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Duplication and Divergence of *fgf8* Functions in Teleost Development and Evolution

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ABSTRACT Fibroblast growth factors play critical roles in many aspects of embryo patterning that are conserved across broad phylogenetic distances. To help understand the evolution of fibroblast growth factor functions, we identified members of the *Fgf8/17/18*-subfamily in the three-spine stickleback *Gasterosteus aculeatus*, and investigated their evolutionary relationships and expression patterns. We found that *fgf17b* is the ortholog of tetrapod *Fgf17*, whereas the teleost genes called *fgf8* and *fgf17a* are duplicates of the tetrapod gene *Fgf8*, and thus should be called *fgf8a* and *fgf8b*. Phylogenetic analysis supports the view that the *Fgf8/17/18*-subfamily expanded during the ray-fin fish genome duplication. In situ hybridization experiments showed that stickleback *fgf8* duplicates exhibited common and unique expression patterns, indicating that tissue specialization followed the gene duplication event. Moreover, direct comparison of stickleback and zebrafish embryonic expression patterns of *fgf8* co-orthologs suggested lineage-specific independent subfunction partitioning and the acquisition or the loss of ortholog functions. In tetrapods, *Fgf8* plays an important role in the apical ectodermal ridge of the developing pectoral appendage. Surprisingly, differences in the expression of *fgf8a* in the apical ectodermal ridge of the pectoral fin bud in zebrafish and stickleback, coupled with the role of *fgf16* and *fgf24* in teleost pectoral appendage show that different *Fgf* genes may play similar roles in limb development in various vertebrates. *J. Exp. Zool. (Mol. Dev. Evol.) 308B:730–743, 2007.*

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A fundamental question in evolutionary developmental biology is the genetic basis of phenotypic variation and the origins and mechanisms of biodiversity. Genome duplication is thought to be an important engine of diversification (Ohno, '70). Genome-scale studies coupled with functional data generated from microarray analyses show that gene duplication can facilitate tissue specialization and functional diversification (Li et al., 2005). If gene duplication enables phenotypic diversity, then the now well-supported hypothesis of a whole-genome duplication early in the ray-fin fish lineage (Amores et al., '98; Postlethwait et al., '98; Wittbrodt et al., '98; Meyer and Schartl, '99; Christoffels et al., 2004; Naruse et al., 2004; Meyer and Van de Peer, 2005) might help explain how the teleosts, the largest group of vertebrates with about 23,600 species (Nelson, '94), are so phenotypically diverse. Until recently, however, the timing of the ray-fin fish-specific genome duplication was unclear, so a causal effect could only be speculated. Support for a causal effect comes from the analysis of gene content in teleosts and nonteleost actinopterygians in an evolutionary context. A phylogenetic analysis of three loci place

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their duplication after the teleosts diverged from other ray-fin fish (Hoegg et al., 2004). The timing of the genome duplication is further supported by a phylogenetic analysis based on three *Hox* genes sampled from all but one major basal actinopterygian group and several teleostei species (Crow et al., 2006). Strikingly, the number of species found in the two first teleost lineages branching off after the genome duplication is in clear contrast with the species richness of the nonteleost actinopterygians (Hoegg et al., 2004). Nevertheless, Crow et al. (2006) point out that duplication events themselves perhaps more than gene number *per se* correlate with diversification by opening a ''window of evolvability'' that would be out of reach without the duplication event.

Recent developments in the theory of duplicated gene evolution provide a testable link between the gene duplication event and its subsequent effect on phenotypic diversification. The classical model assumes redundancy of gene function(s) after duplication, with relaxed selection on one member of the pair of duplicates leading to silencing of that gene (nonfunctionalization). Alternatively, positively selected mutations in one copy could promote functional divergence (neofunctionalization) (Ohno, '70). In a third model, both duplicates are retained through the partitioning of ancestral functions owing to neutral fixation of complementary degenerative mutations (subfunctionalization) (Force et al., '99, 2005). In this context, nonfunctionalization, subfunction partitioning, and neofunctionalization, occurring independently in separate lineages, could lead to different functions, differential subfunction partitioning, relaxation of pleiotropic constraints, and speciation (Lynch and Force, 2000; Taylor et al., 2001; Postlethwait et al., 2004; Force et al., 2004, 2005). Empirical studies have shown great support for the molecular mechanisms of the subfunctionalization model (Lister et al., 2001; Altschmied et al., 2002; Cresko et al., 2003; Liu et al., 2005).

The most striking evidence for the ray-fin fish-specific genome duplication comes from the analysis of *Hox* gene clusters. Vertebrate *Hox* clusters show a pattern of evolution by two first rounds of duplication that occurred after the divergence of urochordates and vertebrates (see Bourlat et al., 2006; Delsuc et al., 2006) but before the split between chondrichthyans and osteichthyans, plus an additional genome duplication early in the ray-fin fish lineage leading to eight *Hox* clusters in stem teleosts in contrast to four clusters in tetrapods (Amores et al., '98, 2004; Aparicio, 2000; Aparicio et al., 2002). Additional genes showing a pattern of evolution consistent with the ray-fin fish-specific genome duplication have also been identified (Venkatesh, 2003; Postlethwait et al., 2004).

The fibroblast growth factors (FGFs) are small extracellular signaling proteins that regulate various aspects of cell proliferation, migration, and differentiation (Ornitz and Itoh, 2001). The FGF-superfamily shows signatures of expansion through large-scale duplications consistent with the 2R hypothesis of genome duplications early in the vertebrates (Itoh and Ornitz, 2004; Popovici et al., 2005). The FGF-superfamily is also thought to have expanded by gene duplication after the split between protostomes and deuterostomes (Itoh and Ornitz, 2004; Popovici et al., 2005). Comparison of gene content for the FGF-superfamily between tetrapods and teleosts, however, does not clearly reflect the hypothesis of a genome duplication specific to the ray-fin fish, possibly because of extensive individual gene losses in this family or because not all FGFs have yet been identified in zebrafish. Comparison between human and zebrafish genomes reveals that the zebrafish has two paralogs for about 20% of identified human genes (Postlethwait et al., 2000), and even fewer in *Tetraodon* (Jaillon et al., 2004), indicating that nonfunctionalization does in fact occur at a high rate.

Fgf genes play critical roles in many aspects of embryo patterning. Moreover, previous studies on a small number of model organisms suggest that FGF functions are stereotypical and conserved across broad phylogenetic distances. For example FGF8 is required for tetrapod limb development (Lewandoski et al., 2000). FGF8, FGF17 and FGF18 are expressed at the midbrain-hindbrain boundary (MHB) and induce midbrain development (Sato et al., 2004), and FGF18 is required for skeletal development (Liu et al., 2002; Ohbayashi et al., 2002). In zebrafish, Fgf24 acts in concert with Fgf8 to promote mesodermal development (Draper et al., 2003). FGF8, FGF17 and FGF18 are part of a subfamily (Ornitz and Itoh, 2001) that also includes Fgf24 in zebrafish (Itoh and Ornitz, 2004; Popovici et al., 2005). Investigation of the FGF content in the urochordate *Ciona intestinalis* revealed only six *Fgf* genes, with one being clearly ancestral to the *Fgf8/17/18*-subfamily (Satou et al., 2002). In zebrafish, *fgf17a* (Reifers et al., 2000a) and *fgf17b* (Cao et al., 2004) were thought to be co-orthologs of the mammalian *Fgf17*. The zebrafish *fgf17* genes exhibit different

patterns of expression, and probably have distinct functions. Fgf17a is thought to act as an organizer of the MHB (Reifers et al., 2000a), whereas Fgf17b seems to play a role in the anteroposterior patterning of the neurectoderm (Cao et al., 2004). The zebrafish *fgf17a* gene was named because of conserved synteny for three loci surrounding *Fgf17* on mouse chromosome 14 and *fgf17a* on zebrafish linkage group 1 (Reifers et al., 2000a), despite showing more sequence similarity with *fgf8* (Reifers et al., 2000a; Cao et al., 2004). As many zebrafish genes were named before the ray-fin fish genome duplication was identified, some may have been misannotated. Examining gene family evolution in several fish species will help reveal true orthologies, improve annotation, and hence advance our understanding of the evolution of developmental mechanisms related to this gene family.

In this study, we identified members of the *Fgf8/17/18*-subfamily in the three-spine stickleback *Gasterosteus aculeatus*, and investigated evolutionary relationships within this subfamily. We showed that *fgf17b* is the ortholog of tetrapod *Fgf17* whereas *fgf17a* and *fgf8 are* teleost duplicates of tetrapod *Fgf8*. Compatible with the teleost whole-genome duplication, this *fgf8* duplication occurred before the divergence of lineages leading to zebrafish and other teleosts. We used in situ hybridization to investigate the expression patterns of stickleback *fgf8a* and *fgf8b*. Results showed that both duplicates exhibit common and unique patterns of expression, indicating that tissue specialization followed the duplication event. Moreover, direct comparison of stickleback and zebrafish *fgf8* expression patterns suggested independent lineage-specific subfunction partitioning and the acquisition or loss of functions between orthologs in these two species. Surprisingly, differences in the expression of zebrafish and stickleback *fgf8a* in the apical ectodermal ridge (AER) of the pectoral fin bud raises the question of a conserved mechanism of limb development in vertebrates.

MATERIALS AND METHODS

Isolation of stickleback fgf genes

We screened our stickleback fosmid genomic library (see Cresko et al., 2003 for details) by polymerase chain reaction (PCR) using degenerate primers designed from the first exon of the zebrafish *fgf8*, *fgf17b*, *fgf18*, and *fgf24* sequences. (Note that we use here the official gene and protein nomenclature conventions of zebrafish for stickleback; we use the mouse convention for generalized chordate genes or species without official conventions, and the human convention for human genes. See http://zfin.org/zf_info/nomen.html for details.) Several positive clones were obtained, and assigned to unique groups by restriction enzyme analysis. A representative clone from each unique group was chosen for shotgun subcloning and sequencing. We sheared and subcloned each fosmid clone using a nebulizer and subclone ends were repaired using a mixture of T4 polymerase and Klenow Fragment. Blunted subclones were then ligated into the PCR4-BLUNT cloning vector (Invitrogen TOPO Shotgun Subcloning Kit, Cat. No. K7000-01, K7010-01, K7050-01 and K7060-01). The average size of inserted DNA was 2–5 kb. Subclones were arrayed into 96-well plates as single clones and then screened via PCR with the same first exon primers described above. Positive clones were sequenced from both ends of the vector, allowing assembly of several thousand base pairs (kb) for each gene. We obtained nearly full-length sequences, including introns and 3′UTR, for the corresponding genes but could not identify the first exon, presumably because of high divergence between the stickleback and zebrafish genes for this exon. We isolated a single sequence for *fgf17b* (6,120 bp), *fgf18* (2,999 bp), and *fgf24* (7,825 bp), but isolated two similar sequences of approximately 10 kB from clones 2C11 and 6H10, respectively, when screening for *fgf8*. After confirming that the sequences were similar, but in fact different, the fosmid clones 2C11 and 6H10 were fully sequenced at the Advanced Center for Genome Technology at the University of Oklahoma (Bodenteich et al., '93; Chissoe et al., '95) and assigned Genbank accession numbers AC148777 and AC148608, respectively. Accession numbers for *fgf17, fgf18, and fgf24* genomic sequences are EF445896, EF445897, and EF445898. Gene structures for each *fgf* gene were predicted using the Genscan (Burge and Karlin, '98) web server (http://genes.mit.edu/GENSCAN.html).

Genetic mapping on a haploid mapping panel

We mapped each clone to stickleback linkage groups using a haploid mapping panel constructed as described for zebrafish (Streisinger et al., '81). After activating a clutch of eggs from a single female from a polymorphic Rabbit Slough oceanic

population (see Cresko et al., 2004 for details) with UV irradiated sperm from a Rabbit Slough male to cross-link the sperm's DNA to prevent it from contributing to the developing zygote, these gynogenetic haploid embryos were allowed to develop until 8 days post fertilization (dpf), at which time all 48 fish were sacrificed and their DNA was extracted using standard phenol-chloroform procedures. We maintain the Rabbit Slough line as a polymorphic colony through repeated outcrosses to retain genetic diversity. Thus, most regions of the genome of the female parent of the cross were heterozygous for polymorphic markers, allowing simple PCR assays for segregating alleles. Using the full-length sequence obtained from clones 2C11 and 6H10, we designed several microsatellite markers for each clone. Segregation of these markers allowed us to map clones 2C11 and 6H10 to linkage groups LGVI and LGIX, respectively.

Phylogenetic analysis

Zebrafish Fgf sequences (*fgf8* (NM_131281), *fgf17a* (NM_182856), *fgf17b* (NM_214808), *fgf18* (NM_001013264), *fgf18-like* (NM_001012379), and *fgf24* (NM_182871)) were used in TBLASTN (Altschul et al., '97) searches of the genomes of *Takifugu rubripes* (Aparicio et al., 2002), *Tetraodon nigroviridis* (Jaillon et al., 2004), and *Oryzias latipes* (The Medaka Genome Sequencing Project, National Institute of Genetics, unpublished) using BLAST servers at the Institute of Molecular and Cell Biology, the Genoscope, and the Shared Information of Genetic Resources. Tetrapod *Fgf* sequences are available in Genbank (NM_010205, NM_008004, NM_008005, AF520763, NM_003867, AF075292, U55189, NM_204714). Exon/intron boundaries were determined first from Genscan predictions and then by sequence comparisons with the DNA sequence of the corresponding zebrafish *fgf*. This strategy identified complete sequences for *Fugu fgf17a* and *Fugu fgf8*. Other sequences lack all or part of exon 1, except for *Tetraodon fgf17a* and *fgf8*, which are only 47 and 57% complete relative to their zebrafish ortholog. Our BLAST search did not identify *fgf17a*, *fgf17b,* and *fgf24* in the medaka genome database. The only sequences with high similarity retrieved by BLAST using Fgf18-like were those found at the same genomic positions when using Fgf18. Reciprocal BLASTs were performed for all newly identified fish *fgf* genes against the zebrafish genome database using the zebrafish BLAST server available at the National Center for Biotechnology Information and it retrieved the sequence used during the original BLAST search as the best hit. All the sequences used in this study are available on the University of Oregon Stickleback server (http://stickleback.uoregon.edu).

We reconstructed phylogenetic relationships among these *Fgf* genes using maximum parsimony (MP), maximum likelihood (ML), and Bayesian methods with *Ciona intestinalis* Fgf8/17/18 (NM_001032476) as outgroup (Satou et al., 2002). Protein sequences were aligned by eye using BioEdit (Ibis Biosciences, Carlsbad, CA) (Hall, '99) and ambiguous positions were removed before to phylogenetic analysis. For MP analysis, we performed a heuristic search using PAUP * 4.0b10 (Swofford, '98). Tree space was searched using random stepwise addition with 100 replicates and with 10 trees retained at each step for branch swapping with tree bisection and reconnection. Confidence was assessed by bootstrapping in full heuristic mode with 500 replicates (Felsenstein, '85; http://www.cmbi.ru.nl/bioinf/PHYLIP/main.html). We used metropolis-coupled Markov chain Monte Carlo (MCMCMC) implemented in Mr Bayes v3.1 (Ronquist and Huelsenbeck, 2003) assuming a JTT model of protein evolution (Jones et al., '92), with five independent runs of 1,000,000 generations each and four chains. Trees were sampled every 100 generations and posterior probabilities were determined after discarding the first 41 trees (''burn-in''). ML analysis was performed using the PROML program as part of the PHYLYP package (Felsenstein, '89) also assuming a JTT model of evolution. Confidence was assessed with 500 bootstrap replicates analyzed with the same parameters.

Gene expression

We used in situ hybridization to detect gene expression of zebrafish and stickleback *fgf8a* and *fgf8b*. RNA probes were made by in vitro transcription of linearized genomic clones and labeled with digoxigenin-UTP. Stickleback *fgf8a* and *fgf8b* probes were synthesized from TOPO/Not-1 linearized clones using T3 RNA polymerase. Both probes are about 900 bp long and cover the last exon and the 3′UTR of the gene. Probes for zebrafish *fgf8a* and *fgf8b* were as described (Reifers et al., '98, 2000a).

Embryos were fixed with 4% paraformaldehyde at 4°C for at least 2 days before being dechorionated by hand under a dissecting microscope. In

situ hybridizations were performed as described (Yan et al., 2002) with several individuals for each developmental stage. Stickleback embryos were staged relative to zebrafish embryos using the zebrafish staging series (Kimmel et al., '95). In our system, stickleback development at 20°C is roughly 2.5 times slower than for zebrafish at 27.5°C (Cresko et al., 2003). The University of Oregon IACUC approved experiments for this study.

RESULTS

fgf8 is present in two duplicate copies in the teleost fish lineage

Using primers designed from the zebrafish *fgf8* sequence, we screened a genomic library for the three-spine stickleback and identified two clones, 2C11 and 6H10, which contain sequences with strong sequence similarity to *fgf8*. These two stickleback *fgf* genes encode predicted proteins 69.5% identical to each other at the amino acid level and we mapped them to LGVI and LGIX, respectively, indicating that the two clones are different loci and not isoforms of the same locus; thus, they are unlikely to have originated by tandem duplication. All of our phylogenetic analyses of the *Fgf8/17/18*-subfamily (MP, ML, and MCMCMC) using *Ciona intestinalis* Fgf8/17/18 (Satou et al., 2002) as an outgroup resulted in essentially the same topology (Fig. 1A). With the possible exception of Fgf24, we found strong support for the monophyly of the groups formed by the Fgf8, Fgf17a, Fgf17b, and Fgf18 sequences. MP gives strong support for an Fgf24 clade where zebrafish Fgf24 is the sister-taxon of a clade formed by the other teleost Fgf24 sequences, whereas MCMCMC gives strong support for zebrafish Fgf24 branching off after the divergence of the other teleost Fgf24 sequences and being the sister-taxon of all other FGF proteins in the *Fgf8/17/18*-subfamily. It is noteworthy, however, that ML gives the same topology as MCMCMC regarding the position of zebrafish Fgf24 but that this topology is poorly supported. Other differences included relationships within the teleost Fgf8 and Fgf18 clades. In almost all cases, conflicting nodes between the three methods were poorly supported either by posterior probabilities or bootstrap values. For instance, the conflict between MP on the one hand and ML and Bayesian on the other regarding the relationships within the teleost Fgf8 or Fgf18 clades is poorly supported by MP bootstraps. In fact, the only conflict for which alternative topologies have good support lies in the position of zebrafish Fgf24.

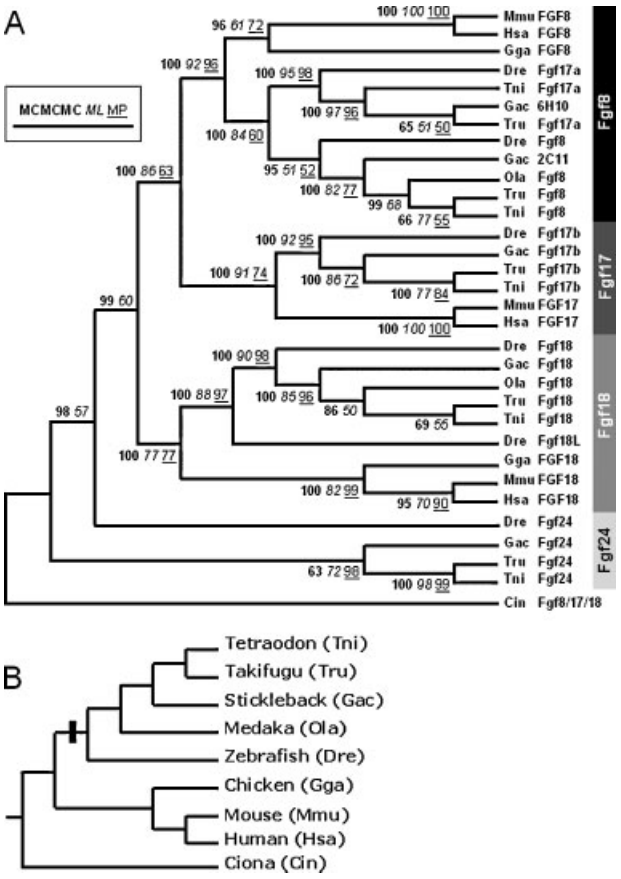

Fig. 1. (**A**) Phylogenetic tree obtained using metropolis-coupled Markov chain Monte Carlo depicting evolutionary relationships in the Fgf8/17/18 subfamily. Posterior probabilities are indicated in bold. Bootstrap values obtained for maximum likelihood and maximum parsimony analyses are also indicated at each node compatible with the metropolis-coupled Markov chain Monte Carlo topology and are shown respectively in italics and underlined. All supports are given in percent values. The teleost gene formerly called *fgf17a* is an *fgf8* duplicate whereas *fgf17b* is an ortholog of tetrapod *Fgf17*. (**B**) Compatible with the whole-genome duplication hypothesis, the *fgf8* duplication (black bar) is specific to the teleost lineage. *Cin*: *Ciona intestinalis*, sea squirt; *Dre*: *Danio rerio*, zebrafish; *Gac*: *Gasterosteus aculeatus*, three-spine stickleback; *Gga*: *Gallus gallus*, chicken; *Hsa*: *Homo sapiens*, human; *Mmu*: *Mus musculus*, mouse; *Ola*: *Oryzias latipes*, medaka; *Tni*: *Tetraodon nigroviridis*, pufferfish; *Tru*: *Takifugu rubripes*, pufferfish.

Importantly, Fgf17a falls clearly within the Fgf8 clade along with other teleost *fgf8* genes, which together form a clade that is the sister-group of the tetrapod Fgf8 clade (Fig. 1A). This genealogical pattern strongly supports the notion that *fgf17a* has been misannotated, and is not an ortholog of *Fgf17* as thought previously (Reifers

et al., 2000a), but is in fact a co-ortholog of *Fgf8* and a duplicate of the annotated zebrafish *fgf8* gene. Moreover, the *fgf8* duplication is specific to the teleost lineage and is compatible with the whole-genome duplication in the ray-fin fish lineage. The clade occupied by zebrafish Fgf17b is the sister-group to the tetrapod Fgf17 clade forming an Fgf17 clade that shows strong support. As these results indicate that *fgf17b* is the sole teleost ortholog of the tetrapod *Fgf17* gene, we suggest that teleost *fgf8* be renamed *fgf8a*, and *fgf17a* be called *fgf8b*. Consequently, *fgf17b* should be renamed *fgf17*. The nomenclature for *fgf18* genes is unaffected. Our results indicate that the stickleback *fgf* genes in fosmids 2C11 and 6H10 are, respectively, the co-orthologs *fgf8a* (AC148777) and *fgf8b* (AC148608). Thus, from this point onward, we will use this terminology to designate these two sequences and their zebrafish orthologs.

Stickleback fgf8 duplicates have overlapping but distinct expression patterns

Given our new understanding of orthology relationships provided by phylogenies with stickleback *fgf*s, we wondered about the partitioning of expression domains between the duplicates in zebrafish and stickleback. We performed in situ hybridizations at different developmental stages to visualize co-ortholog-specific expression dynamics over time and space and used these expression patterns as a proxy for gene function. Stickleback *fgf8a* and *fgf8b* exhibited temporal and spatial differences in expression patterns whereas retaining common expression domains in some tissues (Fig. 2). Stickleback *fgf8a* was strongly expressed in rhombomeres 2 and 4 and telencephalon earlier in development than *fgf8b* (Fig. 2A, B). We saw *fgf8a* expression at 20 hpf but *fgf8b* expression became strong only after 32 hpf (data not shown). At 36 hpf, stickleback *fgf8a* and *fgf8b* were both expressed in the MHB, whereas *fgf8a* was expressed in the telencephalon and weakly in the eyes and *fgf8b* was expressed in neural crest cells emanating from the hindbrain (Fig. 2C, D). The patterns of expression were strengthened at 60 hpf with modifications. At this stage, stickleback *fgf8a* and *fgf8b* were both expressed in the MHB and in the optic stalk, although somewhat differently, while *fgf8a* showed strong and unique expression in the retina and dorsal diencephalon. Expression of *fgf8b*, but not *fgf8a*, appeared in the neural crest and the heart (Fig. 2E, F). Differences of expression were also apparent in the tail, with *fgf8a* but not *fgf8b*

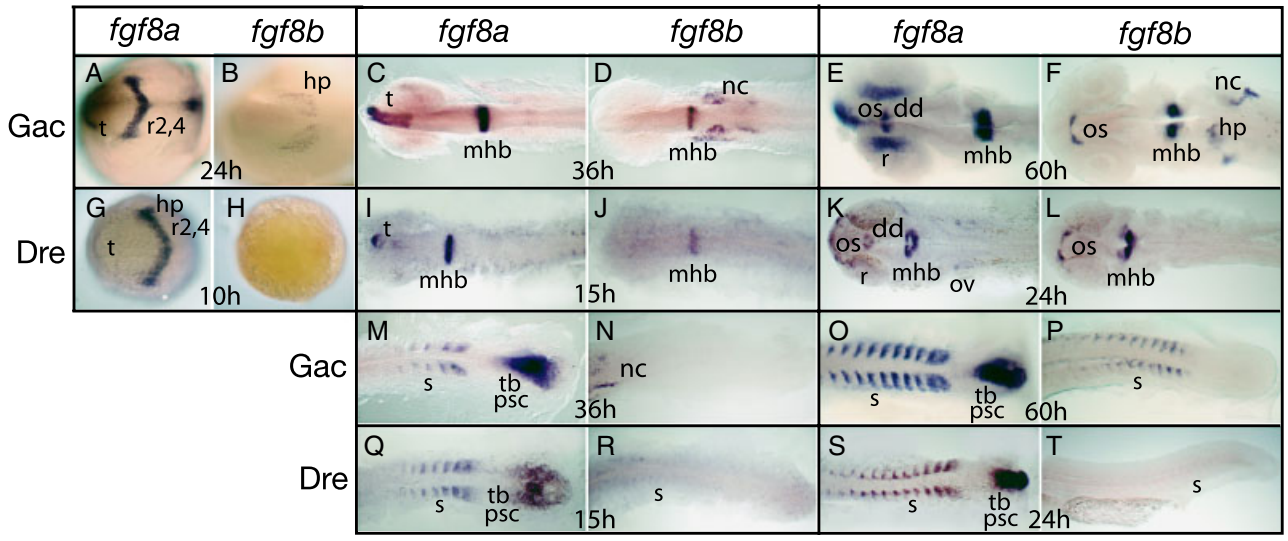

Fig. 2. Comparison of *fgf8a* and *fgf8b* expression patterns in stickleback and zebrafish. The overlapping of expression patterns between *fgf8* orthologs indicates that the partitioning of most subfunctions between co-orthologs occurred before the divergence of the lineages leading to stickleback and zebrafish. Divergent expression patterns between *fgf8* orthologs, however, also suggest independent lineage-specific subfunction partitioning. Embryos are shown from a dorsal view and anterior is oriented to the left. (**A–F** and **M–P**, stickleback; **G–L** and **Q–T**, zebrafish. Ages are given in the figures in hours (h) post fertilization. Abbreviations: dd, dorsal diencephalon; gl, ganglion layer; h, heart; hp, heart primordium; mb, midbrain; mhb, midbrain-hindbrain boundary; n, nose; nc, neural crest; oe, oral ectoderm; opl, outer plexiform layer; os, optic stalks; ov, otic vesicle; pf, pectoral fin; psc, posterior spinal chord; r, retina; r2,4, rhombomeres 2 and 4; s, somites; t, telencephalon; tb, tail bud.

being expressed in the region including the tail bud and the posterior spinal chord (Fig. 2M–P). Quantitative differences were also clear for the optic stalk, the MHB, and the somites (Fig. 2C–F; O,P). These experiments revealed that *fgf8a* and *fgf8b*, the two stickleback co-orthologs of tetrapod *Fgf8*, have overlapping (e.g., MHB) and gene-specific (e.g., posterior spinal cord and reciprocally, the heart primordium) regulatory patterns that arose after the genes duplicated in the teleost genome duplication event. Comparisons with out groups is necessary to distinguish the mechanism as either subfunction partitioning or neofunctionalization.

Stickleback fgf8 co-orthologs have similar and different expression patterns compared with their zebrafish orthologs

After *fgf8* genes duplicated and recombination between homeologous chromosomes was suppressed during rediploidization, *fgf8* co-orthologs could assume independent evolutionary paths. Some of that independent evolution would have occurred before the divergence of extant teleost lineages, and some after the teleost radiation. To determine the extent to which regulatory elements driving *fgf8* co-ortholog expression patterns evolved in these two time intervals, we directly compared the expression patterns of *fgf8* co-orthologs in stickleback and zebrafish. Although expression patterns for several stages of the two zebrafish genes have been published (Reifers et al., 2000b; Cao et al., 2004), it was essential to compare expression patterns at comparable developmental stages. Our results revealed remarkable conservation of expression, but also some important species-specific differences both in time and space. At 36 hpf, stickleback *fgf8b* showed expression in neural crest cells that give rise to cranial ganglia, but no expression is seen in this tissue for the orthologous *fgf8b* gene in zebrafish (Fig. 2D, J, F, L). More strikingly, stickleback *fgf8b* is expressed in the heart at 60 hpf whereas zebrafish *fgf8b* expression was not detected in the heart (Fig. 2F, L). In contrast to stickleback, in zebrafish, *fgf8a* rather than *fgf8b* is expressed in the heart and is required for the expression of cardiac genes (Reifers et al., 2000b). These experiments show that, although the general expression patterns of *fgf8a* and *fgf8b* are shared by stickleback and zebrafish, and hence, parsimoniously would have evolved before lineage divergence, several domains (heart and neural crest) are species-specific, and hence evolved after lineage divergence.

Stickleback fgf8 expression patterns in later embryonic stages

To see if the above conclusions for early developmental stages also hold for older animals, we compared expression patterns of stickleback *fgf8* duplicates at later developmental stages (Fig. 3). At 5dpf, both *fgf8* duplicates in stickleback were expressed in the telencephalon and in the MHB (Fig. 3A, B, E, F), but *fgf8a* showed specific expression in the dorsal diencephalon (Fig. 3A, B, I, J) and in the olfactory primordium (Fig. 3E, F). Differences were also seen in the eye where *fgf8a* was expressed in the ganglion layer and *fgf8b* was expressed in the outer plexiform layer of the retina (Fig. 3A, B, E, F, I, J). Stickleback *fgf8a* and *fgf8b* were both expressed in the oral ectoderm, but showed distinct expression patterns in this region (Fig. 3E, F). Quantitative differences were found for the expression of *fgf8* co-orthologs in the posterior pharyngeal arches, where *fgf8a* showed stronger expression, and in the otic vesicle, where *fgf8b* was more strongly expressed (Fig. 3E, F, I, J).

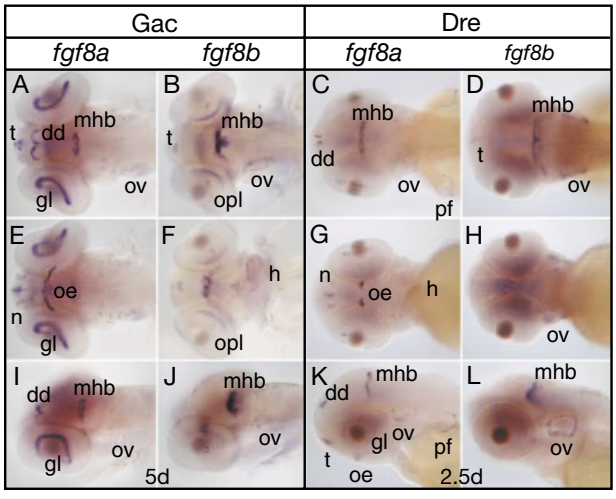

Fig. 3. Stickleback and zebrafish *fgf8a* and *fgf8b* late expression patterns. **A**, **B**, **E**, **F**, **I**, **J**, stickleback (Gac) at 5 days post fertilization (d); **C**, **D**, **G**, **H**, **K**, **L**, zebrafish (Dre) at 2.5 days post fertilization. A–D, dorsal views; E–H, ventral views; I–L, lateral views. Teleost *fgf8* co-orthologs show similar expression patterns but nevertheless exhibit differences, in particular the absence of *fgf8b* expression in the dorsal diencephalon (compare A and B). The expression of zebrafish and stickleback *fgf8* orthologs is remarkably conserved, although the expression pattern of *fgf8a* orthlogs in the oral ectoderm (compare E and G) and in the heart (compare E and G, F and H) differ. Abbreviations as in Figure 2.

Expression patterns of *fgf8a* and *fgf8b* orthologs in zebrafish and stickleback were remarkably conserved overall, but again species-specific differences were apparent. The expression pattern of *fgf8a* in the oral ectoderm was somewhat different between zebrafish and stickleback (Fig. 3E, G), and as noted for earlier stages (Fig. 2), zebrafish and stickleback *fgf8* orthologs showed reciprocal patterns of expression in the heart (Fig. 3F, G).

Taken together, our data show that *fgf8a* and *fgf8b* have common and unique patterns of expression in both stickleback and zebrafish, indicating that tissue specialization by either subfunction partitioning or neofunctionalization followed the duplication event. As most of the expression patterns of stickleback *fgf8a* and *fgf8b* are conserved with their orthologs in zebrafish (Figs. 2 and 3), we conclude that most of the specialization of *fgf8a* and *fgf8b* is ancient, having occurred after the genome duplication event but before the divergence of zebrafish and stickleback lineages (Fig. 1B). Nevertheless, species-specific expression differences in the heart, neural crest, eyes, and other tissues show that *fgf8* orthologs evolved regulatory differences after the divergence of zebrafish and stickleback lineages owing to either the acquisition of new functions or the loss of ancestral functions in one or both lineages.

fgf8 duplicates show expression divergence in the AER of the developing pectoral fins

Previous work in mouse and chicken identified a critical role of Fgf8 for the proper outgrowth of the developing pectoral appendage (Lewandoski et al., 2000; Moon and Capecchi, 2000; Dudley et al., 2002; Sun et al., 2002; Boulet et al., 2004). To explore possible roles of *fgf8* co-orthologs in stickleback and zebrafish, we focused on the timing and position of *fgf8* expression domains in the teleost pectoral appendage. Results showed that *fgf8a* but not *fgf8b* was expressed distally in the developing pectoral fin bud in both teleosts (Fig. 4), indicating that *fgf8a* and *fgf8b* gene duplicates evolved divergent regulation and probably different functions in limb bud development after the duplication event but before zebrafish and stickleback lineages diverged. Expression of *fgf8a* in the developing appendage appeared only after the fin bud formed in stickleback, as in zebrafish (Fig. 4) (Reifers et al., '98). In this respect, the *fgf8a* expression in the appendage bud of stickleback and zebrafish is different from the expression of its tetrapod ortholog, which is

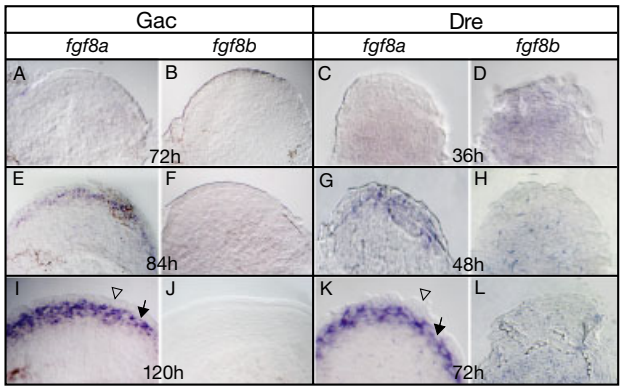

Fig. 4. *fgf8a* and *fgf8b* expression patterns in the pectoral fin of stickleback and zebrafish. The absence of *fgf8b* expression in the pectoral fin (**B**, **F**, **J**, **D**, **H**, **L**) indicates that *fgf8* duplicates have evolved divergent regulation following the gene duplication event but before the divergence between the stickleback and zebrafish lineages. *fgf8a* is expressed only after the limb bud has formed (**E**, **I**, **G**, **K**) and *fgf8a* orthologs are expressed near the margin of the limb bud (I and K, arrow) but are not expressed in the most distal row of cells at the tip of the ridge (I and K, arrowhead).

expressed at early stages of appendage bud development and promotes appendage elongation.

Moreover, the spatial distribution of *fgf8a* expression in the appendage bud in stickleback and zebrafish was different from that of *Fgf8* in tetrapods. Stickleback and zebrafish *fgf8a* orthologs were expressed near the margin of the developing pectoral fin but were not fully expressed in the row of cells in the distal ridge that forms the AER. Instead, *fgf8a* orthologs were expressed on the distal dorsal and ventral surfaces of the developing fin (Fig. 4I, J arrow) leaving a row of cells between these two *fgf8a*-positive cell populations that lacked *fgf8a* expression (Fig. 4I, J arrowhead). These results show that the expression, and hence the likely function, of Fgf8 protein in the pectoral appendage bud is not as conserved as previously thought and is variable among vertebrates.

DISCUSSION

fgf8 duplication in the ray-fin fish lineage

Our phylogeny of the *Fgf8/17/18* subfamily well supports the *Fgf8*, *Fgf17,* and *Fgf18* clades. Moreover, the basal position of *fgf24* implies that the origin of this gene is ancient, before the divergence of ray-fin fish and tetrapods, and that it was subsequently lost in the latter lineage (Fig. 1A). Importantly, our phylogeny reveals,

without ambiguity, that the gene previously annotated as *fgf17a* (Reifers et al., 2000a) is, along with *fgf8a*, a duplicate of the tetrapod *Fgf8* gene, and so, by zebrafish nomenclature conventions (http://zfin.org/zf_info/nomen.html), should be called *fgf8b*. In contrast, our phylogenetic analysis strongly supports the identification of the gene we previously called *fgf17b* (Cao et al., 2004) as an ortholog of the tetrapod *Fgf17* gene. The duplication of *fgf8* occurred before the split between the lineages leading to zebrafish and other euteleosts (Fig. 1B). Furthermore, the position of the zebrafish *fgf18*-like gene, the sister-taxon to the clade formed by the ray-fin fish *fgf18* (Fig. 1A), suggests that *fgf18* was duplicated early in the divergence of the ray-fin fish and was subsequently lost in the genomes we sampled. This is the first evidence that *fgf* genes show a pattern of evolution compatible with the ray-fin fish genome duplication (Amores et al., '98; Postlethwait et al., '98; Wittbrodt et al., '98; Meyer and Schartl, '99; Christoffels et al., 2004; Meyer and Van de Peer, 2005) and suggests either a high rate of gene loss in the *fgf* family in the ray-fin fish or that not all *fgfs* have been identified in this group.

Reifers et al. (2000a) first identified the zebrafish *fgf8b* (previously *fgf17a*) gene by screening a zebrafish genomic library with a mouse *Fgf8* cDNA probe. They assigned orthology to mouse *Fgf17* based on conserved synteny for three markers surrounding *fgf8b* on zebrafish LG1 and *Fgf17* on mouse chromosome 14 and similar expression patterns. As expression patterns evolve independently in different lineages, as shown for the *fgf8a* gene here, for example, gene expression is not a good character for identifying orthologies; rather genomic structure analyses, including phylogenetic analysis and analysis of conserved syntenies are far more reliable for inferring gene histories. Analysis of conserved syntenies (Postlethwait and Jovelin unpublished results) shows that, whereas somewhat distantly linked markers used by Reifers et al. (2000a) support their orthology assignments, genes immediately adjacent to *fgf8b* are orthologous to genes adjacent to *FGF8* in human and in mouse rather than genes adjacent to *FGF17*. In contrast, we assigned orthology of zebrafish *fgf17* based on its proximity to two genes on LG8 orthologous to two genes located very close to *FGF17* on human chromosome 8 (Hsa8) (Cao et al., 2004). As orthology implies a common origin from a single element in the last common ancestor of different lineages, evidence for common origins can come from sequence comparisons and comparison of local conserved syntenies. The greater the genetic distance, the greater the caution needed to adequately interpret conserved syntenies. Likewise, the phenomena of subfunction partitioning and neofunctionalization make conserved expression patterns and conserved functions unreliable characters for use in orthology determination in cases of gene and genome duplication.

fgf8 duplication and subfunction partitioning

Analysis of expression patterns for stickleback *fgf8a* and *fgf8b* clearly reveal that several ancestral subfunctions partitioned between the two duplicates (Figs. 2–4 and Table 1 in supplementary data). Moreover, the extensive overlap of expression patterns of *fgf8* orthologs between zebrafish and stickleback indicates that the partitioning of most subfunctions is ancient and occurred after the gene duplication but before the split between the lineages that led to these two species. Nevertheless, our analysis revealed several divergent expression domains between orthologs, suggesting independent lineage-specific subfunction partitioning.

In mouse, *Fgf8* is required for several aspects of heart development (Abu-Issa et al., 2002; Ilagan et al., 2006). The role of *Fgf8* in heart development seems conserved between ray-fin fish and tetrapods (Reifers et al., 2000b), but in zebrafish, *fgf8a* plays this role (Reifers et al., 2000b) and in stickleback we infer that *fgf8b* plays this role because it is expressed in the heart precursor field rather than *fgf8a* (Fig. 2E, F; Fig. 3F, G). The most parsimonious explanation is that in the last common ancestor of ray-fin and lobe-fin fish, *Fgf8* played a role in heart development and that after the ray-fin fish genome duplication, the heart function was maintained by both *fgf8* duplicates in the last common ancestor of zebrafish and stickleback, but after these lineages diverged, independent evolution of gene regulatory elements led to the independent partitioning of the heart function to different paralogs in the zebrafish and stickleback lineages. As a result, true orthologs now perform nonorthologous functions.

Divergence in Fgf signaling during appendage formation

In vertebrates, appendage development requires the activity of several inducers driving the specification of different axes to form a three-

dimensional structure (Martin, '98). One of these inducers, the AER, promotes the elongation of the proximo-distal axis. The classical view that the AER functions in differentiating the proximal and distal limb elements during the elongation of the limb bud (Summerbell et al., '73; Summerbell, '74) has recently been modified (Dudley et al., 2002; Sun et al., 2002). In the revised model, the proximal and distal limb precursors are differentiated at an early stage in the limb bud and the AER functions to prevent cell death and to maintain proliferation of the different cell populations. Bead transplantation experiments in chicken embryos demonstrated the role of FGF signaling in limb development (Cohn et al., '95). The model predicted that expression of *Fgf8* in the intermediate mesoderm positions the limb field along the antero-posterior axis of the trunk by restricting *Fgf10* expression in the lateral plate mesoderm (LPM) to the limb field. In turn *Fgf10* was thought to promote expression of *Fgf8* in the adjacent ectoderm and interactions between the *Fgf8*-expressing ectoderm and the underlying *Fgf10*-expressing mesoderm was thought to enable the maintenance of the AER and the growth of the limb bud (Crossley et al., '96; Vogel et al., '96; Ohuchi et al., '97). Although genetic studies in mouse have confirmed the requirement of *Fgf10* (Min et al., '98; Sekine et al., '99) and AER-*Fgf8* signaling (Lewandoski et al., 2000; Moon and Capecchi, 2000; Dudley et al., 2002; Sun et al., 2002; Boulet et al., 2004), expression of *Fgf8* in the intermediate mesoderm is not essential for limb development (Boulet et al., 2004). In addition it has been shown in chicken that members of the WNT family are required for *Fgf10* expression in the LPM (Kawakami et al., 2001).

As pectoral limbs and pectoral fins are homologous (Panganiban et al., '97; Wagner and Chiu, 2001; Tanaka et al., 2002), it has often been assumed that mechanisms of limb development known from tetrapod models, in particular the central role of *Fgf8* signaling from the AER, are conserved across all vertebrates. Nevertheless, developmental genetic studies have shown that there can be a dissociation between homology at the phenotypic and molecular levels (Dickinson, '95; Abouheif et al., '97; Wray and Abouheif, '98). Duplication of developmental regulatory genes occurring in separate lineages exacerbates this dissociation resulting in independent modifications of developmental gene networks.

The temporal differences of *fgf8a* expression in the AER between ray-fin fish and tetrapods, and the peculiar spatial expression pattern of *fgf8a* seen in stickleback and zebrafish is a clear example that orthologous developing structures do not necessarily require orthologous genes or processes. Although the exact function of *fgf8a* in AER signaling in teleost appendages remains to be determined, the absence of expression during the initial formation of the pectoral fin bud and during the first steps of its growth shows at least that *Fgf8* signaling is not completely conserved in vertebrates and implies evolutionary divergence of regulatory elements between *fgf8a* in ray-fin fish and its tetrapod ortholog.

Recently, Inoue et al. (2006) have identified several conserved regulatory regions necessary for some functions of *fgf8a* in zebrafish. One of these regulatory regions, S4.2, drives *fgf8a* expression in the somites, the MHB, the optic stalks, and the otic vesicle. The S4.2 region, located downstream of *fgf8a*, includes a genomic sequence called DCR3 that is conserved between chicken, mouse, human, and zebrafish. Interestingly, DCR3 was not found in the downstream sequence of *fgf8a* in the teleosts *T. nigroviridis*, *O. latipes*, *T. rubripes* or in *Fgf8* of the amphibian *Xenopus tropicalis*. The genomic position of DCR3 and the expression patterns under the control of S4.2 suggest that DCR3 may correspond to the conserved sequence named CR3 in the study of Beermann et al. (2006). Beermann et al. (2006) compared human, chicken, mouse, and zebrafish genomes to identify the conserved region CR3 that regulates *Fgf8* transcription in the somites, the MHB, the AER, the commissural plate, and the first branchial arch in mouse. Interestingly, if the regulation by DCR3 and CR3 in zebrafish and mouse are partially consistent, there are also some differences, in particular, the absence of transcription regulation for *fgf8a* in the AER in zebrafish (Inoue et al., 2006). It is possible that DCR3 may be found at other genomic locations in the other teleosts and in the amphibian *X. tropicalis*, and that regulatory differences between DCR3 in zebrafish and CR3 in mouse may be explained by the omission of some enhancers in the reporters used in the two studies (Beermann et al., 2006; Inoue et al., 2006). The differences seen for these conserved genomic sequences (Beermann et al., 2006; Inoue et al., 2006), however, could also be functional and reflect divergence in *Fgf8* regulation within vertebrates. Further investigation is needed to elucidate the biological role of the conserved sequence DCR3/CR3 and to determine if either the absence or the divergence of DCR3 could

explain the divergence of *fgf8a* expression we observed in the AER in stickleback and zebrafish.

The absence or very low levels of *fgf8* transcripts in the AER during the early development of teleost pectoral fins suggests the evolution of FGF signaling and supposes some redundancy and compensation by other members of the FGF family. Interestingly, in zebrafish the *fgf8a* mutation *acerebellar* does not affect normal fin development (Reifers et al., '98), so that despite the expression of *fgf8a* in the zebrafish fin bud, this expression is not essential for normal development. Recently, the Fgf8-related protein Fgf24 (Fig. 1A) (Itoh and Ornitz, 2004; Popovici et al., 2005) has been shown to be essential for pectoral fin development in zebrafish (Draper et al., 2003; Fischer et al., 2003). Fgf24 acts upstream of Fgf10 and induces its expression in the LPM, but *fgf24* expression in the AER occurs after the formation of the limb bud (Fischer et al., 2003). A more distantly related gene, *fgf16*, acts downstream of *fgf10* to induce *fgf4* and *fgf8a* expression in the AER of zebrafish (Nomura et al., 2006). Moreover, *fgf24* in the mesenchyme (Fischer et al., 2003) and *fgf16* in the AER (Nomura et al., 2006) induce sonic hedgehog (*shh*) expression in the mesenchyme underlying the AER. Nomura et al. (2006) also showed that *fgf16* and *shh* maintain and/or induce each other's expression. This is different from the situation in tetrapods, because in mouse and chicken the interaction between the AER and the zone of polarizing activity , the inducer promoting antero-posterior differentiation of the limb, requires interaction between *Fgf8* in the AER and *shh* in the mesenchyme (Laufer et al., '94; Niswander et al., '94; Sun et al., 2002).

A possible scenario is that *Fgf24* and *Fgf8* both played a role in limb formation in the last common ancestor of tetrapods and ray-fin fish. The *Fgf24* gene was subsequently lost in the tetrapod lineage (Fig. 1A) and subfunction partitioning resulted in the loss of this function by *fgf8* but maintenance of the AER function by *fgf24* in the ray-fin fish lineage. As in mouse only *Fgf4*, *Fgf8*, *Fgf9,* and *Fgf17* are known to be expressed in the AER (Niswander and Martin, '92; Heikinheimo et al., '94; Ohuchi et al., '94; Crossley and Martin, '95; Mahmood et al., '95), this suggests complex regulatory evolution and either the loss of function of *Fgf16* in tetrapod limb development or the acquisition of *fgf16* function in pectoral fin formation in ray-fin fish. Regardless of the exact evolutionary scenario, our results and those mentioned above (Reifers et al., '98; Draper et al., 2003; Fischer et al., 2003; Nomura et al., 2006) show that mechanisms of vertebrate limb development are less conserved than previously thought. More generally, these results call into question conclusions regarding the universality of gene function and developmental processes drawn from a few model organisms and point to the need to expand developmental studies beyond model clades and to interpret results in an evolutionary context.

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