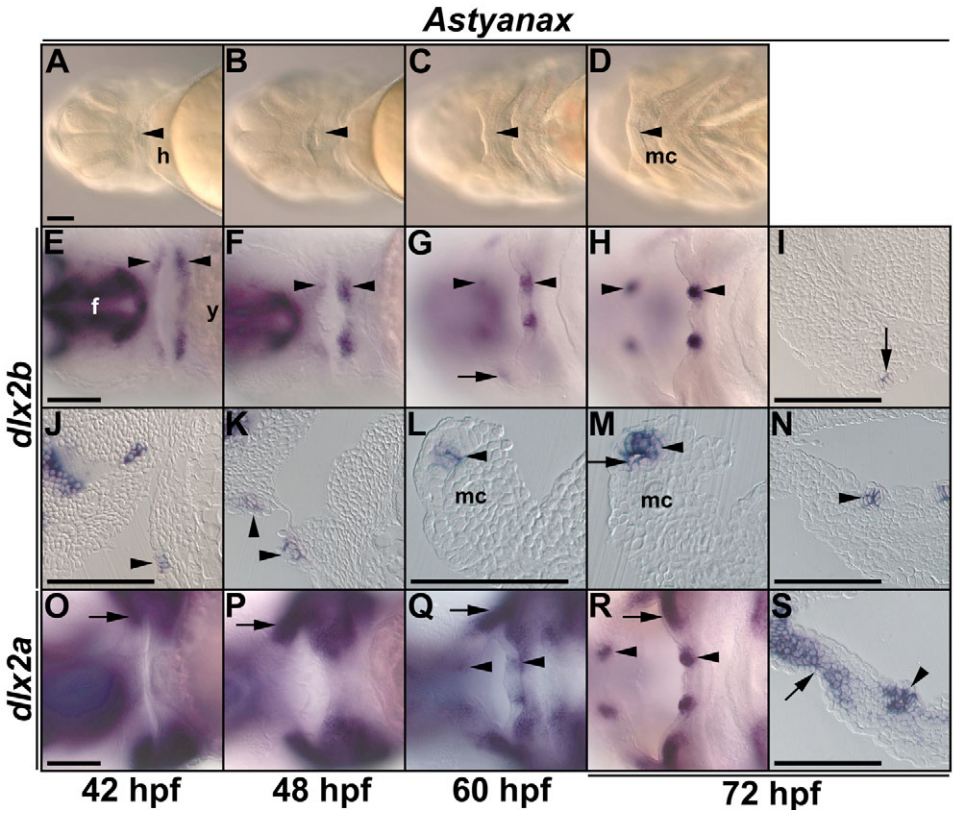

Fig. 3. Expression of *Astyanax* Dlx2 orthologs. (**A-D**) Anterior progression of mouth in live larvae (ventral views, anterior to left). Arrowhead indicates lower jaw. (**E-H**) *dlx2b* expression in ventral whole mount (anterior to left). Arrowheads indicate putative odontogenic epithelium. Arrow indicates additional faint epithelial expression domain in a maxillary process unlikely to be related to dentition. (**I**) Transverse section showing *dlx2b* expression in epithelium of the upper tooth germ (arrow). (**J-M**) *dlx2b* expression in sagittal sections. Odontogenic epithelium labeled as in E-H. Note the mineralization in M (arrow). (**N**) Transverse section showing *dlx2b* expression in epithelium of the mandibular tooth germ (arrowhead). (**O-S**) *dlx2a* expression in ventral whole mount (O-R) and transverse section (S). Arrowheads indicate odontogenic epithelium and arrows expression in lateral jaw mesenchyme. Note expression in the epithelium (arrowhead) and mesenchyme of the tooth germ in S. f, forebrain; h, heart; mc, Meckel's cartilage; y, yolk. Scale bars: 100 μM.

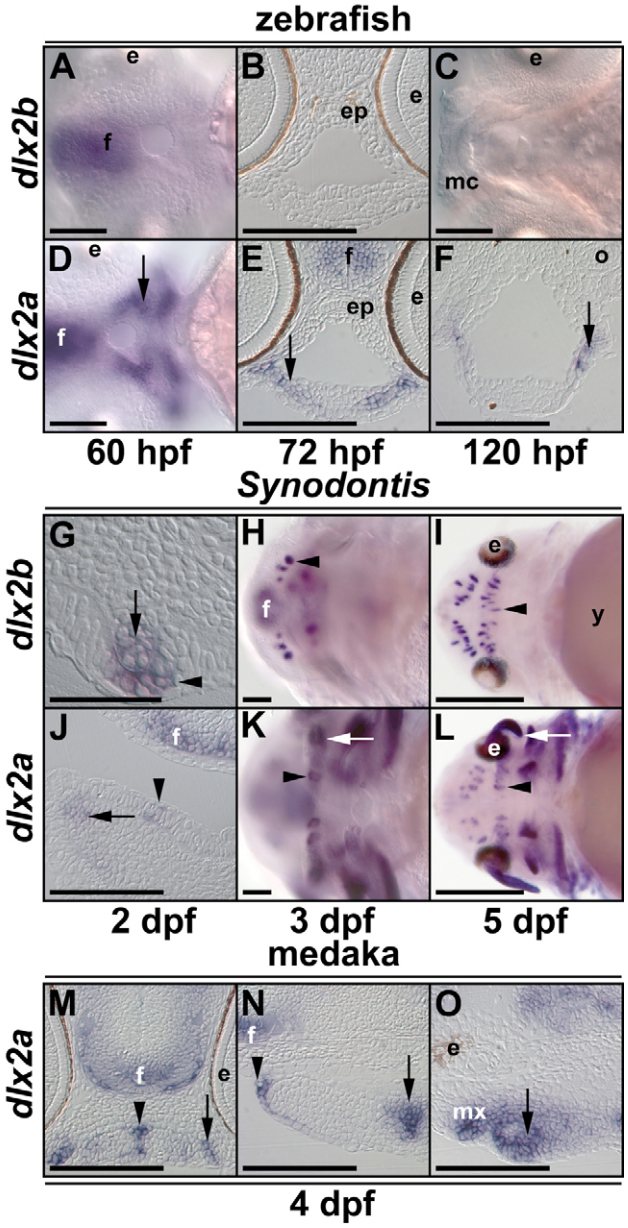

Fig. 4. Expression of Dlx2 orthologs in zebrafish, *Synodontis* and medaka. (**A-C**) *dlx2b* expression is absent at all stages from the zebrafish oral region, but present in forebrain (f). (**D-F**) *dlx2a* expression is absent from zebrafish oral epithelium at all stages, but present laterally in jaw mesenchyme (arrows) and forebrain. (**G-L**) *dlx2a* and *dlx2b* are expressed in tooth germs of *Synodontis*, while only *dlx2a* is expressed laterally in the jaw mesenchyme (arrows in J,K) and barbels (arrow in L). Both genes are expressed (arrowheads) in tooth germ epithelium (cytodifferentiation stage premaxillary germ in G; initiation stage dentary germ in J), and *dlx2b* was additionally detected in tooth germ mesenchyme (arrow in G). Arrowheads indicate one of three premaxillary germs per side in H, one of two dentary germs per side in K, and one of numerous germs visible in upper and lower jaws of I,L. (**M-O**) Medaka *dlx2a* is expressed in oral epithelium (arrowheads in M,N) and mesenchyme (arrows in M-O). Transverse section (M) indicates epithelium expression is in a medial band and sagittal sections (N,O) reveal that mesenchymal expression is lateral to this. Indicated stage is before visible signs of tooth initiation. All whole mounts in ventral view; transverse sections in B,E-G,J,M; sagittal sections in N,O. Abbreviations as in Fig. 1. f, forebrain; mx, maxillary process; y, yolk. Scale bars: 100 μm in A-H,J-K,M-O; 500 μm in I,L.

similarly. Oral epithelial expression was detected at the earliest stage examined and progressed from a broad uniform domain (Fig. 5A,D) to more intense expression in the tooth germs themselves (Fig. 5B,C,E,F).

Mouse *Shh* is localized to the epithelium of initiating tooth germs, but at earlier stages is expressed broadly in pharyngeal endoderm and stomodeal ectoderm (Keränen et al., 1999; Sarkar et al., 2000; Cobourne et al., 2004; Jeong et al., 2004; Moore-Scott and Manley, 2005). Faint mandibular arch expression of *Astyanax shh* could be detected at 42 hours, and, by 48 hours, expression was strong in patches on either side of the midline of upper and lower jaws (Fig. 4G,J). Although tooth germ expression was detected at 72 hpf (Fig. 5I,L), it remains unclear whether expression at earlier stages includes odontogenic epithelium (Fig. 5G,H,J,K). In general, *shh* was more posteriorly (lingually) restricted in expression than *pitx2* and may be adjacent but lingual to *dlx2b* expression before definitive tooth germ expression at 72 hpf (Fig. 3K,L; Fig. 5D,E,J,K).

pitx2 and *shh* are expressed in zebrafish oral epithelium and considered to be markers of ectoderm and endoderm, respectively (Miller et al., 2000; Miller et al., 2004). We examined the expression of these genes in detail to determine whether it resembled expression in *Astyanax* tooth germs. Both *shh* and *pitx2* exhibited broad expression that was continuous across the medial-lateral axis at 60 hpf (Fig. 5M,P). Sagittal sections at a variety of stages revealed that *shh* expression was lingual to that of *pitx2*, with little, if any, overlap (Fig. 5N,Q). Transverse sections showed *shh* expression throughout the mediolateral axis of the lower jaw and in a position dorsal to Meckel's cartilage (Fig. 5R). This pattern persisted through the latest stage examined (120 hpf), with no evidence of focal expression resembling tooth germs. *pitx2* was similarly expressed across the mediolateral axis of the lower jaw (Fig. 5O), with no evidence of focal expression, before becoming undetectable at 120 hpf. Interestingly, expression was ventral to Meckel's cartilage, suggesting the absence of a domain co-expressing *pitx2* and *shh* that characterizes *Astyanax* tooth germs.

Induction of mesenchymal Pax9 and Lhx6 expression by Fgf signaling occurs in zebrafish and *Astyanax*

Absence of evidence for a dental lamina in the zebrafish oral cavity led us to examine an initiation pathway that acts in the mouse before the physical appearance of tooth germs. In this pathway, epithelially expressed *Fgf8* induces the expression of multiple transcription factors in the underlying mesenchyme, including the paired domain-containing *Pax9* and the LIM-homeodomain-containing *Lhx6* (Neubüser et al., 1997; Trumpp et al., 1999). We found *fgf8* expression in the *Astyanax* oral region from the earliest stage examined. This expression was located in the lateral epithelium of presumptive upper and lower jaws (Fig. 6A,D,G,J). As had been observed for *dlx2b*, the left and right *fgf8* expression domains move medially between 42 and 48 hpf. However, *fgf8* expression was more lateral than that of *dlx2b*, being adjacent to *dlx2b* expression through 48 hpf and discontinuous with this dental epithelial marker after 60 hpf (Fig. 6M).

Astyanax pax9 and *lhx6* are expressed in the mesenchyme of upper and lower jaws (Fig. 6B,C,E,F,H,I,K,L,N,O). Although their expression underlies that of *fgf8* and extends further medially, it is concentrated laterally to developing tooth germs. In the case of *pax9*, simultaneous analysis with *shh* or *dlx2b* as markers of the dental epithelium revealed that *pax9* expression at 60-72 hpf was very weak

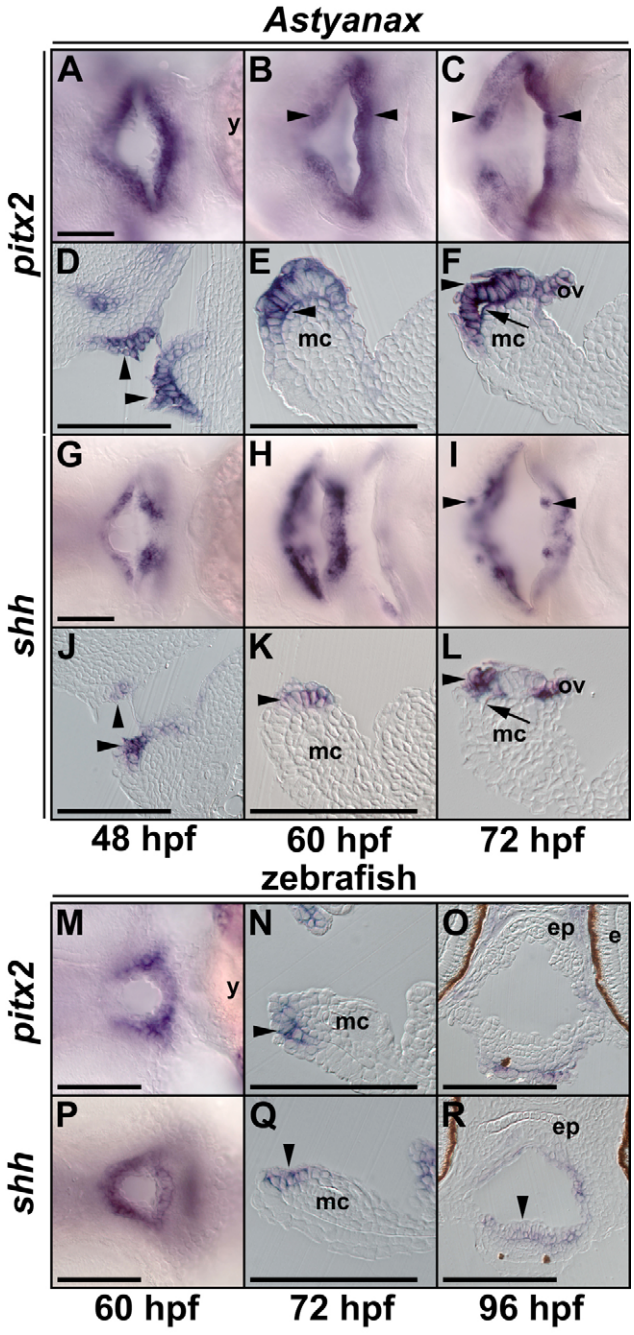

Fig. 5. Expression of Pitx2 and Shh in *Astyanax* and zebrafish. (**A-C**) *pitx2* is expressed broadly and uniformly in the *Astyanax* oral region at early stages, later becoming strongest in tooth germs (arrowheads). (**D-F**) Sagittal sections reveal epithelial *pitx2* expression (arrowheads) that includes morphologically visible tooth germs (arrow indicates mineralized matrix) and the oral valve (ov). (**G-I**) *Astyanax shh* expression is initially broad, becoming discrete in tooth germs by 72 hpf (arrowheads). (**J-L**) Sagittal sections reveal *shh* expression (arrowheads) to be epithelial and more posteriorly (lingually) restricted than *pitx2*. (L) At 72 hpf, *shh* expression is discontinuous between tooth germ (arrow) and oral valve. Arrow indicates mineralized matrix. (**M-O**) *pitx2* is broadly expressed in zebrafish oral epithelium. Sagittal (N) and transverse (O) sections indicate labially restricted expression (arrowhead). No discrete expression corresponding to tooth germs is seen at any stage. (**P-R**) *shh* is expressed in zebrafish oral epithelium (arrowheads), showing little overlap with *pitx2* expression, to which it is lingual. Abbreviations as in Fig. 1. y, yolk. Scale bars: 100 μm.

in the tooth germ mesenchyme (Fig. 6N). Finally, we found that *Astyanax pax9*, but not *lhx6*, was expressed in oral epithelium (Fig. 6H,I,K,L,N).

Expression of zebrafish *fgf8* has been described in the early stomodeum (Eberhart et al., 2006). We examined its later expression, and that of zebrafish *lhx6* and *pax9*, finding no significant differences with *Astyanax*. Expression of all three zebrafish genes persisted in oral jaws through to the latest stage examined (Fig. 6P-R).

To determine whether Pax9 and Lhx6 are regulated by Fgf signaling in teleost oral jaws, as they are in the mouse, we applied the Fgf receptor inhibitor SU5402 to embryos and larvae of *Astyanax* and zebrafish. Treatment of *Astyanax* from 36-84 hpf (Fig. 7B-D,G-I) severely reduced or abolished mesenchymal expression of *pax9* (n=6/8 versus 0/7 in controls) and *lhx6* (n=7/8 versus 0/8 in controls). Similar results were obtained following treatment of zebrafish with SU5402 from 32-56 hpf for *pax9* (n=7/7 reduced or absent versus 0/8 in controls) and *lhx6* (n=6/7 versus 0/7 in controls), revealing the conservation of Fgf signaling to oral mesenchyme in fish and mammals. By contrast, epithelial expression of Pax9 was not affected by SU5402 in either fish species (Fig. 7B,C,G,H).

Inhibition of Fgf signaling in *Astyanax* partially phenocopies zebrafish oral epithelium

Pharyngeal tooth expression of zebrafish *dlx2a* and *dlx2b* is Fgf dependent (Jackman et al., 2004), whereas oral epithelial expression of mouse *Fgf8*, *Shh* and *Pitx2* is not (Mandler and Neubüser, 2001). As *dlx2a* and *dlx2b* expression is absent from zebrafish oral epithelium, while *fgf8*, *shh* and *pitx2* expression is present, we explored the hypothesis that the loss of Fgf signaling led to the loss of oral Dlx2 expression in cypriniforms. We examined whether Fgf signaling is required for the development of oral teeth in *Astyanax* by treating larvae with SU5402 from 30 or 36 hpf through 108 hpf. Both treatments blocked tooth formation (Fig. 7A,F), as assayed by skeletal staining (n=9/12 and 11/13 completely lacking oral teeth from the treatments, respectively, versus n=1/12 in DMSO-treated controls). Varying degrees of cranial cartilage reduction and malformation were also observed, with effects being more severe with early treatments. For this reason, reported gene expression data are from treatments at 36 hpf, unless otherwise indicated.

SU5402 treatment did not prevent oral expression of *shh* (n=7/7) or *pitx2* (n=6/6) in *Astyanax*, although focal expression corresponding to tooth germs was completely eliminated for *shh* and reduced (n=3/6) for *pitx2* (Fig. 7M,N,R,S). Treatment at 30 hpf completely eliminated focal expression of *pitx2* (n=6/6). Similar results were obtained by treating zebrafish with SU5402 from 24 to 56 hpf, which failed to eliminate oral *shh* (n=10/10) or *pitx2* (n=9/9) expression. Unexpectedly, given that oral *Fgf8* expression in mouse was found to be Fgf independent (Mandler and Neubüser, 2001), SU5402 treatment resulted in a severe reduction or absence of oral Fgf8 expression in *Astyanax* (Fig. 7E,J; n=4/4 with treatment at 30 hpf, 6/8 at 36 hpf) and zebrafish (n=9/10).

Finally, we found oral expression of *dlx2a* and *dlx2b* to be Fgf dependent in *Astyanax* (Fig. 7K,L,P,Q). SU5402 treatment eliminated epithelial *dlx2b* (n=6/7) and *dlx2a* (n=8/8) expression, with both results being significantly different from controls (n=2/8, P<0.05; n=1/6, P<0.01, respectively; Fisher's exact test). Although neither gene is expressed in zebrafish oral epithelium, SU5402 treatment reduced or eliminated lateral mesenchymal expression of Dlx2a in both *Astyanax* (Fig. 7K,P; n=8/8) and zebrafish (Fig. 7O,T; n=6/6).

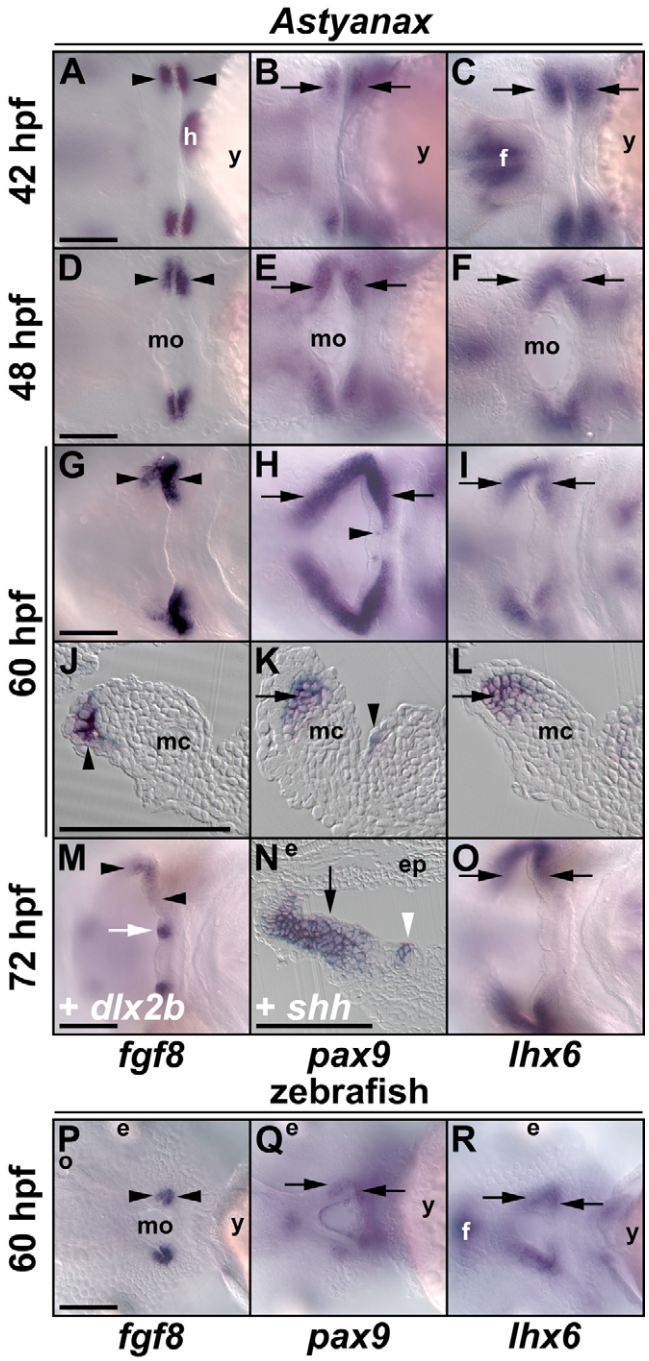

Fig. 6. Expression of putative Fgf signaling pathway components in *Astyanax* and zebrafish. (**A-R**) *fgf8*, *dlx2b*, *pax9* and *lhx6* expression. (A,D,G,J) *Astyanax fgf8* is expressed in maxillary and mandibular epithelium of the developing mandibular arch (arrowheads), with expression domains moving medially between 42 and 48 hpf. (M) Medial limits of *fgf8* expression (arrowheads) do not overlap with *dlx2b* expression (arrow) when detected simultaneously. (P) Zebrafish *fgf8* expression resembles that in *Astyanax*. (B,E,H,K) *Astyanax pax9* is expressed predominantly in the maxillary and mandibular mesenchyme (arrows), but is also found in patches in the oral epithelium (arrowheads). (N) Simultaneous detection of *shh* (arrowhead) and *pax9* (arrow) indicates that *pax9* mesenchymal expression is strongest lateral to the developing tooth germ (arrowhead). (Q) Zebrafish *pax9* expression (arrows) resembles that in *Astyanax*. (C,F,I,L,O) *Astyanax lhx6* is expressed in the maxillary and mandibular mesenchyme (arrows). (R) Zebrafish *lhx6* expression (arrows) resembles that in *Astyanax*. (J-L) Sagittal sections of mandible; (N) transverse section. Abbreviations as in Fig. 1. f, forebrain; h, heart; mo, mouth opening; y, yolk. Scale bars: 100 μm.

DISCUSSION

A dental lamina does not form in the zebrafish mouth

The earliest morphological evidence of vertebrate tooth initiation is epithelial thickening to form a dental lamina (Tucker and Sharpe, 2004; Zhang et al., 2005). Histological analysis failed to reveal such thickening in the zebrafish oral cavity at any developmental stage. For the dental lamina markers examined, the most distinctive difference between zebrafish and *Astyanax* was a complete absence of Dlx2 ortholog expression in zebrafish oral epithelium. Of these genes, at least *dlx2b* is expressed in *Astyanax* oral epithelium well before tooth germs appear, suggesting that differences between the two species arise early in development.

Two other dental lamina markers, *pitx2* and *shh*, are expressed in zebrafish mandibular epithelium. However, both are expressed broadly in oral epithelium prior to tooth initiation in the mouse (Mucchielli et al., 1997; Keränen et al., 1999; Sarkar et al., 2000; Cobourne et al., 2004) and rainbow trout (Fraser et al., 2004), and we found a similar pattern in *Astyanax*. Expression of zebrafish *pitx2* and *shh* was broadly distributed at all stages, with no obvious focal expression corresponding to initiating tooth germs. We suggest that this expression is unrelated to tooth formation, but rather reflects other known functions of these genes in vertebrate craniofacial development (e.g. Lu et al., 1999; Liu et al., 2003; Hu et al., 2003; Moore-Scott and Manley, 2005; Wada et al., 2005).

Fgf signaling to oral mesenchyme occurs in zebrafish but is independent of tooth initiation

We found Fgf signaling to mandibular mesenchyme, an event considered to be the earliest stage of mouse tooth initiation (Neubüser et al., 1997; Tucker and Sharpe, 2004), to be conserved in the zebrafish oral region. Zebrafish *fgf8* is expressed in oral epithelium, and orthologs of mesenchymal transcription factor targets in the mouse (*Pax9* and *Lhx6*) are expressed in mandibular arch mesenchyme in an Fgf-dependent fashion. However, although Fgf8 and Pax9 are required for mammalian tooth development (Peters et al., 1998; Trumpp et al., 1999; Stockton et al., 2000), our analysis of their expression in *Astyanax* suggests this may not be the case in teleosts.

Both Fgf8 and Pax9 are expressed in rodents in regions considered to be presumptive dental epithelium and mesenchyme, respectively, and their expression persists in tooth germs through the bud stage (Neubüser et al., 1997; Peters et al., 1998; Keränen et al., 1999). We found *Astyanax fgf8* expression to be consistently lateral to the expression of *dlx2b*, a probable marker of odontogenic epithelium, and absent from tooth germs once they became morphologically apparent. Although *pax9* expression does extend into early tooth germ mesenchyme, it does so only weakly, with stronger expression located laterally. This lack of association between *fgf8* and *pax9* expression and tooth development in *Astyanax* parallels the absence of expression of both genes in zebrafish pharyngeal teeth (Jackman et al., 2004). We speculate that *fgf8* and *pax9* expression in the zebrafish oral region does not

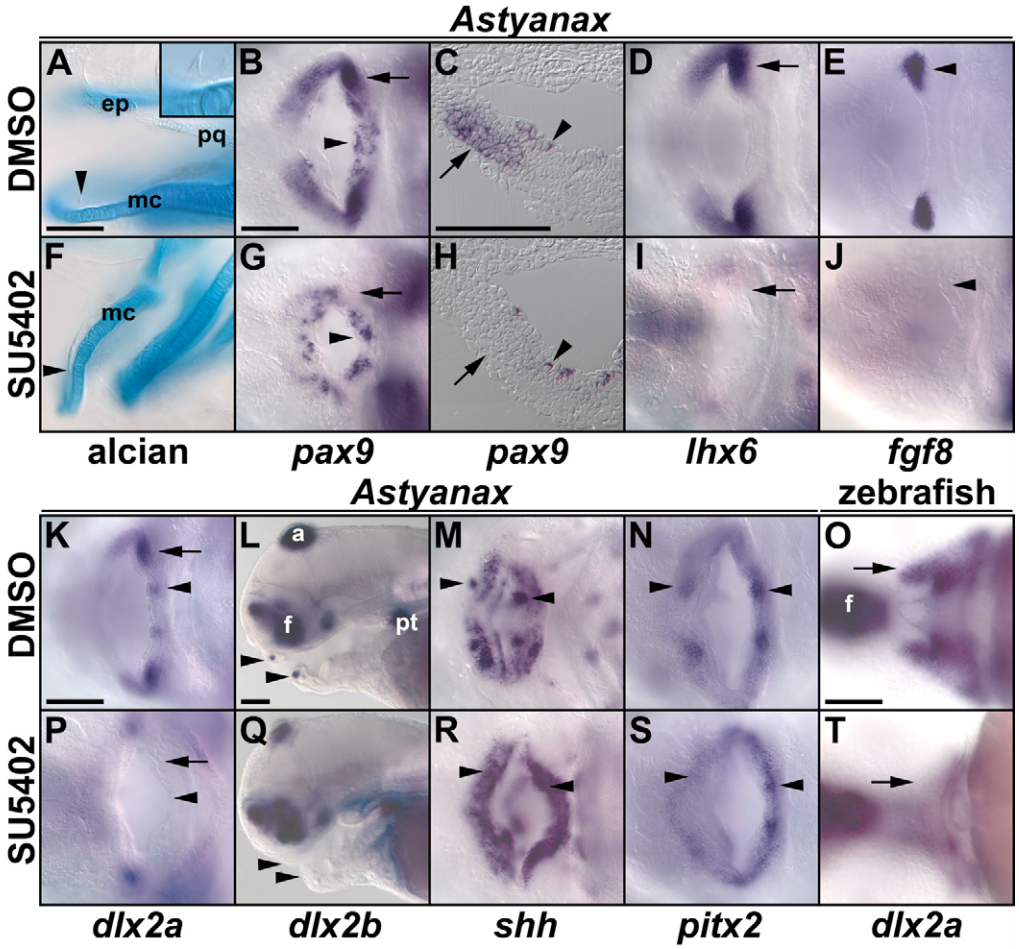

Fig. 7. Targets of Fgf signaling investigated by SU5402 treatment, with DMSO control. (**A**,**F**) Alcian green staining reveals a loss of oral teeth (arrowhead and inset in A) following SU5402 treatment. Arrowhead indicates dentary bone in F. (**B**,**C**,**G**,**H**) Mesenchymal (arrow), but not epithelial (arrowhead), expression of *Astyanax pax9* is absent after SU5402 treatment. Mesenchymal expression of *lhx6* (**D**,**I**, arrows), epithelial expression of *fgf8* (**E**,**J**, arrowheads) and *dlx2b* (**L**,**Q**, arrowheads), and both mesenchymal (arrow) and epithelial (arrowhead) expression of *dlx2a* (**K**,**P**) in the oral region are absent after SU5402 treatment in *Astyanax*. (**M**,**N**,**R**,**S**) Oral epithelial expression of *shh* and *pitx2* remains after SU5402 treatment in *Astyanax*. (**O**,**T**) Mandibular arch mesenchymal expression of *dlx2a* (arrow) in the zebrafish is absent after SU5402 treatment. (B,D-E,G,I-K,M-P,R-T) Whole-mount ventral views; (A,F,L,Q) lateral views; (C,H) transverse sections. Abbreviations as in Fig. 1. a, adhesive gland; f, forebrain; pq, palatoquadrate; pt, pharyngeal tooth. Scale bars: 100 μm.

represent residual tooth initiation, but rather reflects other roles in jaw skeletal development (Peters et al., 1998; Trumpp et al., 1999). Interestingly, *Pax9* is required for dental mesenchyme condensation in the mouse (Peters et al., 1998), a process that may not occur in some teleosts (Huysseune and Sire, 1997; Huysseune et al., 1998).

Loss of epithelial Dlx2 expression is associated with cypriniform oral tooth loss, but is unlikely to be the sole cause

Oral epithelial expression of Dlx2 orthologs is absent from zebrafish but present in two ostariophysans with oral teeth (*Astyanax* and *Synodontis*), and in two outgroups, the medaka and the mouse (Thomas et al., 2000). These data are consistent with loss of cypriniform *dlx2a* and *dlx2b* expression in association with oral tooth loss. Several lines of evidence suggest that loss of Dlx2 expression was not the sole cause of tooth loss, however. *Dlx2* does not have a unique function in mouse tooth development, as its inactivation has no effect on dentition (Qiu et al., 1995). Simultaneous inactivation of *Dlx1* and *Dlx2* leads to an early arrest of upper molar teeth, but this effect is believed to reflect their requirement in mesenchyme (Thomas et al., 1997). However, other Dlx genes are expressed in mouse dental epithelium (Zhao et al., 2000), where they may function redundantly with Dlx2. Similarly, injection of morpholino antisense oligonucleotides targeting zebrafish *dlx2a* and *dlx2b* altered cartilage but not pharyngeal tooth development (W.R.J. and D.W.S., unpublished). Finally, coordinate loss of *dlx2a* and *dlx2b* requires only one change in a (common) trans-acting regulator, as opposed to independent changes in the cis-regulatory region of each gene.

Loss of Fgf signaling is a candidate cause of cypriniform oral tooth loss

One trans-acting process whose alteration could have caused cypriniform tooth loss is Fgf signaling. *dlx2a* and *dlx2b* expression in zebrafish pharyngeal teeth is Fgf dependent (Jackman et al., 2004), and we found a similar dependence in *Astyanax* oral teeth. Furthermore, Shh and Pitx2 expression persists after SU5402 treatment in both species, as it does in the mouse (Mandler and Neubüser, 2001). In *Astyanax*, focal expression frequently did not appear, leaving expression domains similar to those found in

untreated zebrafish. These data are consistent with the hypothesis that a loss of Fgf signaling caused the loss of Dlx2 ortholog expression in cypriniform oral epithelium without abolishing *shh* and *pitx2* expression. Our results with other genes assayed after SU5402 treatment suggest, however, that global loss of Fgf expression did not occur in the cypriniform mouth. Mandibular arch expression of *dlx2a*, *lhx6* and *pax9* in mesenchyme, and *fgf8* in epithelium, has been retained in zebrafish but is Fgf dependent in both zebrafish and *Astyanax*. Mandler and Neubüser (Mandler and Neubüser, 2001) found a similar Fgf dependence for mouse *Dlx2*, *Lhx6* and *Pax9*, but not *Fgf8*. However, the different results for Fgf8 may reflect differences in the developmental stage of treatment.

Our data can be reconciled with a loss of Fgf signaling causing cypriniform tooth loss if there are distinct signals separated by (1) the tissue of origin, (2) position along the mediolateral axis, (3) time, and/or (4) the ligand or receptor involved. Such a hypothesis is illustrated in Fig. 8, in which three Fgf signals are present in the jaw of toothed teleosts and one of these has been lost in cypriniforms. Of the conserved signals, one that induces mesenchymal Dlx2a, Lhx6 and Pax9 is likely to originate in the epithelium, as has been shown for the mouse (Neubüser et al., 1997; Trumpp et al., 1999; Abu-Issa et al., 2002). We speculate that a separate conserved signal inducing epithelial Fgf8 originates from the mesenchyme based on the mesenchymal dependence of oral *Fgf8* expression in the mouse (Creuzet et al., 2004) and induction by mouse mesenchymal cells of ectopic *Fgf8* expression in chick facial primordia (Mitsiadis et al., 2003). The Fgf signal we propose to have been lost from cypriniforms, one inducing epithelial Dlx2a and Dlx2b expression, may originate medial to the former signals based on our gene expression comparisons. We speculate that the source of this is the mesenchyme, through analogy with feather development, which exhibits numerous similarities to that of teeth (Pispa and Thesleff, 2003). Specifically, Fgf signaling from the feather mesenchyme to epithelium is required for organ initiation (Mandler and Neubüser, 2004) and the induction of epithelial *Dlx2* expression (Rouzankina et al., 2004).

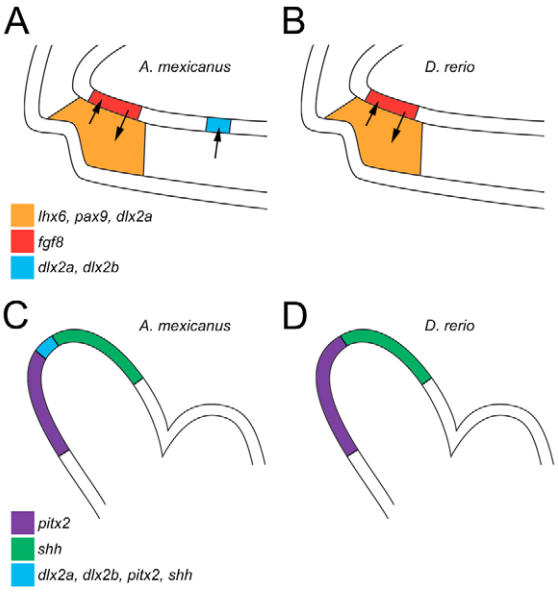

Fig. 8. Comparison of gene expression and hypothesized Fgf signals (arrows) between *Astyanax* and the zebrafish. (**A**,**B**) Transverse views of the left side of the mandible. Lateral epithelial and mesenchymal gene expression common to both species is Fgf dependent. Loss of a medial Fgf signal to the epithelium is hypothesized to have caused cypriniform tooth loss. See text for basis of hypothesized ligand sources. (**C**,**D**) Lateral views of selected features of mandibular epithelial expression. *pitx2* and *shh* expression common to both species is Fgf independent. The zebrafish may lack a domain of overlapping *pitx2* and *shh* expression corresponding to a tooth germ (marked by *dlx2a* and *dlx2b* expression in *Astyanax*).

Multiple paths to tooth loss

The developmental genetic basis of tooth loss has been studied in the chicken (Chen et al., 2000; Mitsiadis et al., 2003; Harris et al., 2006) and the diastema (gap in dentition) of rodents (Keränen et al., 1999). Cypriniform tooth loss appears to differ in mechanism from both of these examples. In contrast to the zebrafish oral region, bud stage rudiments appear in the rodent diastema. In addition, diastemal epithelium exhibits discrete *Shh* and *Pitx2* expression at late stages (Keränen et al., 1999), and *Dlx2* expression at early stages (Thomas et al., 2000). Various (and not mutually exclusive) mechanisms have been proposed for tooth loss in birds, including loss of *Bmp4* signaling from the epithelium (Chen et al., 2000), loss of a mesenchyme-to-epithelium signal (Mitsiadis et al., 2003) and change in the position of an epithelial signaling center (Harris et al., 2006). Although the mandibular arch expression pattern of *Dlx2* has not been described in detail in the chicken, this species differs from the zebrafish in the presence of a rudimentary dental lamina expressing *Shh* and *Pitx2* (Helms et al., 1997; Chen et al., 2000; Harris et al., 2006). We conclude that tooth development proceeds to later stages in birds and the rodent diastema than in cypriniform oral jaws, although such differences may have accumulated after the initial loss of functional teeth.

Is cypriniform tooth loss reversible?

It has been argued that tooth loss in birds is evolutionarily irreversible because of the genetic drift-induced inactivation of tooth-specific genes (Marshall et al., 1994). Consistent with this view, ectopic protein expression and a single gene mutation can produce tooth rudiments in chickens (Chen et al., 2000; Harris et al., 2006) but not fully formed teeth. Even associations of mouse and chicken tissues that form tooth-like structures with dentine fail to form enamel (Mitsiadis et al., 2003).

In contrast to birds, zebrafish retain the genetic information necessary to make pharyngeal teeth and the data available to date suggest that this information is similar to that used in controlling oral tooth development (Jackman et al., 2004; Fraser et al., 2004). The number of genes that must be redeployed in the cypriniform oral region to produce teeth remains unknown, however. We documented differences in the expression of *pitx2*, *shh*, *dlx2a* and *dlx2b* in zebrafish and in teleosts with oral teeth, and showed that all could potentially result from a single upstream genetic change, namely a loss of Fgf signaling. Such a change might be reversible in evolution; for example, through the de novo appearance of an enhancer in a gene in the Fgf pathway. This potential ease of tooth re-acquisition conflicts with evidence for tooth loss having constrained the evolution of cypriniform feeding mechanisms. Although cypriniform fishes exhibit a diversity of feeding modes, large fish-eating forms are rare (Sibbing, 1991), and those that exist may be less efficient predators than teleosts retaining oral teeth (Portz and Tyus, 2004). Investigation of additional genetic pathways of tooth development in the zebrafish oral region and particularly gain-of-function experiments should further help to distinguish between adaptation and developmental constraint as explanations for the pattern of cypriniform dental evolution.

We thank William Jeffery, Allen Strickler, and especially Yoshiyuki Yamamoto for providing many of the *Astyanax* specimens used, and for helping to establish our colony of this species. *Synodontis* specimens were a generous gift from Alexander Cruz. Sarah Wise provided valuable comments on the manuscript. This work was supported by a grant-in-aid from the University of Colorado Council on Research and Creative Work to D.W.S., NSF grants IBN-0092487 and IBN-0446720 to D.W.S., and an NIH National Research Service Award (5F32DE015029) from the NIDCR to W.R.J. In addition, Kenneth Weiss provided support (through NSF SBR 9408402) during early stages of the project.

References

Abu-Issa, R., Smyth, G., Smoak, I., Yamamura, K. I. and Meyers, E. N. (2002). *Fgf8* is required for pharyngeal arch and cardiovascular development in the mouse. *Development* **129**, 4613-4625.

Akimenko, M.-A., Ekker, M., Wegner, J., Lin, W. and Westerfield, M. (1994). Combinatorial expression of three zebrafish genes related to *Distal-less*: part of a homeobox gene code for the head. *J. Neurosci.* **14**, 3475-3486.

Arakawa, H., Nakamura, T., Zhadanov, A. B., Fidanza, V., Yano, T., Bullrich, F., Shimizu, M., Blechman, J., Mazo, A., Canaani, E. et al. (1998). Identification and characterization of the *ARP1* gene, a target for the human acute leukemia *ALL1* gene. *Proc. Natl. Acad. Sci. USA* **95**, 4573-4578.

Cavender, T. M. (1991). The fossil record of the Cyprinidae. In *Cyprinid Fishes: Systematics, Biology and Exploitation* (ed. I. J. Winfield and J. S. Nelson), pp. 34-54. New York: Chapman & Hall.

Chen, Y., Zhang, Y., Jiang, T.-X., Barlow, A. J., St Amand, T. R., Hu, Y., Heaney, S., Francis-West, P., Chuong, C.-M. and Maas, R. (2000). Conservation of early odontogenic signaling pathways in Aves. *Proc. Natl. Acad. Sci. USA* **97**, 10044-10049.

Cobourne, M. T., Miletich, I. and Sharpe, P. T. (2004). Restriction of sonic hedgehog signaling during early tooth development. *Development* **131**, 2875-2885.

Creuzet, S., Schuler, B., Couly, G. and Le Douarin, N. M. (2004). Reciprocal relationships between Fgf8 and neural crest cells in facial and forebrain development. *Proc. Natl. Acad. Sci. USA* **101**, 4843-4847.

Dassule, H. R. and McMahon, A. P. (1998). Analysis of epithelial-mesenchymal interactions in the initial morphogenesis of the mammalian tooth. *Dev. Biol.* **202**, 215-227.

Dowling, T. E., Martasian, D. P. and Jeffery, W. R. (2002). Evidence for multiple genetic forms with similar eyeless phenotypes in the blind cavefish, *Astyanax mexicanus*. *Mol. Biol. Evol.* **19**, 446-455.

Eberhart, J. K., Swartz, M. E., Crump, J. G. and Kimmel, C. B. (2006). Early Hedgehog signaling from neural to oral epithelium organizes anterior craniofacial development. *Development* **133**, 1069-1077.

Ellies, D. L., Stock, D. W., Hatch, G., Giroux, G., Weiss, K. M. and Ekker, M. (1997). Relationship between the genomic organization and the overlapping embryonic expression patterns of the zebrafish *dlx* genes. *Genomics* **45**, 580-590.

Essner, J. J., Branford, W. W., Zhang, J. and Yost, H. J. (2000). Mesendoderm and left-right brain, heart and gut development are differentially regulated by *pitx2* isoforms. *Development* **127**, 1081-1093.

Fink, S. V. and Fink, W. L. (1996). Interrelationships of ostariophysan fishes (Teleostei). In *Interrelationships of Fishes* (ed. M. L. J. Stiassny, L. R. Parenti and G. D. Johnson), pp. 209-249. New York: Academic Press.

Fraser, G. J., Graham, A. and Smith, M. M. (2004). Conserved deployment of genes during odontogenesis across osteichthyans. *Proc. R. Soc. Lond. B Biol. Sci.* **271**, 2311-2317.

Gage, P. J., Suh, H. and Camper, S. A. (1999). The *bicoid*-related Pitx gene family in development. *Mamm. Genome* **10**, 197-200.

Hanken, J. and Wassersug, R. (1981). The visible skeleton. *Funct. Photog.* **16**, 22-26, 44.

Hardcastle, Z., Mo, R., Hui, C.-C. and Sharpe, P. T. (1998). The Shh signalling pathway in tooth development: defects in *Gli2* and *Gli3* mutants. *Development* **125**, 2803-2811.

Harris, M. P., Hasso, S. M., Ferguson, M. W. J. and Fallon, J. F. (2006). The development of archosaurian first-generation teeth in a chicken mutant. *Curr. Biol.* **16**, 371-377.

Helms, J. A., Kim, C. H., Hu, D., Minkoff, R., Thaller, C. and Eichele, G. (1997). *Sonic hedgehog* participates in craniofacial morphogenesis and is down-regulated by teratogenic doses of retinoic acid. *Dev. Biol.* **187**, 25-35.

Hu, D., Marcucio, R. S. and Helms, J. A. (2003). A zone of frontonasal ectoderm regulates patterning and growth in the face. *Development* **130**, 1749-1758.

Huysseune, A. and Sire, J.-Y. (1997). Structure and development of first-generation teeth in the cichlid *Hemichromis bimaculatus* (Teleostei, Cichlidae). *Tissue Cell* **29**, 679-697.

Huysseune, A. and Sire, J.-Y. (1998). Evolution of patterns and processes in teeth and tooth-related tissues in non-mammalian vertebrates. *Eur. J. Oral Sci.* **106** Suppl 1, 437-481.

Huysseune, A., Van der heyden, C. and Sire, J.-Y. (1998). Early development of the zebrafish (*Danio rerio*) pharyngeal dentition (Teleostei, Cyprinidae). *Anat. Embryol.* **198**, 289-305.

Jackman, W. R., Draper, B. W. and Stock, D. W. (2004). Fgf signaling is required for zebrafish tooth development. *Dev. Biol.* **274**, 139-157.

Jeffery, W. R. (2001). Cavefish as a model system in evolutionary developmental biology. *Dev. Biol.* **231**, 1-12.

Jeffery, W. R. and Martasian, D. P. (1998). Evolution of eye regression in the cavefish *Astyanax*: apoptosis and the *Pax-6* gene. *Am. Zool.* **38**, 685-696.

Jeong, J., Mao, J., Tenzen, T., Kottmann, A. H. and McMahon, A. P. (2004). Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordial. *Genes Dev.* **18**, 937-951.

Jernvall, J. and Thesleff, I. (2000). Reiterative signaling and patterning during mammalian tooth morphogenesis. *Mech. Dev.* **92**, 19-29.

Keränen, S. V. E., Kettunen, P., Aberg, T., Thesleff, I. and Jernvall, J. (1999). Gene expression patterns associated with suppression of odontogenesis in mouse and vole diastema regions. *Dev. Genes Evol.* **209**, 495-506.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

Kumar, S., Tamura, K., Jakobsen, I. B. and Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244-1245.

Liu, W., Selever, J., Lu, M.-F. and Martin, J. F. (2003). Genetic dissection of *Pitx2* in craniofacial development uncovers new functions in branchial arch morphogenesis, late aspects of tooth morphogenesis and cell migration. *Development* **130**, 6375-6385.

Lu, M.-F., Pressman, C., Dyer, R., Johnson, R. L. and Martin, J. F. (1999). Function of Reiger syndrome gene in left-right asymmetry and craniofacial development. *Nature* **401**, 276-278.

Mandler, M. and Neubüser, A. (2001). FGF signaling is necessary for the specification of the odontogenic mesenchyme. *Dev. Biol.* **240**, 548-559.

Mandler, M. and Neubüser, A. (2004). FGF signaling is required for initiation of feather placode development. *Development* **131**, 3333-3343.

Marshall, C. R., Raff, E. C. and Raff, R. A. (1994). Dollo's law and the death and resurrection of genes. *Proc. Natl. Acad. Sci. USA* **91**, 12283-12287.

Meyer, A. and Van de Peer, Y. (2005). From 2R to 3R: evidence for a fish-specific genome duplication (FSGD). *BioEssays* **27**, 937-945.

Miller, C. T., Schilling, T. F., Lee, K.-H., Parker, J. and Kimmel, C. B. (2000). *sucker* encodes a zebrafish Endothelin-1 required for ventral pharyngeal arch development. *Development* **127**, 3815-3828.

Miller, C. T., Maves, L. and Kimmel, C. B. (2004). *moz* regulates Hox expression and pharyngeal segment identity in zebrafish. *Development* **131**, 2443-2461.

Mitsiadis, T. A., Chéraud, Y., Sharpe, P. and Fontaine-Pérus, J. (2003). Development of teeth in chick embryos after mouse neural crest transplantations. *Proc. Natl. Acad. Sci. USA* **100**, 6541-6545.

Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R. and Schlessinger, J. (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955-960.

Moore-Scott, B. A. and Manley, N. R. (2005). Differential expression of Sonic hedgehog along the anterior-posterior axis regulates patterning of pharyngeal pouch endoderm and pharyngeal endoderm-derived organs. *Dev. Biol.* **278**, 3232-3235.

Mucchielli, M.-L., Mitsiadis, T. A., Raffo, S., Brunet, J.-F., Proust, J.-P. and Goridis, C. (1997). Mouse *Otlx2/RIEG* expression in the odontogenic epithelium precedes tooth initiation and requires mesenchyme-derived signals for its maintenance. *Dev. Biol.* **189**, 275-284.

Nelson, J. S. (1994). *Fishes of the World* (3rd edn). New York: John Wiley & Sons.

Neubüser, A., Peters, H., Balling, R. and Martin, G. R. (1997). Antagonistic interactions between FGF and BMP signaling pathways: a mechanism for positioning the sites of tooth formation. *Cell* **90**, 247-255.

Peters, H., Neubüser, A., Kratochwil, K. and Balling, R. (1998). *Pax9*-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes Dev.* **12**, 2735-2747.

Pispa, J. and Thesleff, I. (2003). Mechanisms of ectodermal organogenesis. *Dev. Biol.* **262**, 195-205.

Portz, D. E. and Tyus, H. M. (2004). Fish humps in two Colorado River fishes: a morphological response to cyprinid predation? *Environ. Biol. Fishes* **71**, 233-245.

Qiu, M., Bulfone, A., Martinez, S., Meneses, J. J., Shimamura, K., Pedersen, R. A. and Rubenstein, J. L. R. (1995). Null mutation of *Dlx-2* results in abnormal morphogenesis of proximal first and second branchial arch derivatives and abnormal differentiation in the forebrain. *Genes Dev.* **9**, 2523-2538.

Qiu, M., Bulfone, A., Ghattas, I., Meneses, J. J., Christensen, L., Sharpe, P. T., Presley, R., Pedersen, R. A. and Rubenstein, J. L. R. (1997). Role of the Dlx homeobox genes in proximodistal patterning of the branchial arches: mutations of Dlx-1, Dlx-2, and Dlx-1 and –2 alter morphogenesis of proximal skeletal and soft tissue structures derived from the first and second arches. *Dev. Biol.* **185**, 165-184.

Rouzankina, I., Abate-Shen, C. and Niswander, L. (2004). *Dlx* genes integrate

positive and negative signals during feather bud development. *Dev. Biol*. **265**, 219-233.
Saitoh, K., Miya, M., Inoue, J. G., Ishiguro, N. B. and Nishida, M. (2003). Mitochondrial genomics of ostariophysan fishes: perspectives on phylogeny and biogeography. *J. Mol. Evol.* **56**, 464-472.
Sarkar, L., Cobourne, M., Naylor, S., Smalley, M., Dale, T. and Sharpe, P. T. (2000). Wnt/Shh interactions regulate ectodermal boundary formation during mammalian tooth development. *Proc. Natl. Acad. Sci. USA* **97**, 4520-4524.
Schilling, T. F. (2002). The morphology of larval and adult zebrafish. In *Zebrafish: A Practical Approach* (ed. C. Nüsslein-Volhard and R. Dahm), pp. 59-94. New York: Oxford University Press.
Sibbing, F. A. (1991). Food capture and oral processing. In *Cyprinid Fishes: Systematics, Biology and Exploitation* (ed. I. J. Winfield and J. S. Nelson), pp. 377-412. New York: Chapman & Hall.
Sire, J.-Y. (2001). Teeth outside the mouth in teleost fishes: how to benefit from a developmental accident. *Evol. Dev*. **3**, 104-108.
Sire, J.-Y., Davit-Beal, T., Delgado, S., Van der heyden, C. and Huysseune, A. (2002). First-generation teeth in nonmammalian lineages: evidence for a conserved ancestral character. *Microsc. Res. Tech*. **59**, 408-434.
Stock, D. W. (2001). The genetic basis of modularity in the development and evolution of the vertebrate dentition. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **356**, 1633-1653.
Stock, D. W., Ellies, D. L., Zhao, Z., Ekker, M., Ruddle, F. H. and Weiss, K. M. (1996). The evolution of the vertebrate Dlx gene family. *Proc. Natl. Acad. Sci. USA* **93**, 10858-10863.
Stockton, D. W., Das, P., Goldenberg, M., D'Souza, R. N. and Patel, P. I. (2000). Mutation of *PAX9* is associated with oligodontia. *Nat. Genet*. **24**, 18-19.
Thomas, B. L., Tucker, A. S., Qiu, M., Ferguson, C. A., Hardcastle, Z., Rubenstein, J. L. R. and Sharpe, P. T. (1997). Role of the *Dlx-1* and *Dlx-2* genes in patterning of the murine dentition. *Development* **124**, 4811-4818.
Thomas, B. L., Liu, J. K., Rubenstein, J. L. R. and Sharpe, P. T. (2000). Independent regulation of *Dlx2* expression in the epithelium and mesenchyme of the first branchial arch. *Development* **127**, 217-224.
Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876-4882.
Trapani, J., Yamamoto, Y. and Stock, D. W. (2005). Ontogenetic transition from unicuspid to multicuspid oral dentition in a teleost fish: *Astyanax mexicanus*, the Mexican tetra (Ostariophysi: Characidae). *Zool. J. Linn. Soc*. **145**, 523-538.
Trumpp, A., Depew, M. J., Rubenstein, J. L. R., Bishop, J. M. and Martin, G. R. (1999). Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. *Genes Dev*. **13**, 3136-3148.
Tucker, A. and Sharpe, P. (2004). The cutting-edge of mammalian development; how the embryo makes teeth. *Nat. Rev. Genet*. **5**, 499-508.
Valdéz-Moreno, M. E. and Contreras-Balderas, S. (2003). Skull osteology of the characid fish *Astyanax mexicanus* (Teleostei: Characidae). *Proc. Biol. Soc. Wash*. **116**, 341-355.
Van der heyden, C. and Huysseune, A. (2000). Dynamics of tooth formation and replacement in the zebrafish (*Danio rerio*) (Teleostei, Cyprinidae). *Dev. Dyn*. **219**, 486-496.
Wada, N., Javidan, Y., Nelson, S., Carney, T. J., Kelsh, R. N. and Schilling, T. F. (2005). Hedgehog signaling is required for cranial neural crest morphogenesis and chondrogenesis at the midline in the zebrafish skull. *Development* **132**, 3977-3988.
Wittbrodt, J., Shima, A. and Schartl, M. (2002). Medaka – a model organism from the Far East. *Nat. Rev. Genet*. **3**, 53-64.
Yamamoto, Y., Stock, D. W. and Jeffery, W. R. (2004). Hedgehog signaling controls eye degeneration in blind cavefish. *Nature* **431**, 844-847.
Zardoya, R., Abouheif, E. and Meyer, A. (1996). Evolutionary analyses of hedgehog and *Hoxd-10* genes in fish species closely related to the zebrafish. *Proc. Natl. Acad. Sci. USA* **93**, 13036-13041.
Zhang, Y. D., Chen, Z., Song, Y. Q., Liu, C. and Chen, Y. P. (2005). Making a tooth: growth factors, transcription factors, and stem cells. *Cell Res.* **15**, 301-316.
Zhao, Z., Stock, D. W., Buchanan, A. V. and Weiss, K. M. (2000). Expression of Dlx genes during the development of the murine dentition. *Dev. Genes Evol*. **210**, 270-275.