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RESEARCH ARTICLE

Roles for FGF in lamprey pharyngeal pouch formation and skeletogenesis highlight ancestral functions in the vertebrate head

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ABSTRACT

A defining feature of vertebrates (craniates) is a pronounced head supported and protected by a cellularized endoskeleton. In jawed vertebrates (gnathostomes), the head skeleton is made of rigid three-dimensional elements connected by joints. By contrast, the head skeleton of modern jawless vertebrates (agnathans) consists of thin rods of flexible cellular cartilage, a condition thought to reflect the ancestral vertebrate state. To better understand the origin and evolution of the gnathostome head skeleton, we have been analyzing head skeleton development in the agnathan, lamprey. The fibroblast growth factors *FGF3* and *FGF8* have various roles during head development in jawed vertebrates, including pharyngeal pouch morphogenesis, patterning of the oral skeleton and chondrogenesis. We isolated lamprey homologs of *FGF3*, *FGF8* and FGF receptors and asked whether these functions are ancestral features of vertebrate development or gnathostome novelties. Using gene expression and pharmacological agents, we found that proper formation of the lamprey head skeleton requires two phases of FGF signaling: an early phase during which FGFs drive pharyngeal pouch formation, and a later phase when they directly regulate skeletal differentiation and patterning. In the context of gene expression and functional studies in gnathostomes, our results suggest that these roles for FGFs arose in the first vertebrates and that the evolution of the jaw and gnathostome cellular cartilage was driven by changes developmentally downstream from pharyngeal FGF signaling.

KEY WORDS: Lamprey, FGF, Pharynx, Cartilage, Vertebrate evolution

INTRODUCTION

Based on the fossil record and the morphology of living chordates, it has been proposed that the head skeleton of the first vertebrates consisted of thin bars of flexible cellular cartilage (Mallatt and Chen, 2003; Shu et al., 2003). This primitive skeleton provided elastic recoil for pharyngeal pumping, which facilitated gill ventilation and filter feeding (Gans and Northcutt, 1983). In the gnathostome (jawed vertebrate) lineage, both the composition and organization of the vertebrate pharyngeal skeleton were modified. The simple bars supporting each pharyngeal arch were replaced by multiple articulating elements with complex three-dimensional morphologies. Concurrently, flexible elastin-rich cellular cartilage was replaced with a mixture of rigid collagen-rich cartilage and intervening soft joint tissue. Precisely how these changes occurred is unknown, but they presumably involved alterations to both head skeleton patterning and the genetic program underlying cellular cartilage differentiation.

In jawed vertebrates, the fibroblast growth factors *FGF3* and *FGF8* are expressed in pharyngeal endoderm and ectoderm, and their loss leads to defects in both head skeleton organization and cartilage differentiation. In zebrafish and mouse, these defects are accompanied by disruptions in pharyngeal pouch formation, suggesting that FGFs help pattern the head skeleton in part by mediating pharyngeal segmentation (Abu-Issa et al., 2002; Crump et al., 2004; Walshe and Mason, 2003). Evidence also indicates that FGF signals act directly on skeletogenic cranial neural crest cells (NCCs) to regulate head skeleton development. In zebrafish, chick and mouse, FGF receptors (FGFRs) are expressed by cranial NCCs (Abu-Issa et al., 2002; Sarkar et al., 2001; Yamauchi et al., 2011), and exposure of chick mandibular mesenchyme to ectopic FGFs inhibits expression of *Bapx* and formation of the jaw joint (Wilson and Tucker, 2004). FGFs also perform a patterning function in mouse, in which different levels of FGF8 signaling modulate gene expression and odontogenic potential along the rostral-caudal axis in mandibular arch NCCs (Tucker et al., 1999). In zebrafish, evidence supports a more general role for FGFs in specifying skeletogenic fate. Expression of dominant-negative FGFRs in zebrafish NCCs causes broad downregulation of the chondrogenic NCC marker *dlx2* (Das and Crump, 2012), and treatment with the FGFR inhibitor SU5402 causes loss of *barx* expression in chondrogenic NCCs and complete failure of chondrogenesis (Sperber and Dawid, 2008; Walshe and Mason, 2003). Similarly, in chick, transfection of mandibular NCCs with dominant-negative FGFRs results in locally reduced *Sox9*, *Runx* and *Aggrecan* expression and hypoplastic cartilage elements (Havens et al., 2008). Furthermore, under cell culture conditions, chick NCC-derived prechondrocytes require FGF signaling for survival, *Sox9* expression and overt chondrogenesis, and *Fgf8* is sufficient to induce *Barx* expression (Barlow et al., 1999; Sarkar et al., 2001).

To better understand the evolution of the vertebrate head skeleton we, and others, have been investigating skeletogenesis in the jawless vertebrate, lamprey. Lamprey diverged from gnathostomes shortly after vertebrate origins and is the most basal vertebrate readily accessible to embryonic manipulation. Although lamprey possesses a head skeleton incorporating cellular cartilage it is thought to retain the basic structure and function of the head skeleton of early vertebrates. In contrast to the rigid, jointed cartilage elements of gnathostomes, the pharyngeal component of the lamprey head skeleton consists of thin rods of cellular cartilage that secrete an

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Development

extracellular matrix (ECM) made mainly of elastins and elastin-like proteins, with little fibrillar collagen, the main protein component of gnathostome cellular cartilage ECM (Martin et al., 2009; McBurney and Wright, 1996; McCauley, 2008; Robson et al., 1993; Yao et al., 2011). At the genetic level, although the lamprey head skeleton requires *SoxE* function to develop (Lakiza et al., 2011; McCauley and Bronner-Fraser, 2006), lamprey cellular cartilage does not express *Runx* or *Barx*, which are essential for gnathostome chondrogenesis (Cattell et al., 2011). Finally, while the lamprey head skeleton includes cellular cartilage in the pharynx, the oral region and ventral pharynx is supported by a soft mesenchymal skeletal tissue called mucocartilage (Johnels, 1948). Although the relationship of mucocartilage to bona fide cellular cartilage is still unclear, all mucocartilage expresses *SoxE* genes and some portions express *Runx* and *Barx*, suggesting that it utilizes a cellular cartilage-like gene regulatory network (GRN) (Cattell et al., 2011).

Although lamprey is a modern vertebrate with a highly specialized anatomy and lifestyle, comparisons between lamprey and gnathostomes can identify conserved features of vertebrate development as well as potential novelties. Using gene expression and pharmacological perturbations, we tested whether FGF signaling is involved in lamprey pharyngeal morphogenesis, head skeleton patterning and/or chondrogenesis. At early stages of head development, we observed endodermal expression of FGF signaling components, regulation of *FGF3* and *FGF8/17/18* by retinoic acid (RA) signaling, and a loss of pharyngeal pouch formation in FGFR inhibitor-treated embryos. These data suggest early roles for FGF and RA signaling in pharyngeal segmentation in lamprey. By exploiting the slow development of lamprey, we then show a temporally distinct role for FGFs in the development of lamprey skeletal tissue. We find that after pharyngeal segmentation is complete, lamprey FGFRs are activated throughout skeletogenic NCCs, and FGF signaling inhibition during this period reduces prechondrocyte marker expression and the differentiation of Alcian Blue-reactive chondrocytes. We also find that, as with bona fide cellular cartilage, FGFs are required for the full development of mucocartilage around the mouth, supporting an evolutionary relationship between the two tissues. Finally, we noted a role for FGFs in maintaining proper gene expression in a portion of the oral skeleton, suggesting a gnathostome-type patterning function in the oral region. Taken together, these results reveal conserved roles for FGF signaling in pharyngeal segmentation, chondrogenesis and oral skeleton patterning dating to the first jawless vertebrates. The results suggest that the evolution of the gnathostome head skeleton is likely to have involved changes to FGF target genes such as *Bapx* and *Barx*.

RESULTS
The isolation and expression of lamprey *FGF3* and *FGF8* orthologs in embryos and larvae
Jawed vertebrates possess more than 20 FGFs involved in a range of developmental processes (Itoh and Ornitz, 2008). A few are expressed in the pharyngeal arches (Lea et al., 2009). Of these, only *FGF3* and *FGF8* have functionally validated roles in head skeleton development or pharyngeal morphogenesis. We thus searched the preassembly sea lamprey genome and identified single *FGF3* and *FGF8* cognates. Phylogenetic analyses confirmed the homology of lamprey and gnathostome *FGF3* genes (supplementary material Fig. S1). The lamprey *FGF8* homolog was placed as an outgroup to gnathostome *FGF8* and its paralogs *FGF17* and *FGF18* (supplementary material Fig. S1). We thus designated this gene *FGF8/17/18*.

We then examined the expression of these factors at embryonic and larval stages. Using whole-mount *in situ* hybridization, we
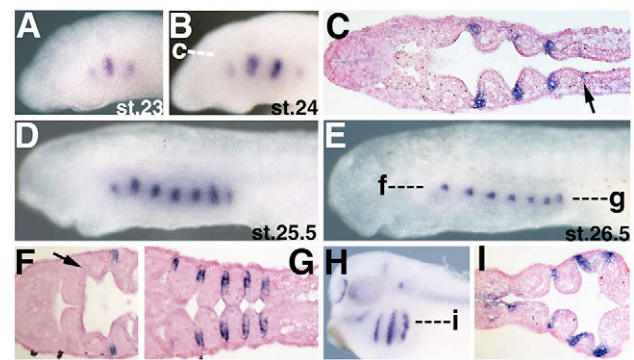

Fig. 1. Expression of lamprey and *X. laevis FGF3*. (A,B,D,E) Lamprey *FGF3* expression at the indicated stages. (C) Section at the level of c in B. Arrow indicates *FGF3* expression in the forming fifth pouch. (F,G) Sections at the levels of f and g in E. First pharyngeal pouch expression has been lost (arrow). (H) *FGF3* expression in *X. laevis* pharynx at st. 33. (I) Section at the level of i in H.

first observed *FGF3* expression in the pharynx at Tahara stage (st.) 23 (Fig. 1A). Sectioning at st. 24 revealed expression in the posterior aspect of each pharyngeal pouch (Fig. 1B,C). *FGF3* transcripts were also observed in a patch of posterior pharyngeal endoderm, presumably presaging formation of the fifth pharyngeal pouch. Pouch expression was observed throughout pharyngogenesis, which ends around st. 26, and persisted until the early stages of chondrogenesis at approximately st. 26.5 (Cattell et al., 2011) (Fig. 1D-G). Although iterated pharyngeal expression of *FGF3* is seen in gnathostomes, we could find no description of lamprey-like expression in the posterior aspect of the pharyngeal pouches. We thus examined *FGF3* expression in *Xenopus laevis* and confirmed posteriorly restricted pharyngeal pouch expression identical to that of lamprey *FGF3* (Fig. 1H,I).

Expression of lamprey *FGF8/17/18* was observed at the early neurula stage (st. 20) in the putative midbrain/hindbrain boundary and presomitic mesoderm (data not shown). Pharyngeal expression begins at st. 21 (data not shown) as a single band, with one to three additional bands added at each subsequent stage until the end of pharyngeal segmentation (Fig. 2A-E). Sectioning at st. 23.5 reveals that transcripts are present throughout the pharyngeal endoderm, but are enriched in the newly formed and nascent pharyngeal pouches (Fig. 2C,I). Endodermal expression persists until st. 26.5, but is lost by st. 27 (Fig. 2F,J). In the epidermal ectoderm, *FGF8/17/18* is first observed in the stomodeal ectoderm. At stage 23.5, spots of expression are observed in pharyngeal ectoderm overlying the first pharyngeal pouch (Fig. 2C). At st. 26.5, transcripts are still apparent in the oral ectoderm (Fig. 2H) and patches of expression are seen overlying every pharyngeal pouch (Fig. 2E,I). These patches are still seen at st. 27, after endodermal expression of *FGF3* has been lost and the pharyngeal pouches are beginning to fuse with the pharyngeal ectoderm, forming the gill slits (Fig. 2F,J). Ectodermal expression around the mouth and gill slits continues until at least st. 28, the latest stage examined (Fig. 2G). Taken together, the pattern of *FGF8/17/18* expression was virtually identical to that seen for *X. laevis* FGF8 (Fig. 2K,L).

The isolation and expression of lamprey FGFRs in embryos and larvae
To help determine which cells and tissues are capable of responding to *FGF3* and *FGF8/17/18* in the pharynx, we examined the expression of FGFRs in lamprey embryos and larvae. An exhaustive
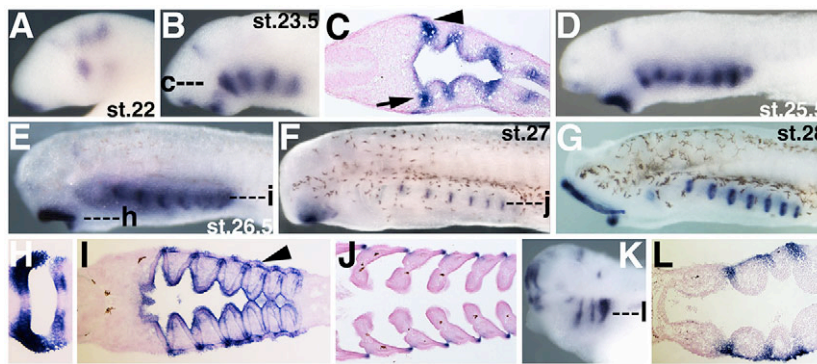
Fig. 2. Expression of lamprey *FGF8/17/18* and *X. laevis* FGF8. (A,B,D-G) Lamprey *FGF8/17/18* expression at the indicated stages. (C) Section through c in B showing endodermal (arrow) and ectodermal (arrowhead) *FGF8/17/18* expression. (H) Section through h in E showing oral ectoderm expression. (I) Section at the level of i in E. Arrowhead indicates ectodermal expression. (J) Section at the level of j in F. (K) Expression of *X. laevis* FGF8 at st. 33. (L) Section through l in K.

search of the lamprey genome identified two lamprey FGFRs, although phylogenetic analyses failed to consistently place the lamprey FGFRs within any single gnathostome paralogy group. We thus designated them *FGFRa* and *FGFRb*. In the youngest embryos examined, namely st. 21 neurulae, *FGFRa* expression was seen throughout the CNS, except for a gap around the mid/hindbrain boundary (data not shown). This broad neural signal was maintained until st. 26. At st. 22, deep expression in the area of the forming pharynx became apparent (Fig. 3A). Sectioning at st. 23.5 reveals *FGFRa* transcripts enriched in the medial pharyngeal arch endoderm and reduced or absent in the apex of the forming pharyngeal pouches (Fig. 3C). At st. 25.5, *FGFRa* transcripts also became apparent in the nascent cranial ganglia, the NCC-derived mesenchyme around the mouth and in the pharyngeal arches, and in the heart (data not shown). At st. 26, *FGFRa* transcripts continue to mark postmigratory NCCs and the nascent heart, while sectioning shows that pharyngeal endoderm expression is reduced (Fig. 3D,E). NCC expression persists into st. 27, when *FGFRa* transcripts mark the condensing prechondrocytes that will generate the pharyngeal gill bars, as well as nascent mucocartilage in the first two arches and oral region (Fig. 3F). As st. 27 progresses, *FGFRa* expression is lost from the gill bars in an anterior to posterior sequence that mirrors the differentiation of the gill bar cartilage (Fig. 3G).

Like *FGFRa*, lamprey *FGFRb* displays broad neural expression and marks the nascent heart from st. 21-26. However, unlike *FGFRa*, strong expression in the pharyngeal endoderm is not observed at these stages. Instead, starting at st. 23.5, *FGFRb*

transcripts are seen in the trigeminal placode, scattered ectodermal cells, ventral pharyngeal mesenchyme (Fig. 4A) and the forming somites (not shown). Later, at st. 24.5, *FGFRb* marks portions of the cranial ganglia, and continues to be expressed in the ventral pharyngeal mesenchyme, which will go on to form mucocartilage in the lower lip and around the endostyle (Fig. 4B,C). At st. 26, *FGFRb* becomes activated in chondrogenic NCCs populating the anterior pharyngeal arches (Fig. 4D). By st. 27, this *FGFRb* expression has spread to chondrogenic NCCs in the posterior arches (Fig. 4E,F). At the latest stage examined, st. 28, *FGFRb* expression around the mouth and in the anterior arches has reduced, although high levels of transcript are still apparent in the anterior CNS, posterior gill bars and posterior mucocartilage (Fig. 4G).

In summary, the expression of lamprey *FGFRa* and *FGFRb* closely mirrored the collective expression of *FGFR1-4* previously reported in gnathostomes, including expression in NCCs, pharyngeal endoderm and brain (Lea et al., 2009).

FGF signaling is necessary for pharyngeal pouch formation in lamprey

In zebrafish, FGF signals are needed for pharyngeal pouch formation (Crump et al., 2004). The spatiotemporal expression of *FGF3*, *FGF8/17/18* and *FGFRa/b* in lamprey suggests a similar role in lamprey pharyngeal segmentation (Fig. 4H). We tested this using SU5402, a potent and specific pharmacological inhibitor of FGFR signaling (Chung et al., 2004; Mandler and Neubüser, 2001; Mohammadi et al., 1997; Pani et al., 2012; Walshe and Mason, 2003). SU5402 is ideally suited for studying the function of FGF during pharyngogenesis as it can be applied at late embryonic and larval stages, avoiding the confounding effects of FGFR inhibition on early processes such as gastrulation and neural induction. We exposed lamprey embryos at various stages before, during and after pharyngogenesis to SU5402 and monitored for disruptions in pharyngeal pouch formation and gene expression. The most extensive treatment, from st. 23-26.5, spanned the entire duration of pharyngogenesis (Fig. 5D). The treatment was tolerated well at early stages, but a lack of detectable pharyngeal segmentation and heart defects became obvious starting at st. 26.5. After st. 28, most of the treated larvae died, probably from cardiovascular defects. Of those that survived, all displayed heart edema and possessed a thin, unsegmented pharynx at st. 30 (4/4). Embryos treated with SU5402 from st. 23-24, when pouches 1-3 form, had a similar failure of pharyngeal segmentation at st. 26.5, as determined by inspection of unstained whole-mount larvae (20/20) and *HhA in situ* hybridization (3/3) (Fig. 5K,L). When the SU5402 treatment window was shifted to st. 24-26.5, the formation of one to three anterior pouches was apparent in unstained larvae (75/75). Closer
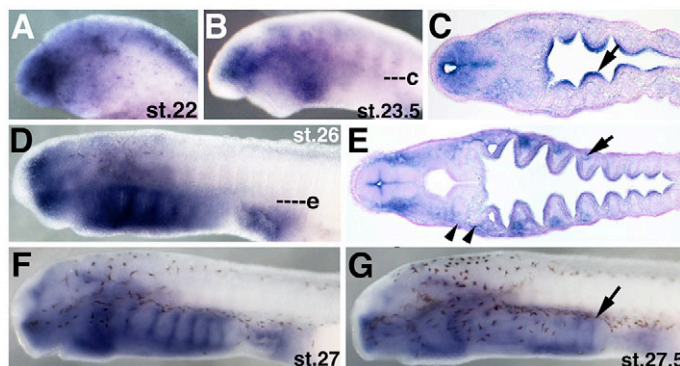

Fig. 3. Lamprey *FGFRa* expression. (A,B,D,F,G) *FGFRa* expression at the indicated stages. (C) Section through c in B shows *FGFRa* expression in medial endoderm (arrow). (E) Section through e in D. Transcripts mark presumptive mucocartilage (arrowheads) and cellular cartilage (arrow). Arrow in G indicates expression in the last forming cellular cartilage bar.
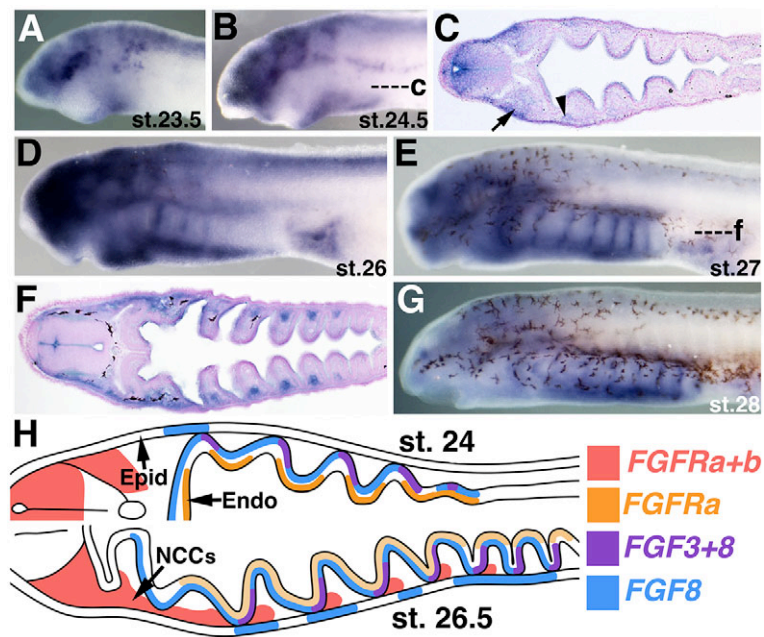
Fig. 4. Lamprey *FGFRb* expression. (A,B,D,E,G) *FGFRb* expression at the indicated stages. (C) Section through c in B showing expression in premaxillary (arrow) and first arch (arrowhead) NCCs. (F) Section at the level of f in E showing expression throughout NCCs. (H) Summary of FGF ligand and receptor expression. Pink indicates expression of FGFR genes in NCCs; orange is high-level expression of *FGFRa* in the pharyngeal pouches; light orange is low-level expression of *FGFRa*; blue and purple depict expression of FGF ligands. Endo, endoderm; Epid, epidermal ectoderm; NCC, neural crest cell.

examination by *HhA in situ* hybridization revealed hypomorphic pharyngeal pouches 1-4 and an unsegmented posterior pharynx (4/4; Fig. 5I,J). All larvae treated with SU5402 from st. 25.5-26.5 displayed outwardly normal pharyngeal development at st. 26.5, as determined by inspection of unstained larvae (~200) and *in situ* hybridization for *HhA*, *SoxB1b* (Cattell et al., 2012), *Mef* and *muscle actin* transcripts (Fig. 5G; supplementary material Fig. S2). Examination of cryosections of *HhA* hybridized embryos revealed slightly hypomorphic pouches and a loss *HhA* expression in their lateral aspect (5/5; Fig. 5H). When embryos exposed to this treatment were fixed at st. 30, pharyngeal segmentation was apparent, although the larvae were generally smaller than wild types (25/25; Fig. 5C). SU5402 exposure from st. 26-26.5, after pouch formation is completed, had no obvious effect on pharyngeal morphology at st. 30 (25/25; Fig. 5B).

Endodermal expression of FGFs is regulated by retinoic acid signaling

In gnathostomes, retinoic acid (RA) signaling is required by the pharyngeal endoderm for pouch formation (Schneider et al., 2001; Wendling et al., 2000). It has been proposed that this might be via regulation of endodermal FGF3 and/or FGF8 signaling. We tested whether increasing or decreasing RA signaling could interfere with pharyngeal segmentation and/or FGF gene expression in lamprey pharyngeal endoderm. First, we exposed lamprey embryos and larvae to the RA synthesis inhibitor diethylaminobenzaldehyde (DEAB) at multiple time points during pharyngeal segmentation. Inhibiting RA synthesis from st. 24-26.5 reduced the number of fully formed pharyngeal pouches from eight (Fig. 6A,D) to an average of six (n=15; Fig. 6B,E). In addition, we observed a loss of *FGF3* expression in the last, partially formed pharyngeal pouch (Fig. 6E). Similarly, treatment with DEAB from st. 25.5-26.5 reduced the number of fully formed pouches from eight to six or seven, with *FGF3* expression lost from the most posterior, partially formed pouch (n=18; Fig. 6C,F). A similar effect was seen on *FGF8/17/18* expression (data not shown).

We next treated lamprey embryos and larvae with 1 hour pulses of exogenous RA at various points during pharyngeal segmentation. RA pulses at st. 24 and st. 25 caused a consistent anteroposterior

compression of the pharynx, accompanied by the occasional fusion of adjacent arches (10/10 larvae for both treatments; Fig. 6H,I). RA pulses at st. 24, st. 25 and st. 25.5 also caused an expansion of *FGF3* expression into the anterior aspect of the pharyngeal pouches (10/10 for all three treatments; Fig. 6K,L). No similar expansion of *FGF8/17/18* expression was observed. Interestingly, the pouches showing *FGF3* expansions were consistently the two or three most recently formed pouches at the time of the RA pulse. For example, in embryos treated at st. 24, anterior *FGF3* expansion is seen in pouches 2 and 3, but not 4-8, whereas in larvae treated at st. 25.5, anterior *FGF3* expansion is seen in pouches 5-7, but not 1-4 or 8. We observed a similar anterior expansion of *FGF3* in larvae treated with talarozole, an inhibitor of RA metabolism, although the effect was delayed compared with RA treatment. Thus, larvae in which talarozole treatment was started at st. 25.5 show an anterior expansion of *FGF3* only in pharyngeal arch 8 (Fig. 6M). This is likely to reflect the lag time between when talarozole begins inhibiting RA metabolism by Cyp26, and when enough endogenous RA has accumulated to perturb *FGF3* expression.

FGF signals are required for NCC-derived cellular cartilage development

Work in zebrafish and chick suggests that FGF signals are directly required by chondrogenic NCCs to express *Dlx2*, *Sox9* and *Runx* and to secrete Aggrecan and Alcian Blue-reactive ECM (Das and Crump, 2012; Sarkar et al., 2001; Walshe and Mason, 2003). This implies essential roles of FGFs in the specification and later differentiation of NCC-derived chondrocytes. We tested whether these roles are also features of lamprey cellular cartilage development. We monitored gene expression and cartilage differentiation in lamprey embryos and larvae treated with SU5402 for various intervals during prechondrocyte specification and differentiation. First, we noted that the early treatments that disrupted pharyngeal pouch formation also led to the loss of cellular cartilage, suggesting that, as in gnathostomes, proper pharyngeal pouch formation is a precondition for chondrogenesis in lamprey. In later treatments starting at, or after, st. 25.5, when pharyngeal pouch formation is almost complete, various effects on gene expression and differentiation in NCC-derived prechondrocytes were observed.
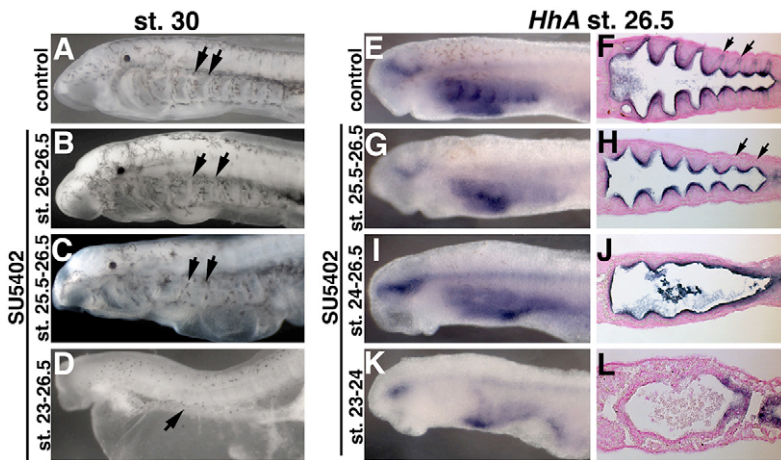
Fig. 5. FGF signaling inhibition disrupts pharyngeal pouch development in lamprey. (A) Pharyngeal arches (arrows) in wild-type larvae. (B-D) Larvae exposed to SU5402 from st. 26-26.5 (B) or from st. 25.5-26.5 (C) have a segmented pharynx (arrows), and SU5402 from st. 23-26.5 prevents pharyngeal segmentation (D, arrow). (E-L) Untreated (E,F) and SU5402-treated (G-L) larvae fixed at st. 26.5 and probed for *HhA* transcripts. F, H, J and L are horizontal sections through E, G, I and K, respectively. SU5402 treatments starting at st. 23 or st. 24 prevent pharyngeal pouch formation. SU5402 treatment from st. 25.5-26.5 inhibits *HhA* expression in the lateral pouches (compare arrows in F and H).

Our most extensive treatment spanned st. 25.5-28, when FGFR gene expression was observed in NCCs. Larvae treated this way displayed a complete loss of cellular cartilage (5/5; Fig. 7B), although pharyngeal segmentation was still apparent, supporting separate roles for FGFs in pharyngeal segmentation and skeletogenesis.

We then examined whether FGF signaling was functioning during the specification and/or differentiation phases of cartilage development. *SoxE1*, which is the lamprey homolog of the chondrogenic specifier *Sox9*, is expressed in prechondrocytes from st. 25.5 until st. 28, and is required for lamprey pharyngeal skeleton development. *Ednra* is also a marker for newly formed NCC-derived prechondrocytes in both lamprey and gnathostomes. Short treatments with SU5402 starting at st. 25.5 or 26, when postmigratory pharyngeal NCCs form a loose, undifferentiated mesenchyme, caused clear reductions in *SoxE1* (8/8) and *Ednra* (5/5) expression at st. 26.5, suggesting that FGFs are needed for the expression of these early markers of chondrogenic fate (Fig. 7E-L;

supplementary material Fig. S3A-C). To confirm this, we exposed larvae to UO126, an inhibitor of MEK kinases, which are major downstream effectors of FGF signaling (Favata et al., 1998; Kourakis and Smith, 2007). A similar reduction in *SoxE1* expression was observed (supplementary material Fig. S3D-I).

Next, we next investigated whether FGFs are necessary for later stages of cartilage development by exposing lamprey larvae to SU5402 before overt differentiation, but well after the onset of *SoxE1* and *Ednra* expression. To do this, we performed two SU5402 treatments, one from st. 27-28 and one from st. 27-28.5. The shorter treatment caused the loss of posterior cartilage bars in a few larvae (2/7; Fig. 7C). The longer treatment, which extended into the early stages of overt cartilage differentiation, consistently caused the complete loss of Alcian Blue-reactive cellular cartilage (8/8; Fig. 7D). This shows that FGF signals are required by lamprey NCC-derived prechondrocytes well after their presumed specification, when they are condensing (st. 27) and differentiating (28.5) (as summarized in Fig. 8).
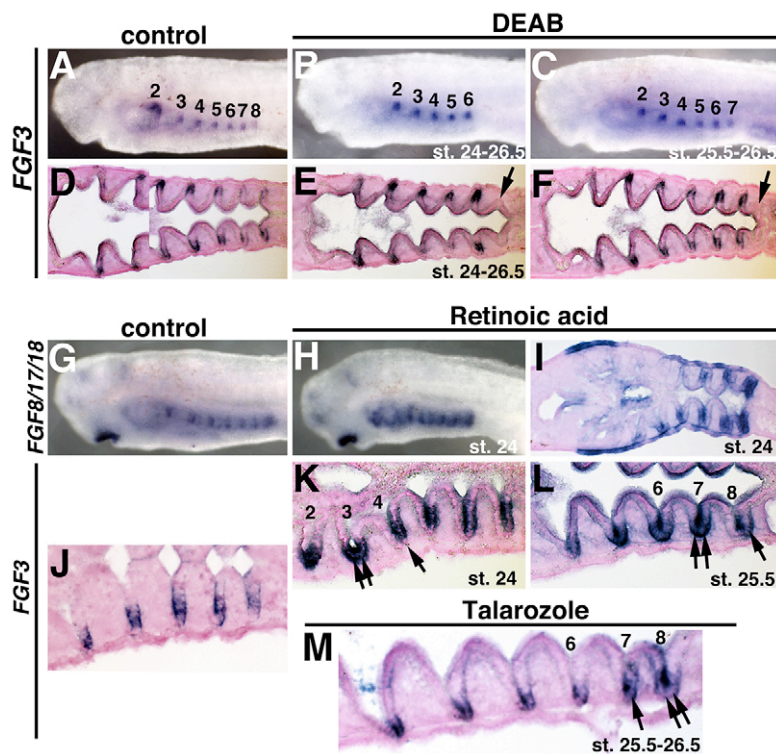

Fig. 6. Retinoic acid signaling regulates *FGF3* and *FGF8/17/18* expression and pharyngeal pouch formation in lamprey. (A,D) *FGF3* expression at st. 26.5. (B,E) DEAB treatment from st. 24-26.5 causes an average loss of two pouches. (C,F) DEAB treatment from st. 25.5-26.5 causes the loss of one or two pouches. Horizontal sections (D-F) show loss of *FGF3* transcripts in partially formed pouches (arrows). (G) *FGF8/17/18* expression at st. 26.5. (H,I) A 1-hour retinoic acid (RA) pulse at st. 24 causes compression of the pharynx. (J) Polarized *FGF3* expression in pharyngeal pouches. (K-M) 1-hour RA pulses at st. 24 (K) or st. 25.5 (L) or talarozole treatment from st. 25.5 (M) causes symmetrical expression of *FGF3* (double arrows). Pouches forming before or after the treatments are unaffected (single arrows).
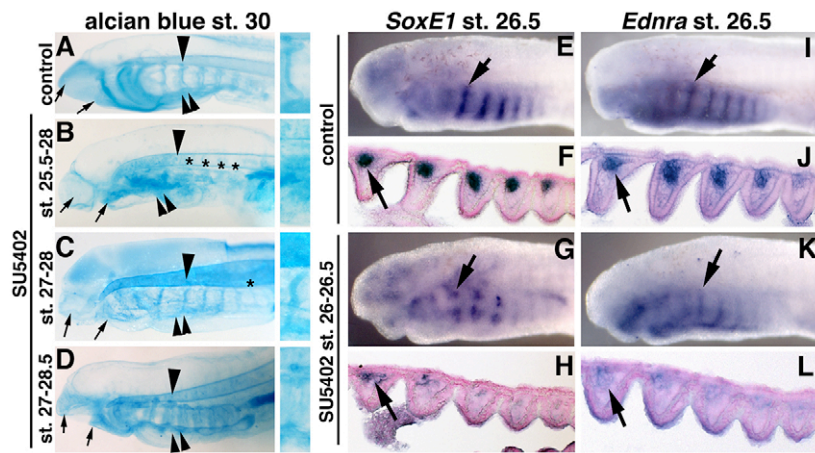
Fig. 7. SU5402 disrupts skeletal development in lamprey. (A-D) Alcian Blue staining of wild-type and SU5402-treated larvae at st. 30 showing oral mucocartilage (arrows), pharyngeal mucocartilage (double arrowheads) and cellular cartilage bars (large arrowheads, right panels). Arches lacking cartilage are indicated with asterisks. SU5402 exposure from st. 25.5-28 (B) or st. 27-28.5 (D) causes a loss of cellular cartilage and a reduction in mucocartilage, while SU5402 treatment from st. 27-28 causes a loss of posterior cartilage bars (C). (E-L) Wild-type expression of *SoxE1* (E,F) and *Ednra* (I,J) in prechondrogenic NCCs (arrows) is reduced by SU5402 (G,H and K,L).

FGF signaling is needed for the full development of mucocartilage in the oral region and ventral pharynx

In gnathostomes, FGFs play a role in patterning the NCC-derived skeletal tissue of the first and second arches, including positioning the jaw joint. In lamprey, the first and second arches, as well as the ventral pharynx, are supported by a cellular cartilage-like mesenchymal skeletal tissue called mucocartilage (Cattell et al., 2011; Johnels, 1948; Martin et al., 2009; Yao et al., 2011). Since lamprey mucocartilage also expresses FGFRs we asked what function FGF signaling might have in mucocartilage specification, differentiation or patterning. Unlike its effect on cellular cartilage, no SU5402 treatment caused a complete loss of mucocartilage, although extended SU5402 treatment from st. 25.5 to st. 28 resulted in a reduction of mucocartilage around the mouth and in the ventral pharynx (5/5; Fig. 7). These results suggest that FGFs are not necessary for the specification of mucocartilage, but might play a role in the proliferation and/or differentiation of mucocartilage cells. Consistent with this, markers of upper lip and ventral first arch (lower lip) mucocartilage, *Alx* (5/5) and *Barx* (5/5), were expressed normally in larvae treated with SU5402 from st. 25.5-26.5 or st. 26-26.5, even as *Alx* expression in cellular cartilage in the posterior arches was greatly reduced (supplementary material Fig. S4A-F). Hand, which marks nascent mucocartilage in the ventral pharynx, was also present in these larvae, but was reduced (10/10; supplementary material Fig. S4J-L). Notably, SU5402 treatment from st. 26-26.5 caused the complete loss of *Runx* expression in the mucocartilage at the lateral edges of the mouth (3/3; supplementary material Fig. S4G-I), suggesting that this particular subpopulation of mucocartilage is unusually dependent on FGF signals.

DISCUSSION

An ancestral role for intra-endodermal FGF signaling in vertebrate pharyngeal pouch morphogenesis

In zebrafish, gene knockdowns and grafting experiments have shown that *fgf3* and *fgf8* are needed for the formation of all pharyngeal pouches, although their precise roles appear to differ in the anterior versus posterior pharynx (Crump et al., 2004). During morphogenesis of the first and second pouches, Fgf3 and Fgf8 emanating from the neural tube and lateral mesoderm appear to act as chemoattractants, directing the lateral migration of pharyngeal endoderm cells. In the region of the more posterior pouches, expression of *fgf3* and *fgf8* in the brain and mesoderm is not seen, indicating that FGFs are acting via a different mechanism. As in zebrafish, we find that FGF signaling is required for pharyngeal segmentation in lamprey. However, unlike zebrafish, lamprey pharyngeal endoderm does not contact the neural tube, and neither *FGF3* nor *FGF8/17/18* is expressed in head mesoderm during pharyngeal segmentation. Instead, in lamprey both *FGF3* and *FGF8/17/18* are expressed in the pharyngeal endoderm along with *FGFRa*, suggesting that FGF signaling within the pharyngeal endoderm is driving pharyngeal pouch formation. The precise mechanism by which this occurs is unclear. Interestingly, *FGFRa* expression in medial pharyngeal endoderm alternates with *FGF3* and high levels of *FGF8/17/18* in the outpocketing pouch endoderm. Considering that FGFs are well-known regulators of growth and proliferation, it is possible that *FGF3* and *FGF8/17/18* drive the lateral outgrowth of the pharyngeal pouches by inducing increased proliferation of medial pharyngeal endoderm. Polymerization of actin cables in the apical aspect of pharyngeal endoderm cells has
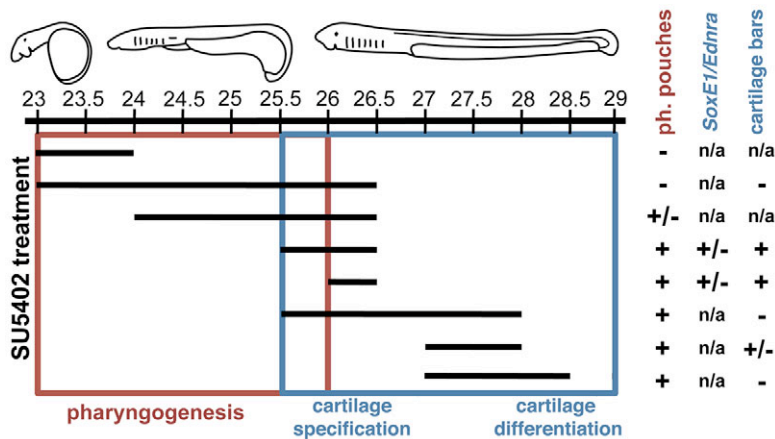

Fig. 8. Summary of SU5402 treatments performed. FGFs are required at different times for pharyngeal pouch formation, prechondrogenic NCC marker expression and cellular cartilage differentiation.

been shown to drive pharyngeal pouch formation by constraining endodermal expansion in the lateral (distal) pharyngeal pouch (Quinlan et al., 2004). Segmental FGF signals might also be involved in the iterated polymerization of these fibers.

To determine if the pattern of *FGF3* and *FGF8* expression we observed in lamprey pharyngeal endoderm was conserved in any jawed vertebrate, we re-examined *FGF3*, *FGF8* and *FGFR* expression in *X. laevis* during pharyngeal morphogenesis. We confirmed that the expression of *FGF3*, *FGF8* and *FGFR3* (data not shown) in pharyngeal endoderm was virtually identical to that of lamprey, as previously reported (Lea et al., 2009). We also noted that, like lamprey and unlike zebrafish, *FGF8*-expressing neural tube cells in *X. laevis* do not contact pharyngeal endoderm during pharyngeal segmentation. Together, these results suggest that the ancestral mechanism of vertebrate pharyngeal segmentation involved intra-endodermal FGF signaling. In this evolutionary context, the role of neural and mesodermal *fgf3* and *fgf8* in the morphogenesis of the zebrafish first and second pouches might represent a lineage-specific novelty. The reduction in head mesoderm at pharyngula stages in zebrafish, and the resulting dorsoventral compression of the head, might have allowed neural Fgf8 signals to compensate for primitively endodermal and ectodermal FGF3 and FGF8 signals. It is not known how FGFs control pharyngeal pouch formation in the posterior arches of zebrafish, although our model would predict a lamprey-type mechanism involving intra-endodermal FGF signals.

If the iterated expression of FGFs and FGFRs is driving pharyngeal segmentation in lamprey, how is this expression established? Inhibiting FGF and RA signaling causes similar disruptions in pharyngeal segmentation in both mouse and zebrafish, and in mouse RA signaling is necessary for pharyngeal expression of *Fgf3* and *Fgf8* (Abe et al., 2008; Kopinke et al., 2006; Wendling et al., 2000). Based on these results, it has been proposed that RA and FGFs might cross-regulate to drive pharyngeal segmentation (Kopinke et al., 2006). Consistent with this, we found that lamprey pharyngeal morphogenesis requires RA signaling, and this requirement corresponds to a loss of *FGF3* and reduction of *FGF8/17/18* expression in the forming pharyngeal pouches. Furthermore, we found several putative retinoic acid response elements near the transcriptional start sites of lamprey *FGF3* and *FGF8/17/18* (supplementary material Table S1) (Sandelin et al., 2004), and observed expression of a lamprey retinoic acid receptor in pharyngeal endoderm (supplementary material Fig. S5). Although not conclusive, these data raise the possibility that RA signaling might directly regulate FGFs in lamprey. Regardless of the precise mechanism, it is also worth noting that the RA and FGF signaling pathways interact during somitogenesis. This suggests that mechanisms similar to those operating during paraxial mesoderm segmentation might also underlie pharyngeal segmentation.

Although RA was necessary for normal FGF gene expression, we found that excess RA signaling did not expand *FGF8/17/18* expression or increase the size or number of pouches formed. Instead, we noted a compression of the pharynx with the earliest RA treatments, as described previously (Kuratani et al., 1998). A similar shortening of the pharynx is seen in RA-treated amphioxus larvae, supporting an ancestral role for RA signaling in setting the posterior limit of the chordate pharynx (Schubert et al., 2005). With later RA treatments, we did observe an anterior expansion of *FGF3* in newly formed pouches, resulting in *FGF3* transcripts in both the anterior and posterior walls of affected pouches. This loss of anteroposterior polarity is consistent with results from the skate (Gillis et al., 2009),

a basal cartilaginous gnathostome, demonstrating at RA signaling establishes AP polarity in the developing gill arches. Taken together, our perturbations indicate that RA signaling is needed both to initiate FGF gene expression in the nascent pharyngeal pouches and also to establish polarized *FGF3* expression within the pouches.

Pharyngeal gill slits are a defining feature of chordates and an ancestral feature of deuterostomes. Recent molecular phylogenies have revealed that urochordates are the closest invertebrate relatives of the vertebrates (Delsuc et al., 2006). FGF inhibition prevents gill slit formation in the urochordate *Ciona savignyi*, indicating that FGFs might have mediated pharyngeal morphogenesis in the last common ancestor of urochordates and vertebrates (Kourakis and Smith, 2007). However, *C. savignyi* gill slits form when neither *FGF3* nor *FGF8/17/18* transcripts are detectable in the endoderm, indicating that endodermal FGFs are unlikely to control pharyngeal segmentation in this species. By contrast, the invertebrate chordate amphioxus deploys both *FGF8/17/18* and *FGFA* (a likely ortholog of *FGF3*) in patterns virtually identical to their vertebrate orthologs, with both genes displaying iterated expression in the pharyngeal endoderm during gill slit formation (Bertrand et al., 2011; Meulemans and Bronner-Fraser, 2007). Given the basal phylogenetic position of amphioxus, these observations support a deeply conserved role for endodermal FGFs in chordate pharyngeal segmentation. A critical test of this will be establishing whether FGFs are necessary for amphioxus gill slit formation, and if they are regulated by RA. It will also be informative to determine whether FGFs are involved in pharyngeal gill slit formation in hemichordates, the most basal deuterostomes with vertebrate-type pharyngeal perforation. FGF-dependent gill slit formation in this group would indicate that this mechanism pre-dates chordates.

Roles for FGFs in the specification and differentiation of lamprey cellular cartilage

Evidence from zebrafish and chick suggests that FGFs act through FGFRs on migrating cranial NCCs to specify chondrogenic fate by regulating *Dlx2*, *Runx*, *Barx* and *Sox9* expression (Das and Crump, 2012; Sarkar et al., 2001; Sperber and Dawid, 2008; Walshe and Mason, 2003). Whereas lamprey does not express *Runx* or *Barx* in NCC-derived prechondrocytes (Cattell et al., 2011), the lamprey *Sox9* homolog *SoxE1* is strongly expressed in pharyngeal NCCs and is required for their development into cellular cartilage (Meulemans and Bronner-Fraser, 2007; Lakiza et al., 2011; McCauley and Bronner-Fraser, 2006). Consistent with a role for FGFs in specifying chondrogenic fate, we observed coexpression of *SoxE1* and FGFRs in lamprey pharyngeal NCCs starting shortly after their migration into the pharynx. Furthermore, when FGF signaling was inhibited during this period, we noted a strong reduction in *SoxE1* expression. This effect was evident even after relatively short treatments with SU5402 (st. 26-26.5, ~1 day), indicating a tight regulatory relationship between FGF signaling and *SoxE1* expression. In both jawed vertebrates and lamprey, Endothelin receptors (Ednrs) are robust markers for prechondrogenic NCCs (Cerny et al., 2010; Kimmel et al., 2003). Recent work in *X. laevis* has revealed an essential role for Endothelin signaling in maintaining the NCC specification state and regulating *Sox9* (Bonano et al., 2008). We found that FGF inhibition also caused a strong reduction in the expression of lamprey *Ednra* in pharyngeal NCCs. Taken together, our findings support an early role for FGF signals in chondrogenic NCC specification through activation and/or maintenance of *SoxE1* and *Ednra* expression.

Cultured chick NCCs will only form cartilage nodules when exposed to FGF signals for a prolonged period extending past the
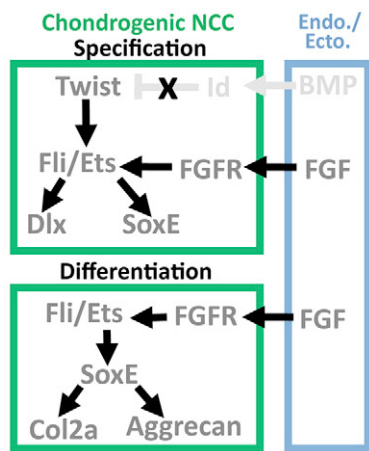
Fig. 9. Reconstruction of the GRN underlying cellular cartilage development in the first vertebrates. Conserved roles for FGFs and *SoxE* and the similar expression of *Twist*, *Id*, *Dlx* and *Fli/Ets* in lamprey and gnathostomes support a two-phase GRN for cellular cartilage development in early vertebrates. Endo./Ecto., endoderm/ectoderm.

first day of culture (Sarkar et al., 2001), whereas disrupting FGF signaling in zebrafish embryos not only causes a reduction in chondrogenic NCC marker expression, but also a failure of chondrogenesis (Walshe and Mason, 2003). These observations suggest that FGFs are needed both to initiate chondrogenic specification and to drive the later steps of cartilage differentiation in gnathostomes. Consistent with a late role for lamprey FGFs in cartilage differentiation, we found that lamprey FGFR gene expression persists in cranial NCCs until the onset of overt differentiation, several days after the initiation of *SoxE1* expression, and that FGF inhibition at these stages can prevent their differentiation into cartilage. Like FGFs, Sox9 proteins have been shown to have multiple roles in cartilage development, first as specifiers of chondrogenic fate and then as direct regulators of fibrillar collagen and ECM proteins (Lee et al., 2004; Mori-Akiyama et al., 2003; Yan et al., 2002). In lamprey, FGFs and *SoxE1* transcripts are coexpressed throughout development and FGFs are required for *SoxE1* expression at st. 26.5. It is thus possible that both the early and late functions of FGF in lamprey cartilage are mediated by SoxE1.

Reconstructing the ancestral GRN for cellular cartilage development

Collagen-based cellular cartilage is considered a defining feature of vertebrates and its development has been widely studied in gnathostomes. Although agnathans possess cellular cartilage, its development, histology and ECM composition differ significantly from gnathostomes. Our work suggests that, despite these differences, a core conserved GRN for vertebrate cellular cartilage development operates in both vertebrate lineages (Fig. 9). Recent work in zebrafish suggests that chondrogenic NCC specification is initiated as NCCs migrate into the pharynx and away from epidermal BMP signals. This results in the downregulation of the bHLH transcriptional inhibitor Id, which allows *twist* to activate *fli/ets* expression. FGF signals then work through FGFRs and *fli/ets* to activate *dlx* expression and specify chondrogenic fate (Das and Crump, 2012). In lamprey, *Id* expression is lost by cranial NCCs soon after they enter the pharynx, whereas *Twist*, *Fli/Ets*, *Dlx* and *FGFR* are upregulated (Bronner-Fraser et al., 2003; Meulemans and

Bronner-Fraser, 2007; Neidert et al., 2001). FGFs are also needed during this period for expression of the cartilage specifier *SoxE1*, supporting the conservation of a zebrafish-type chondrogenic specification mechanism. In chick, FGFs are needed to maintain *Sox9* expression in chondrogenic NCCs and also to drive the later steps of cartilage differentiation (Sarkar et al., 2001). Both of these functions appear conserved in lamprey, as SU5402 inhibition at later stages prevents overt cartilage differentiation. Based on these consensus features of vertebrate cellular cartilage development, we propose a two-phase model for cellular cartilage development in early vertebrates. In the first phase, chondrogenic NCCs are specified by a zebrafish-type mechanism involving *Id* downregulation, *Twist*, *Fli/Ets* and FGF-mediated activation of *Dlx* and *SoxE*. This is followed by a later differentiation phase in which the expression of cartilage effector genes (i.e. collagen and aggrecans) is driven by FGF signaling and *SoxE*.

The evolutionary origin of vertebrate cellular cartilage

Urochordates are the closest invertebrate relatives of vertebrates and have migratory mesoderm cells and non-migratory melanocytes with some of the molecular and developmental properties of NCCs (Abitua et al., 2012; Jeffery et al., 2008). However, urochordates lack any collagen-based skeletal tissue resembling vertebrate cellular cartilage. By contrast, the basal chordate amphioxus possesses an acellular pharyngeal skeleton composed of chondroitin sulfate proteoglycans and fibrillar collagen (Azariah, 1973; Meulemans and Bronner-Fraser, 2007). Although the development of this tissue is poorly described, the first evidence of its formation is likely to be the expression of fibrillar collagen in the pharyngeal mesoderm of early larvae. Nascent amphioxus pharyngeal mesoderm also expresses *Id*, *Twist* and *Fli/Ets* and is in close contact with pharyngeal endoderm expressing *FGF8/17/18* (Meulemans and Bronner-Fraser, 2007). These similarities with NCC-derived cartilage suggest that the chondrogenic specification program deployed by vertebrate cranial NCCs might have been coopted, in part, from pharyngeal mesoderm. Although an attractive hypothesis, a central component of this program in vertebrates, *SoxE*, is not expressed in amphioxus pharyngeal mesoderm, at least at early larval stages. It is possible that *SoxE* might have been incorporated into this program solely in the vertebrate lineage, or is activated in skeletogenic mesoderm later in amphioxus development.

Similarities in lamprey mucocartilage and cellular cartilage development are consistent with common evolutionary origins

Although lamprey possesses cellular cartilage bars in its posterior arches, the skeleton of the mouth and first and second arches consists of a mass of mesenchymal skeletal tissue called mucocartilage (Johnels, 1948; Wright and Youson, 1982). The ECM of mucocartilage reacts with many of the classical stains for vertebrate cellular cartilage, but mucocartilage cells have an irregular mesenchymal morphology that is distinct from the compact polygonal cells characteristic of cellular cartilage. Furthermore, mucocartilage is apparently lost during metamorphosis and replaced with typical cellular cartilage in adult lampreys (Armstrong et al., 1987). These differences have led to speculation that mucocartilage might represent a form of connective tissue that is unrelated to cellular cartilage, that it is a larval cartilage type unique to lamprey, or is an evolutionary precursor to cellular cartilage (Armstrong et al., 1987; Johnels, 1948; Wright and Youson, 1982). Recent gene expression studies show that mucocartilage expresses all core regulators of cellular cartilage development shared by gnathostomes

Development

and lamprey cellular cartilage including *Id*, *Twist*, *Fli/Ets*, *Dlx* and *SoxE* (Lakiza et al., 2011; Neidert et al., 2001; Sauka-Spengler et al., 2007). The present study extends these results, showing that mucocartilage expresses FGFRs and requires some FGF signaling to fully develop, although it is less sensitive to FGF inhibition than cellular cartilage. Together, these data support common evolutionary origins for mucocartilage and cellular cartilage.

FGF signaling and the evolution of the gnathostome head skeleton

The evolution of gnathostomes from ancient agnathans is likely to have involved changes in both the composition and patterning of the head skeleton. FGF signaling has key roles in both skeletal differentiation and patterning and is an attractive candidate for a signaling pathway that might have been altered during gnathostome evolution. Despite divergent skeletal morphologies, we found striking conservation of pharyngeal FGF expression and function across the vertebrates, with lamprey FGFs performing gnathostome-like roles in both pharyngeal segmentation and chondrogenesis. Furthermore, we show that *Runx* expression in the mesenchyme flanking the mouth is completely eliminated by FGF inhibition. Interestingly, these cells contact oral epithelium expressing *FGF8/17/18*, suggesting that they require high levels of FGF signal to transcribe *Runx*. Thus, graded FGF signaling might also perform a gnathostome-like patterning function in the lamprey oral skeleton. Recent work has revealed remarkable conservation of early cranial NCC patterning along the anteroposterior and dorsoventral axes in gnathostomes and lamprey despite differences in the head skeletons that they generate (Cerny et al., 2010; Medeiros and Crump, 2012; Takio et al., 2007). Our results are consistent with this broad conservation, implying that the origin and diversification of the gnathostome head skeleton were likely to have been driven by changes developmentally downstream of these mechanisms. *Barx* and *Bapx* are regulated by FGFs in gnathostomes and are essential for proper positioning of the jaw joint. However, in lamprey, *Barx* is not regulated by FGF signaling, and *Bapx* is not expressed in FGFR-positive skeletogenic mesenchyme (Cerny et al., 2010), precluding its direct regulation by FGF signaling. Thus, changes to FGF signaling targets in the gnathostome lineage might have been crucial in the evolution of the jaw and gnathostome-type cartilage.

MATERIALS AND METHODS
Isolation of lamprey and frog *FGF3*, *FGF8* and *FGFR* homologs
Embryos and larvae of the sea lamprey *Petromyzon marinus* were obtained as reported previously (Nikitina et al., 2009) and staged according to Tahara (Tahara, 1988). *Xenopus laevis* embryos were collected according to standard methods (Sive et al., 2000). The lamprey preassembly genome was searched using *X. laevis* and *Danio rerio* FGF3, FGF8 and FGFR1-4 protein sequences. cDNA fragments were obtained via RACE as previously described (Cattell et al., 2011). *X. laevis FGF3*, *FGF8* and *FGFR* fragments were amplified directly from st. 33/34 cDNA.

In situ hybridization and histology
In situ hybridizations and cryosectioning were performed as previously described (Cattell et al., 2011; Cerny et al., 2010), although for *X. laevis* lower stringency hybridization and post-hybridization wash solutions were used (5× SSC pH 7.0 instead of 1.3× SSC pH 5.0). Alcian Blue staining on whole-mount larvae was as previously described (Martin et al., 2009).

SU5402 and UO126 treatments
To inhibit FGF signaling, lamprey embryos and larvae were treated with 25, 50 and 100 μM SU5402 (Tocris Bioscience) and 10-200 μM UO126 (Tocris

Bioscience). Stocks of both drugs were made in undiluted DMSO. Treatments were performed in 0.05× Marc's Modified Ringer's (MMR). Controls were performed by incubating sibling larvae in 0.05× MMR with DMSO added to the concentration of the experimental treatments. Embryos were treated in groups of 20-25 in 4-ml Petri dishes. Dead larvae were removed and solutions were changed every 2 days.

Retinoic acid signaling perturbations
Embryos were treated with 0.5 μM retinoic acid (all-trans retinoic acid, Sigma-Aldrich), 50 μM DEAB (Sigma-Aldrich) and 50 μM talarozole (Active Biochem) in 0.5% DMSO/0.05× MMR.

All procedures were performed with the approval of the University of Colorado, Boulder Institutional Animal Care and Use Committee.

Author contributions
D.M.M. conceived of the project and prepared the manuscript prior to submission. D.J. designed and performed experiments, analyzed the data, and helped edit the manuscript. M.B.H. and T.A.S. designed and performed experiments. R.C. and M.V.C. performed experiments.

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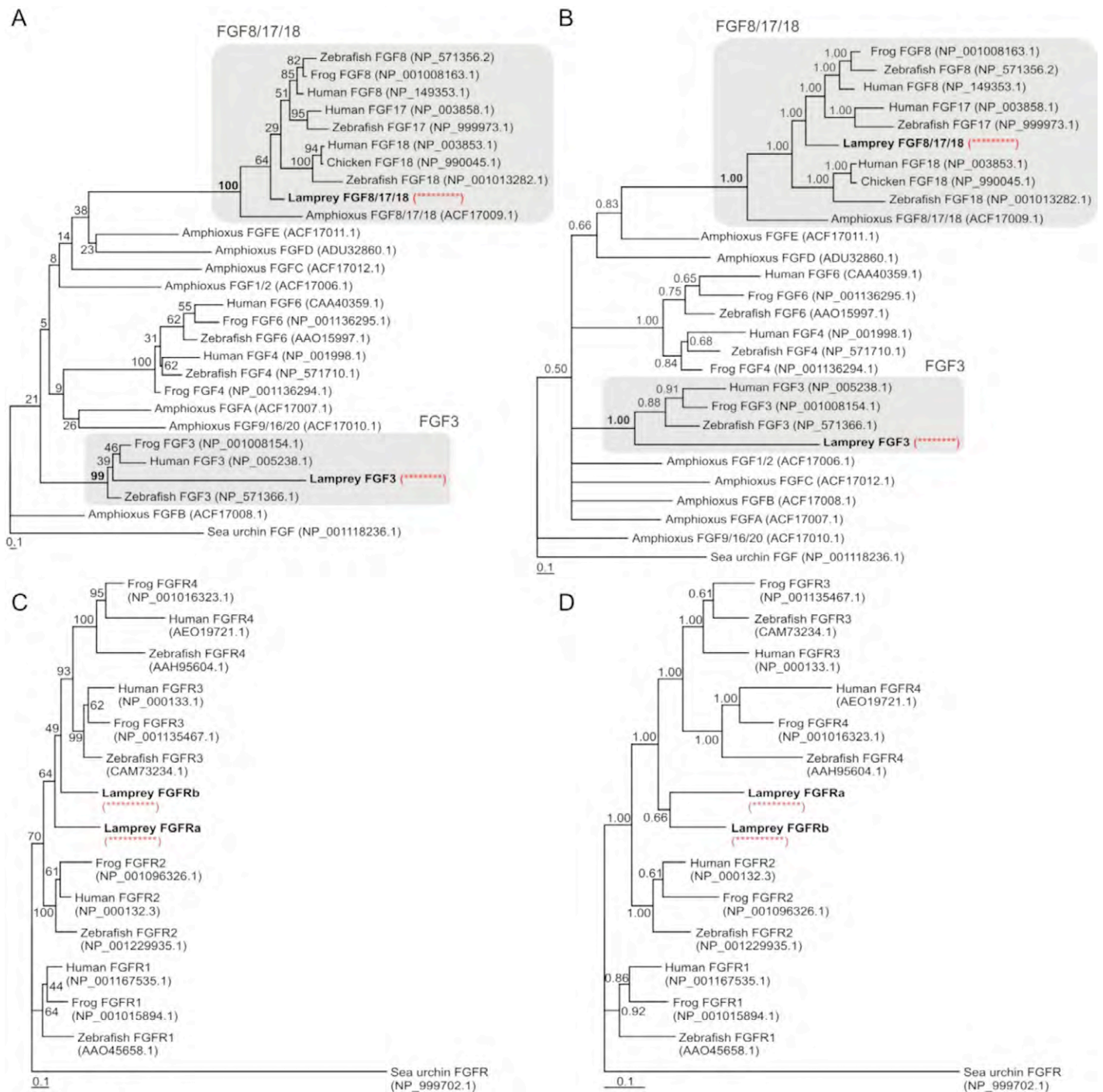
Fig. S1. Phylogenetic trees of the vertebrate *FGF3/4/6*, *FGF8/17/18*, and *FGFR* gene families. Amphioxus (*Branchistoma lanceolatum*) and sea urchin (*Strongylocentrotus purpuratus*) homologs were used as outgroups. (A,B) Lamprey *FGF3* and *FGF8/17/18* form well-supported clades with their gnathostome cognates. (C,D) Lamprey *FGFR*s cluster with gnathostome *FGFR*s, though their placement within any one paralogy group is not supported. Maximum likelihood phylogenies (A,C) were reconstructed in PhyML 3.0 (Guidon and Gascuel, 2003) using the best of the NNI and SPR branch swapping. Branch support was calculated as bootstrap values based on 100 resampled data sets. The Bayesian phylogenies (B,D) were calculated in MrBayes 3.2 (Ronquist et al., 2012) using the mixed model of amino acid evolution. Analyses were performed with two runs and four chains for 2,000,000 generations sampling every 100th tree. Majority-rule consensus trees were produced and the posterior probabilities calculated as the frequency of samples recovering any particular clade.
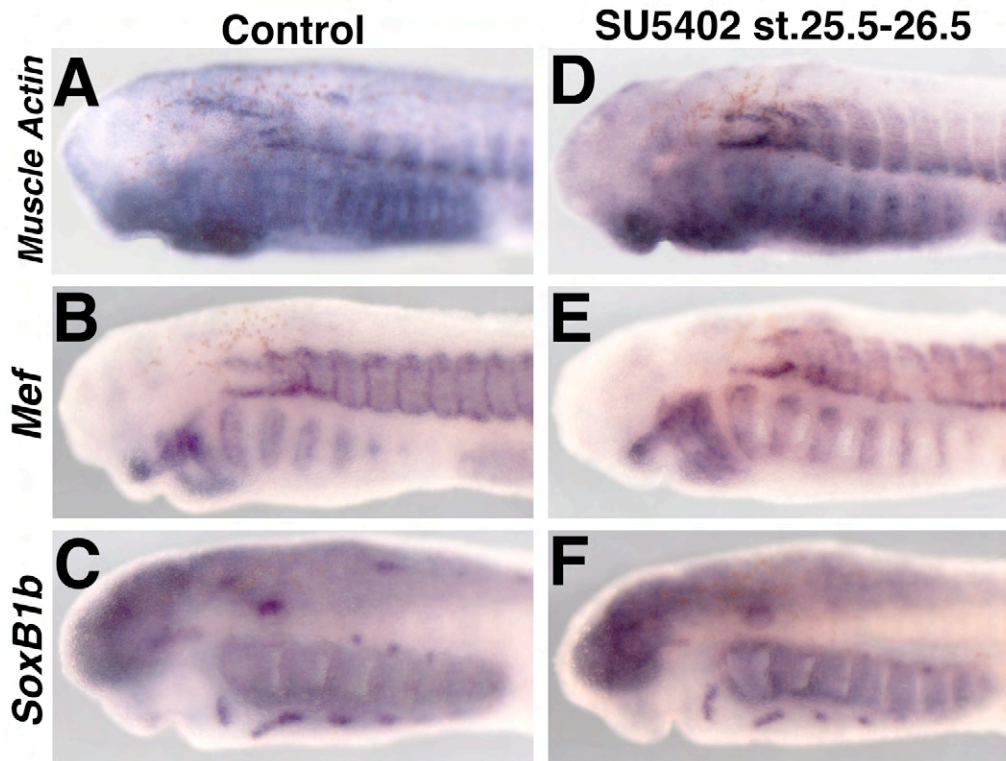
Fig. S2. Treatment with 50uM SU5402 from st. 25.5 to 26.5 does not significantly alter the expression of mesodermal or endodermal markers. (A) Muscle actin expression in a wildtype larva at st. 26.5. (B) Muscle actin expression in SU5402-treated larvae at st. 26.5. (C) Expression of a lamprey myocyte enhancer factor (*Mef*) homolog at st. 26.5. (D) Similar expression is seen in SU5402-treated larvae. (E) Expression of *SoxB1b* in the pharyngeal endoderm, neural tube, and epidermal sensory neurons as st. 26.5 as previously described(Cattell et al., 2012). (F) *SoxB1b* expression in SU5402-treated larvae is largely the same as in wild-type larvae.
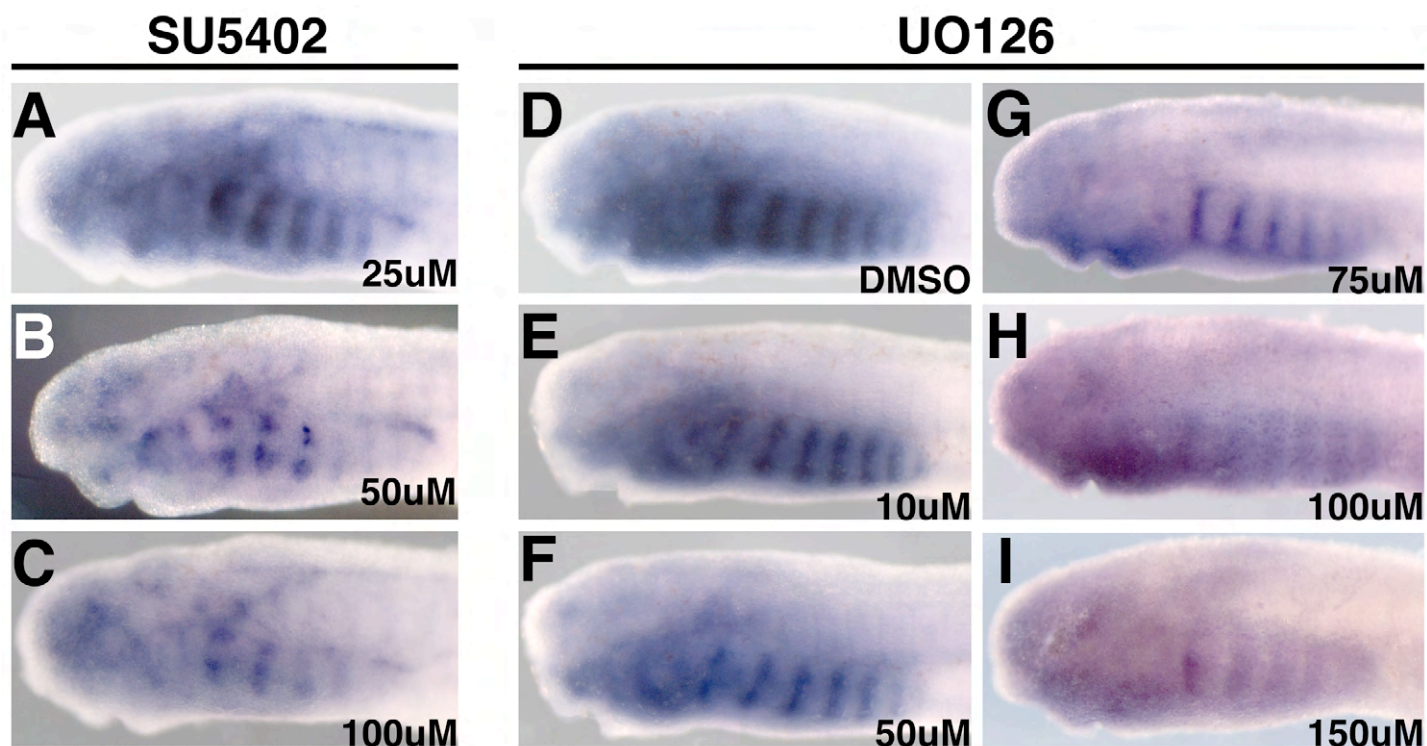
Fig. S3. *SoxE1* **expression in st. 26.5 lamprey larvae treated with various concentrations of SU5402 and MEK kinase inhibitor UO126 from st. 25.5-st. 26.5.** (A) 25uM SU5402 has little or no effect on *SoxE1* expression (10/10 larvae). (B,C) 100uM SU5402 resulted in a reduction in *SoxE1* expression similar to that of 50uM (10/10 larvae). (D-F) Treatment with 10uM or 50uM UO126 caused no detectable change in *SoxE1* expression relative to DMSO-only controls. (H) Treatment with 75uM UO126 resulted in a general developmental delay, as determined by body shape and pigmentation, and little or no change in the *SoxE1* expression pattern. (I) 100uM UO126 resulted in a developmental delay similar to the 75uM treatment with a clear reduction in *SoxE1* expression in 9/11 larvae. (I) 150uM resulted in developmental delay and a reduction in *SoxE1* expression in 5/5 larvae.
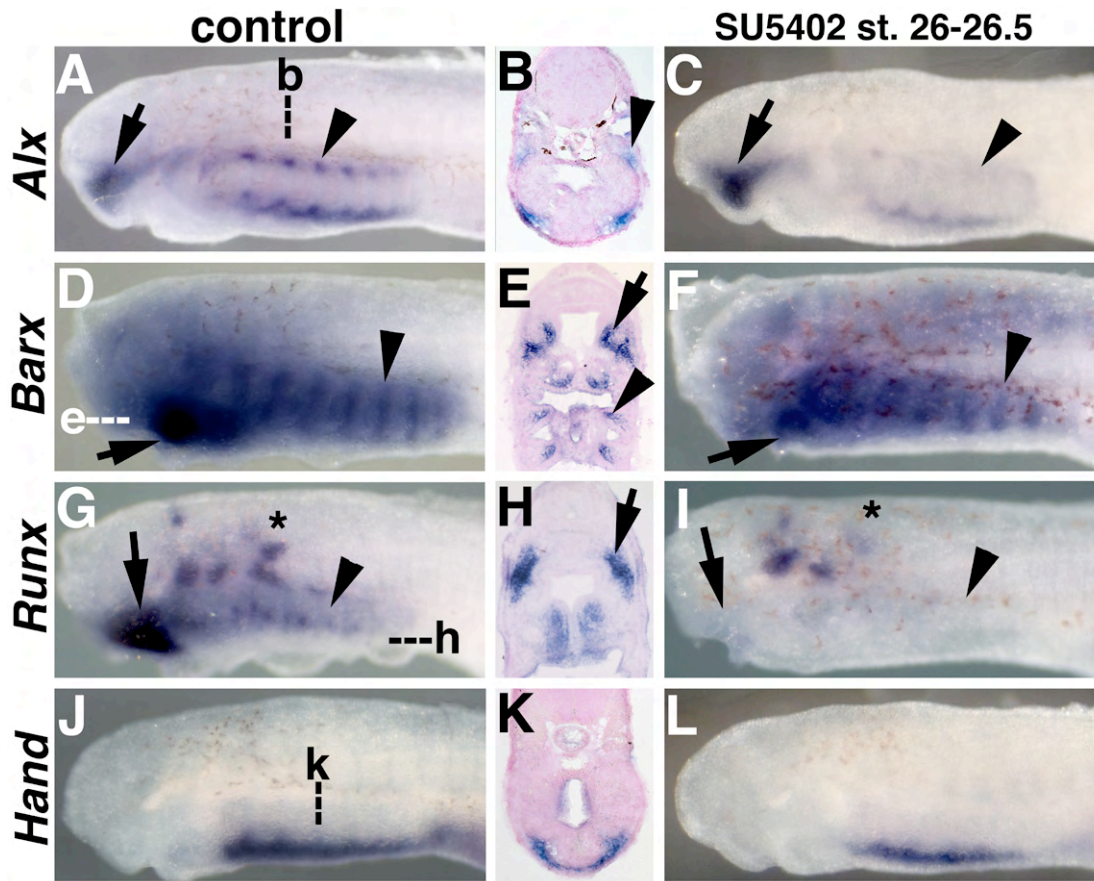
Fig. S4. Effects of *FGF* inhibition on gene expression in nascent mucocartilage of the oral skeleton and ventral pharynx. (A) *Alx* expression at st. 26.5 in the upper lip mucocartilage (arrow) and in the dorsal (arrowhead) and ventral aspects of the forming cellular cartilage bars. (B) Section at the level of b in A showing cellular cartilage expression. (C) Treatment with SU5402 from st. 26-26.5 has no effect on *Alx* expression in the upper lip (arrow), but causes a reduction in cellular cartilage expression, especially in the dorsal domain. (D) Expression of *Barx* in lower lip mucocartilage (arrows) and NCC in the medial aspect of the pharyngeal arches (arrowheads). (E) Horizontal section at the level of e in F showing expression in mucocartilage. (F) SU5402 has no detectable effects on pharyngeal *Barx* expression in the lower lip (arrow) or posterior pharynx (arrowhead). (G) In wildtype larvae, *Runx* is expressed in the mucocartilage at the edges of the mouth, pharyngeal mesoderm (arrowhead) and cranial ganglia, including the epibranchial and lateral line ganglia (asterisk). (H) Horizontal section at the level of h in G showing *Runx* expression in oral mucocartilage (arrow). (I) SU5402 treatment from st. 26-26.5 causes a complete loss of *Runx* expression in the oral mucocartilage, pharyngeal mesoderm, epibranchial, and lateral line ganglia (asterisk). *Runx* transcripts are still apparent in the trigeminal ganglion. (J,K) Hand expression in the nascent ventral mucocartilage. (L) SU5402 treatment causes a clear reduction of Hand expression in the ventral pharynx at st. 26.5.
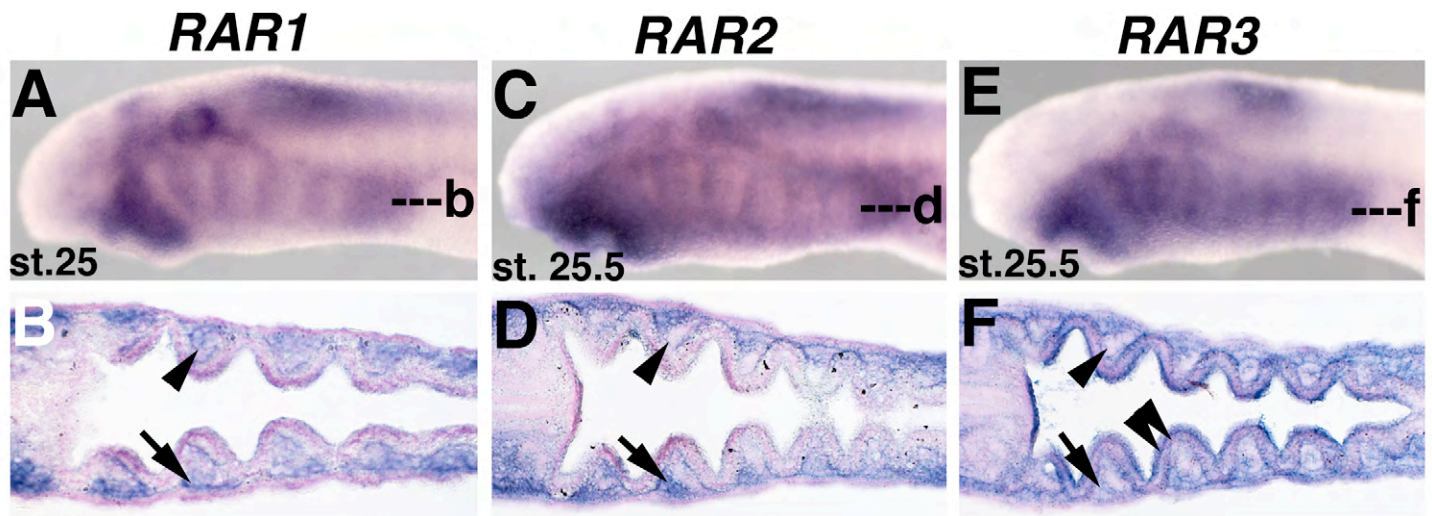
Fig. S5. Expression of lamprey retinoic acid receptors (*RARs*) during pharyngogenesis. We searched the preassembly *P. marinus* genome and identified exons corresponding to 3 RARs. We isolated cDNA fragments of all 3 genes and examined their expression at st. 25 and st. 25.5, when the posterior pharyngeal pouches are being formed. (A) Lamprey *RAR1* is expressed in the neural tube, pharynx, and oral region at st. 25. (B) Sectioning shows pharyngeal expression includes the mesodermal core of the pharyngeal arches (arrowhead) as well as NCC. (C) Lamprey *RAR2* is also expressed in the neural tube, pharynx and oral region at st. 25.5. (D) Sectioning reveals pharyngeal expression is limited to NCC. (E) Lamprey *RAR3* is observed in the pharynx, around the mouth, and in the neural tube at st. 25.5, though the neural expression is restricted relative to *RAR1* and *RAR2*. (F) Sectioning shows expression in NCC (arrow) and pharyngeal endoderm (double arrowhead), but little or no expression in pharyngeal mesoderm (arrowhead).

Locus	Predicted element	Sequence	Location (assembly, contig:basepair)
FGF3	Transcription Start	ATG (GATGTAGCTCACAGC)	petMar2, GL487047:4814
	RARE	AGGTGTGGGGAAGGTCA	petMar2, GL487047: 231
	RARE	GGATAAGCGAGAAGTCA	petMar2, GL487047:2163
	RARE	AGGTGGGCAGCAGGTGA	petMar2, GL482471:846911
	RARE	GGGTCAGAGAGCTGGGA	petMar2, GL482471: 847673
FGF8/17/18	Transcription Start	ATG (CGACCCTCGCACTGC)	petMar1, 44966:3745
	RARE	AGCTCACCACGAGTTCA	petMar1, 44966:5898
	RARE	GGTACAGCAGAAGTTCA	petMar1, 44966:6601
	RARE	GGTTCAGAAAATCGTCA	petMar1, 44966:7856
	RARE	GGGTCACCACAAAGACA	petMar1, 44966:10312

Table S1. Predicted retinoic acid response elements (RAREs) at the lamprey *FGF3* and *FGF8/17/18* loci. Using our *FGF3* RACE cDNA sequence as a scaffold, we linked contigs GL487047 and GL482471 of the September 2010 sea lamprey genome assembly (petMar2, WUGSC v7.0). We also identified a 7000 bp contig (44966) in the March 2007 preassembly lamprey genome (petMar1, WUSTL v.3.0) that contained all of our *FGF8/17/18* cDNA sequence. We then searched these contigs for consensus RAREs using the JASPAR database (www.jaspar.genereg.net) (Sandelin et al., 2004) with the score threshold set to the default of 80%. Limiting our search to a maximum of 10,000 kb upstream or downstream of the transcriptional start site, we found 4 matches to the RARE consensus motif at both loci. Likely transcriptional start sites are shown on the table with an additional 5 codons in parentheses.