A shift in anterior–posterior positional information underlies the fin-to-limb evolution

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Abstract

The pectoral fins of ancestral fishes had multiple proximal elements connected to their pectoral girdles. During the fin-to-limb transition, anterior proximal elements were lost and only the most posterior one remained as the humerus. Thus, we hypothesised that an evolutionary alteration occurred in the anterior–posterior (AP) patterning system of limb buds. In this study, we examined the pectoral fin development of catshark (Scyliorhinus canicula) and revealed that the AP positional values in fin buds are shifted more posteriorly than mouse limb buds. Furthermore, examination of Gli3 function and regulation shows that catshark fins lack a specific AP patterning mechanism, which restricts its expression to an anterior domain in tetrapods. Finally, experimental perturbation of AP patterning in catshark fin buds results in an expansion of posterior values and loss of anterior skeletal elements. Together, these results suggest that a key genetic event of the fin-to-limb transformation was alteration of the AP patterning network.

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Introduction

Regulatory interactions between transcriptional factors play important roles for interpreting a morphogen gradient as positional information (Balaskas et al., 2012). Changes in these regulatory interactions may therefore be key players for patterning changes during morphological evolution. The fin-to-limb transformation is a prominent but still unsolved example of morphological evolution. 150 years ago Carl Gegenbaur subdivided the skeletal elements of shark pectoral fins into three segments along the anterior–posterior (AP) axis: propterygium, mesopterygium, and metapterygium (Gegenbaur, 1865) (Figure 1A), which are also found in the majority of chondrichthians, non-teleost actinopterygians, placoderms, and acanthodians (Orvig, 1962; Coates, 1994, 2003). Therefore, possession of propterygium, mesopterygium, and metapterygium is considered to be a plesiomorphic state for gnathostomes. In the sarcopterygians (lobe-finned fishes including tetrapods), the propterygium and mesopterygium have been lost (Coates, 2003), thus, suggesting that anterior positional values have been lost or reduced during tetrapod evolution.

In mouse limb buds, Hand2, Gli3, and Shh are key genes for controlling AP patterning (Riddle et al., 1993; Te Welscher et al., 2002). One of the earliest patterning events is the mutual
transcriptional repression between Gli3 in the anterior tissue and Hand2 in the posterior (Te Welscher et al., 2002; Osterwalder et al., 2014). This early polarity in expression contributes to the subsequent posterior localized expression of Shh, and this morphogen in turn reinforces the anteriorly restricted Gli3 protein activity (Gli3R) provides cells with information about their position along the ‘anterior–posterior’ (or thumb-to-little finger) axis of the bud. This protein regulates several genes that are involved in limb development, and this results in different genes being expressed in cells along the anterior–posterior axis. For example, Alx4 is only expressed in a small area at the anterior end of the bud, while Hand2 expression is found in a large area towards the posterior part.

Gli3R is also found in a fish called the catshark, but it is not clear how it controls the formation of fins. Onimaru et al. show that the pattern of gene expression in the catshark fin bud is different to that of the mouse limb bud. For example, Alx4 is expressed in a larger area of the fin bud that extends further towards the posterior, while Hand2 is only found in a much smaller area at the posterior end of the bud. The experiments also suggest that Gli3R is active in a much larger area of the fin bud than in the limb bud.

Next, Onimaru et al. used a drug on the catshark embryos to increase the activity of another protein that can inhibit Gli3R. The fin buds of these shark had anterior shift in several gene expression domains, and the fins that formed were missing several anterior bones and had only a single bone connected to the pectoral girdle. Onimaru et al.’s findings suggest that during the evolution of the tetrapods, there may have been a shift in the anterior–posterior patterning of the fin bud to form a limb. An important area for future work will be to use genome-wide studies to study the fin/limb buds of other species.

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Results and discussion
To investigate changes in AP patterning during the fin-to-limb transition, we first cloned a number of AP patterning genes from the non-model species Scyliorhinus canicula (Figure 1B–H and Figure 1—figure supplement 1 for phylogentic analyses). In the mouse limb bud, Alx4, Pax9, Hand1, and Zic3 are positively regulated by Gli3R (Te Welscher et al., 2002; Fernandez-Teran et al., 2003; McGlinn et al., 2005; Vokes et al., 2008) and thus are expressed in a localized anterior domain (one-third of the axis), while Hand2 and Tbx2 show broad posterior expression domains (two-thirds and one-half of the axis, respectively). In stage 30 S. canicula embryos (staged according to Ballard et al., 1993), we found instead that the anterior genes Alx4, Pax9, Hand1, and Zic3 were expressed in broad domains, which extend more posteriorly than in the mouse (half the fin bud for Alx4, two-thirds for Pax9 and Hand1, and the whole axis for Zic3, Figure 1B–E). By contrast, the Hand2 and Tbx2...
Figure 1. Anterior–posterior patterning in Scyliorhinus canicula pectoral fin buds. (A) Skeletal patterns of S. canicula pectoral fin and mouse limb. Blue colours, homologous elements. (B–G) In situ hybridisation for Alx4 (B), Pax9 (C), Hand1 (D), Zic3 (E), Hand2 (F), and Tbx2 (G) in S. canicula pectoral fin buds at stage 30 and mouse limb bud at E11.5 (left panel in G). Arrowheads in G, anterior boundary of posterior Tbx2 expression. Dorsal view; anterior is to the top. Scale bars, 100 μm. (H) Schematic of the gene expression patterns. Arrowheads, Hand2 expression boundary. Expressions of mouse limb buds at E11.5 are after EMBRYS database (Yokoyama et al., 2009; Fernandez-Teran et al., 2003).

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The following figure supplements are available for figure 1:

Figure supplement 1. Molecular phylogenetic trees of relevant S. canicula genes.
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Figure supplement 2. Temporal expression analysis of Alx4, Pax9, Hand2, and Hoxa13 in S. canicula pectoral fins.
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Figure supplement 3. Temporal expression analysis of Alx4, Pax9, Hand2, and Hoxa13 in chick limb buds.
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domains were more posteriorly restricted in S. canicula fin buds than in mouse limb buds (Figure 1F,G).
All of these 6 AP patterning genes show the same trend—their expression boundaries are more posterior in S. canicula fin buds (Figure 1H), apparently reflecting a gross shift in the AP coordinate system. We chose 3 of these genes to test at multiple time-points to determine whether this was a transient gene expression state (Figure 1—figure supplements 2, 3), but in all cases these shifts were observed from stage 29 to stage 31 (which covers ~30 days of S. canicula development). In particular, stage 29 is a stage where Sox9 expression (a prechondrogenic marker) starts in the proximal part of the pectoral fin buds (Figure 1—figure supplement 2E), which suggest that the observed shift of AP values would affect proximal skeletal elements as well as distal.

Since the above AP patterning genes are regulated by Shh–Gli3 pathway (Te Welscher et al., 2002; Fernandez-Teran et al., 2003; McGlinn et al., 2005; Galli et al., 2010), we cloned Gli3 from S. canicula fin buds (Figure 2A and Figure 2—figure supplement 1A for phylogenetic tree) and analysed its expression in pectoral fin buds. In striking contrast to tetrapod limb buds (Büsscher et al., 1997; Schweitzer et al., 2000), Gli3 expression is not restricted to the anterior region—thus again indicating a general posterior shift of AP positional values in the S. canicula. To address whether this situation is conserved in other chondrichthians, we also cloned and analysed the expression of Gli3 in pectoral fin buds of a holocephalian, Callorhinchus milii. This strongly suggests that the Hand2–Gli3 mutual inhibition seen in tetrapods is weak or non-existent in chondrichthians.

In chick and mouse limb buds, Gli2 does not play a major role in AP patterning because of its weak processing efficiency to produce its repressor form (Wang et al., 2000). However, in zebrafish, Gli2 does indeed act as a repressor (Maurya et al., 2013), so we checked whether Gli2 could be playing the repressor role in S. canicula fin buds. First, we analysed Gli2 expression in S. canicula embryos and found it to be uniform until stage 29 (Figure 2C) and then subsequently restricted to the posterior region (Figure 2C). Second, we checked whether Gli3 and Gli2 of S. canicula have the repressor function, by measuring their processing efficiencies. We analysed the processing determinant domain (PDD), which determines the differential processing efficiencies of Gli3 and Gli2 in mice and humans (Pan and Wang, 2007). We inserted the PDDs from human Gli2 and S. canicula Gli2 or Gli3 into the human Gli3 PDD region (Figure 2D and Figure 2—figure supplement 1 for the amino acid sequences), transfected these constructs into HEK293 cells, and treated the cells with forskolin (FSK) to induce Gli processing. Human and S. canicula Gli2 PDD did not induce Gli3R, whereas their Gli3 PDDs did (Figure 2E). Thus, in S. canicula (as in chick and mouse), Gli3, but not Gli2, plays the major role in repressor production.

We next wished to explore if a genetic explanation could be found for the lack of Gli3 repression in the posterior part of pectoral fin buds of S. canicula and C. milii. To compare Gli3 enhancers in chondrichthians and tetrapods, we used the VISTA enhancer browser (Visel et al., 2007) and found a limb-specific Gli3 enhancer, element 1586, which replicates anterior Gli3 expression in mouse limb buds. We identified the homologues of element 1586 in S. canicula and C. milii and compared them with those from other vertebrates. Consistent with the slow evolutionary rate of chondrichthians and coelacanths (Amemiya et al., 2013; Renz et al., 2013; Venkatesh et al., 2014), element 1586 is conserved in tetrapods, coelacanths, and chondrichthians, but not in gar, medaka, and zebrafish (Figure 3A). To assess whether the element 1586 in different species has different functionalities, we cloned this element from chick, S. canicula, and C. milii in front of a basal promoter followed by a GFP reporter (Ochi et al., 2012; Figure 3B). These constructs were electroporated into chick forelimb buds with a constitutively active RFP vector (to determine the spatial efficiency of electroporation). As with endogenous Gli3 expression (Büsscher et al., 1997), the chick element 1586 drove GFP expression specifically in anterior tissue and was repressed in the posterior region, even though RFP was expressed throughout the buds (Figure 3C). The element 1586 from both S. canicula and C. milii also drove GFP expression in the chick limb buds, confirming that its general activity is conserved from sharks to tetrapods. However, in both cases, the specific posterior repression observed in the chick element was absent (Figure 3D,E). Thus, the differential activity of this enhancer (with tetrapods showing posterior repression, and chondrichthians not) recapitulates the differences in Gli3 expression within these groups. Furthermore, by recombining S. canicula and chick enhancers, we identified a sequence that can exert the posterior repression when inserted into the S. canicula enhancer (Figure 3F,G and Figure 3—figure supplement 1).
This sequence contains tetrapod or sarcopterygian-specific sequences, suggesting that the posterior repressive activity would have been acquired in a stepwise fashion.

Finally, we wished to address whether changes to AP positional information could modify skeletal arrangement of the propterygium and mesopterygium in catshark. For this purpose, we explored methodologies for performing manipulative experiments on this very slow-developing non-model fish (see ‘Materials and methods’). We treated *S. canicula* embryos with retinoic acid (RA) to increase Shh-signalling activity (at stage 29 with 1 μg/ml of RA for 4 days). Activation of Shh signalling by RA is known to be conserved among vertebrate limbs/fins (Riddle et al., 1993; Hoffman et al., 2002; Dahn et al., 2007), and as expected, the most reliable Shh target gene, *Ptch1* expression (Marigo et al., 1996; Vokes et al., 2008; and see Figure 1—figure supplement 1F for phylogenetic analysis) was increased and expanded anteriorly (Figure 4A). Consistent with this, *Hand2* expression also extended anteriorly (Figure 4B)—probably due to inhibition of Gli3 repressor formation by ectopic activation of
Shh signalling (as revealed by the extended Ptch1 expression). On the other hand, Pax9 expression (an anterior marker) was significantly downregulated and showed only weak expression in the anterior part of the fin buds (Figure 4C). The most anterior regions may not be sensitive to this treatment, as expression of Alx4 was not significantly shifted (Figure 4D), and this may be due to the lack of inhibitory regulation from Hand2 to Gli3 described above. To test whether the results of RA treatment were due to specific effects on AP patterning or instead due to a more general interference with limb development, we examined a marker for proximal-distal (PD) patterning in mouse and chick limb buds—Hoxa13 (Tamura et al., 1997; Mercader et al., 2000; Yashiro et al., 2004). In RA-treated pectoral fin buds, Hoxa13 expression was weaker than in control, but a shift in its expression domain was not seen (Figure 4E), showing that the impact of RA in these experiments is primarily on the AP patterning (the shifts of Ptch1, Hand2, and Pax9, Figure 4A–C), rather than on PD patterning or a general impact on development. Most intriguingly, we examined skeletal patterns of S. canicula pectoral fins in these partially ‘posteriorised’ fin buds (Figure 4F). Phenotypes varied from mild to severe but in all cases the appearance of distinct anterior elements (propterygium and mesopterygium) was lost. In the mild cases, a proximal element anterior to the metapterygium is attached to the pectoral girdle (single asterisk in Figure 4F). This proximal element may result from...
a fusion of the proximal parts of propterygium and mesopterygium. Whereas, the severe cases still have a fused element anterior to the metapterygial axis (double asterisk in Figure 4F), but this element is not directly attached to the pectoral girdle, indicating that the pectoral fin of the severe phenotype has lost the anterior proximal elements. By contrast, the posterior metapterygium itself was larger than normal, but retained its strong identity as the primary axis from which radial branching was observed. Although RA treatment potentially cause non-specific effects, given the clear affect on AP patterning (the shifts of \textit{Ptch1}, \textit{Hand2}, and \textit{Pax9}, Figure 4A–C), while causing no obvious effects on PD patterning, the main cause of the phenotype is likely caused by the AP pattern change.

In the present study, we have found that \textit{S. canicula} pectoral fin buds have a gross posterior shift in the AP coordinate system compared to mouse limb buds. We show that \textit{S. canicula} and \textit{C. milii} lack a specific enhancer activity for \textit{Gli3}, which in tetrapods mediates the posterior repression, and that this genetic difference likely contributes to the shift of AP positional information. Finally, RA treatment analyses suggest that a partial posteriorisation of \textit{S. canicula} fin buds leads to a loss of anterior proximal elements (propterygium and mesopterygium). Thus, while the loss of the anterior proximal
elements during evolution was associated with cis-regulatory changes of Gli3 in the RA experiments, it was driven by a Shh-mediated affect on the Gli3 protein itself, but in both cases achieving similar phenotypic changes by anterior shift in AP pattern. In support of our observations, a recent study also showed that anterior extension of Shh signalling accompanied with an anterior shift of Gli3 expression resulted in a loss of anterior skeletal elements in mouse limbs (Li et al., 2014). Considering all these data together, we therefore propose that one of the key events during the fin-to-limb transition was an anterior shift of AP positional information (a posteriorisation), which caused the loss of anterior proximal elements (Figure 4G).

In the RA treatment experiments, we also observed that the anterior distal radials reduced and only metapterygial radials retained, suggesting that anterior shift in AP positional information may also have had an impact on the distal radials during the fin-to-limb transformation. Interestingly, nearly 30 years ago, a classic study proposed that the distal end of the metapterygial axis (which has a uniformly posterior position in chondrichthians) bent anteriorly during acquisition of digits—the so-called digital arch model (Shubin and Alberch, 1986; Oster et al., 1988). Although the detailed validity of this model is unclear (Wagner and Larsson, 2007), there is a possibility that an AP shift in molecular patterning was involved in the acquisition of digits. In addition to our RA treatment analysis, knockdown analyses of actinotrichia proteins, which are components of fin rays and lost in tetrapod, show an anterior shift in several gene expressions in zebrafish pectoral fin buds (Zhang et al., 2010). Therefore, it is interesting to speculate that AP positional information may have shifted several times until the acquisition of digits.

We have shown that the Gli3 regulatory region of S. canicula and C. milii lacks the tetrapod-specific repressive element, which is likely needed for the Gli3–Hand2 interaction in mouse limb buds. In mice, Gli3−/−; Hand2−/− limbs show a severe dysplastic humerus (some of them have ectopic protrusion in humerus; Osterwalder et al., 2014), suggesting that the Gli3–Hand2 interaction has an important role for patterning the proximal elements. However, how Gli3 regulates the proximal skeletal pattern is not well understood even in mice. Although Gli3 is involved in the stylopod (humerus/femur) formation in mice, the phenotype in stylopod always appears with combination of other gene knockouts. For example, Gli3−/−; Plzf−/− mice lack a femur, and Gli3−/−; Alx4−/− mice exhibit humerus malformation (Barna et al., 2005; Panman et al., 2005). These facts suggest that evolutionary modification of Gli3 regulation is likely necessary, but additional regulatory modifications are required for the loss of the anterior elements. Since Alx4 and Hand2 are expressed in S. canicula pectoral fin bud, and Plzf is involved only in hindlimb development, currently there is no obvious candidate that would be involved in the loss of propterygium and mesopterygium. Although S. canicula genome has not been sequenced, systematic studies at genome-wide level such as ChIP-seq in S. canicula pectoral fin buds would be invaluable to provide a more complete picture of evolutionary mechanism of the loss of the anterior elements in the future.

In conclusion, by taking advantage of the slow evolutionary rates of chondrichthyian genomes, we were able to precisely compare the gene expression, function and regulation between pectoral fin and limb development, and discover a key difference between them. In particular, our study suggest that changes in morphogen interpretation by gene regulatory network mutations may have a major impact on morphological evolution.

Materials and methods

Animals

Experiments were performed in accordance with guidelines for animal experiments of Tokyo Tech and CRG, and experiments involved in mice were approved by animal ethics committees of CRG (JMC-07-1001P3-JS). Catshark (S. canicula) eggs were incubated at 12–16˚C in seawater and staged according to (Ballard et al., 1993). C. milii eggs and embryos were collected as described (Takagi et al., 2012) and staged according to (Didier et al., 1998). C52BL/6 (Charles River Laboratories, Wilmington, MA) mouse timed-pregnant females were sacrificed at different days after gestation E11.5. Chicken (Gallus gallus) eggs were incubated at 38˚C in a humidified incubator until the desired Hamburger–Hamilton (HH) stage (Hamburger and Hamilton, 1951) was reached. For in situ hybridisation, embryos were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline, dehydrated in a graded methanol series, and stored in 100% methanol at −20˚C.
Gene isolation and phylogenetic analysis

Total RNA was extracted from stage 24 to 29 S. canicula embryos, stage 28 chick embryos and E11.5 mouse embryos using an RNeasy kit (Qiagen, Netherlands). cDNA was synthesised by reverse transcription and used as a template for PCR. Extraction of total RNA and cDNA synthesis from C. milii embryos were carried out as described (Takagi et al., 2012). To clone S. canicula and C. milii genes, we used primers that were based on the nucleotide sequences of putative C. milii orthologues found in the Elephant Shark Genome Project database (http://esharkgenome.imcb.a-star.edu.sg/) (Venkatesh et al., 2007) for Pax9, Alx4, and Gli3; SkateBase (http://skatebase.org/) (Wang et al., 2012) for Hand1, Zic3, Tbx2, and Ptc1; and GenBank for Gli2 (EU196410) and Sox9 (EU241880): S. canicula Alx4, 5′-AGGAATGAAACCGCCGAGACTTG-3′ and 5′-TCATGTTGCCCAAGATATACG-3′; S. canicula Pax9, 5′-GCTTGTGCAGCAAGATACTTG-3′ and 5′-CCGCAGTGTGCTAGT-3′; S. canicula Gli3, 5′-CAGCCCAAGCAGAATACTACC-3′ and 5′-GAGATCTCAGCGCCATGTAGT-3′; S. canicula Gli2, 5′-GTAAGCTTACTCAGCTCG-3′ and 5′-CGGAGTACGGCAGCTGTAGT-3′; S. canicula Sox9, 5′-CCAGGTTGCTGAGGGATACT-3′ and 5′-GGCAGGATGCTGCAACTC-3′; S. canicula Hand1, 5′-GAGACATCAAACAGCCATTCGC-3′ and 5′-TTCCCCTGGCTCTAACTCCGT-3′; S. canicula Zic3, 5′-GTGGCCATGCCGAGTCTGAGT-3′ and 5′-GGTCTCAGCCGCTG-3′; S. canicula Tbx2, 5′-GACACAGAACCCGCTGATC-3′ and 5′-GAAACGAGATCCACAACTTTGAG-3′; S. canicula Ptc1, 5′-GAGGTTTCCACTCTCGATGGGATAACC-3′ and 5′-CCATATGCGCTGTTTCGATG-3′; C. milii Gli3, 5′-GAGATCTCCAGGAGCAATTGAGT-3′ and 5′-GAGATCTCAGCGCCATTGATG-3′. To clone chick and mouse genes, we used primers that were based on the nucleotide sequences of Pax9 that were described (Muller et al., 1999, Hoxa13 (NM_204139), and Tbx2 (NM_009324) (Bollag et al., 1994a): chick Pax9, 5′-TGAGCCGACACCTCGTGAC-3′ and 5′-GTTTCTCGCCGTCG-3′; chick Hoxa13, 5′-GTGGGATCCTGACACAACTT-3′ and 5′-GTTGCGGATCCTGACACC-3′; mouse Tbx2, 5′-ATCCTGAACTCCATGACACC-3′ and 5′-TGGGAAATGGAGGTTGAGG-3′; S. canicula Tbx2, 5′-GGTGGCTATGGAGATGCTG-3′ and 5′-GTTTCTCGCCGTCG-3′; S. canicula Alx4, 5′-GAACTGCTGCCCATGGCTG-3′ and 5′-GCTGTGTCAGCAAGATACTGG-3′; C. milii Gli3, 5′-GAGATCTCAGCGCCATTGATG-3′ and 5′-GAGATCTCAGCGCCATTGATG-3′.

Probe synthesis and in situ hybridisation

Chick Alx4 (NM_204162) was kindly provided by Dr Toshihiko Ogura. Riboprobes for Hand2 (AY057890) and Hoxa13 (EU005550) of S. canicula and for chick Alx4 were synthesised as described (Takahashi et al., 1998; Tanaka et al., 2002a; Sakamoto et al., 2009a). The cloned genes described above were used as templates for riboprobe synthesis. Whole-mount in situ hybridisation was carried out as described (Tanaka et al., 2002a). Sox9 expressions were scanned with Optical Projection Tomography (OPT) as described (Sharpe et al., 2002) and analysed with Volviewer (Lee et al., 2006).

Gli processing analysis

Human Gli3 (clone name: pFN21AE1055) and Gli2 (Roessler et al., 2005) were obtained from the Kazusa DNA Research Institute (Nagase et al., 2008) and Addgene, respectively, pCAGGS was kindly provided by Dr Toshihiko Ogura and originated from Dr Jun-ichi Miyazaki (Niwa et al., 1991). For Western blotting analysis, the N-terminal HaloTag in the human Gli3 construct was replaced with a 6×Myc tag (Myc-hsGli3). Then, the human Gli3 PDD (amino acids 644–842) (Pan and Wang, 2007) was replaced with the homologous domain from human Gli2 and S. canicula Gli2 and Gli3 by a combination of PCR (Wurch et al., 1998) and restriction enzyme digestions. The HEK293 cell line was kindly provided by Dr Masayuki Komada. HEK293 cells were grown in Dulbecco’s modified Eagle medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% foetal bovine serum (Gibco, Thermo Fisher Scientific Inc., Waltham, MA) and penicillin/streptomycin (Sigma–Aldrich) at 37°C. For Western blot analysis, cells were plated in 6-well plates without penicillin/streptomycin and transfected with 4 µg of constructs using polyethylenimine (GE Healthcare, England) for 3 h. After the transfection, the medium was changed, and cells were cultured for
24 hr and then treated with 50 μM forskolin (FSK; Sigma) in Dimethyl sulfoxide (DMSO) DMSO or with DMSO alone for 24 hr. Whole-cell extracts were prepared by solubilisation in lysis buffer containing 50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM ethylenediaminetetraacetic acid (EDTA); 1% Triton X-100; 0.1% Sodium Dodecyl Sulfate (SDS); 1% sodium deoxycholate; and protease inhibitor cocktail (Roche, Switzerland). Whole-cell lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and analysed by Western blotting and anti-c-Myc (Sigma–Aldrich), anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA), and enhanced chemiluminescence detection (GE Healthcare).

Enhancer analysis

The limb-specific GlI3 enhancer was found with VISTA enhancer browser (http://enhancer.lbl.gov/) (Visel et al., 2007). The enhancer ID is hs1586, which is located in GlI3 intron 3 in the human genome (hg19). For alignment, GlI3 intron 3 sequences from mouse (Mus musculus), chick (G. gallus), frog (Xenopus tropicalis), coelacanth (Latimeria chalumnae), gar (Lepisosteus oculatus), medaka (Oryzias latipes), and zebrafish (Danio rerio) from the Ensembl and Pre Ensembl genome browsers (http://www.ensembl.org/, http://pre.ensembl.org/) were collected. The element 1586 homologue from elephant shark (C. mili) was retrieved from the genome assembly (http://esharkgenome.imcb.a-star.edu.sg/) (Venkatesh et al., 2007) by using human element 1586 sequence as the query. The GenBank accession number of the C. mili element 1586 is AAVX01295166. The S. canicula counterpart of element 1586 was amplified by PCR with primers designed from conserved sequences of the upstream exon and the distal part of element 1586: 5′-AGTGGACCCCGAAATGGCTACATGGACC-3′ and 5′-GAACATCTTCTAATTTACTGGAATCCCAG-3′. The amplified fragment was then cloned into pBluescript SK−. The sequence of S. canicula element 1586 was deposited in GenBank under accession number KF297619. The alignment was carried out with the SLAGAN method, and overall sequence similarities in the alignment were visualised with mVISTA (Mayor et al., 2000; Brudno et al., 2003; Frazer et al., 2004).

For functional analysis, the element 1586 homologues were isolated from chick and C. mili genomes by PCR. The following forward and reverse primers were used: chick element 1586, 5′-CGAGCTCTCCTCTCAGTCTTCTTGC-3′ and 5′-TGTTGAGACATCTACTTTGATC-3′; C. mili element 1586, 5′-GAGCTCTGTTACGTAGTGAATGTTG-3′ and 5′-GAGATTTTCGAGTCTCCTTTATGTC-3′. The amplified DNA fragments were cloned into pBluescript SK−. To subclone the S. canicula 1586 fragment, we used the following primers: 5′-CCGCTCTAGAAGACTGTCTATGATTGGTGTGGAGTGAATGTTG-3′ and 5′-CAGGGGGATCCACTAGCTATGGTGGAGTGAATGTTG-3′. The element 1586 sequence from each species was subcloned in front of a chicken β-actin basal promoter that is followed by a GFP reporter (Ochi et al., 2012). Recombined enhancers were created by PCR. In ovo, electroporation was carried out as described (Suzuki and Obara, 2008). A DNA solution was prepared with Maxi Prep (Qiagen). pCAGGS-RFP was kindly provided by Dr Cheryll Tickle. GlI3 limb enhancers and empty β-actin basal promoter–GFP at ~6 μg/μl, coloured with ~3% fast green, and co-electroporated with pCAGGS-RFP (~2 μg/μl) into the presumptive forelimb field of stage 13–14 embryos. A CUY21EDIT II electroporator (BEX Co., Ltd., Japan) was used. Electric pulses consisted of one short pulse (25 V; 0.05 ms) and a 0.1-ms interval, followed by five long pulses (8 V; 10 ms) with 1-ms intervals. The electric pulses were applied during injection of the DNA solution.

RA treatment

S. canicula embryos were removed from their egg shells, then placed into 6-well plates. 4–6 ml of artificial seawater containing penicillin/streptomycin was used for culturing embryos. RA was dissolved in DMSO to 2 mg/ml as a stock solution and diluted in the artificial seawater to 1–2 μg/ml. 1% DMSO in the artificial seawater was used as negative controls. Embryos at stage 28–29 were cultured with RA for 4 days for gene expression analyses. For alcian blue staining, embryos at stage 28–29 were cultured with 1–2 μg/ml RA for 20 days and additional 10–18 days after removing RA. Note that effect of RA is highly dependent on individual embryos. Some batches of embryos were lethal at 2 μg/ml of RA, probably due to season or parents’ condition. In this case, embryos were treated with 1 μg/ml of RA.

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Tickle for comments; and K Munakata, S Ueda, and N Suda for technical advice. Sequences for Alx4, Pax9, Gli3, Hand1, Tbx2, Zic3, Ptc1, and element 1586 from S. canicula and Gli3 from C. milii are deposited in GenBank under accession numbers KC507187–9, KF748129, KP055651-KP055653, KF297619, and KF297620. Correspondence and requests for materials should be addressed to M Tanaka (mitanaka@bio.titech.ac.jp) and J Sharpe (james.sharpe@crg.eu). This work was supported in part by the Global COE Program ‘Evolving Education and Research Center for Spatio-Temporal Biological Network’ from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to KO and MT, the Japan–Australia Research Cooperative Program to SH, the Grant-in-Aid for Scientific Research on Innovative Areas, the Grant-in-Aid for Scientific Research (B) and the Inamori Foundation to MT, ICREA and the Spanish Ministry of Economy and Competitiveness, ‘Centro de Excelencia Severo Ochoa 2013-2017’, SEV-2012-0208 to JS and the Centre for Genomic Regulation to KO and JS.

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**Author contributions**

KO, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article, Contributed unpublished essential data or reagents; SK, Acquisition of data, Analysis and interpretation of data, Contributed unpublished essential data or reagents; WT, SH, Acquisition of data, Contributed unpublished essential data or reagents; JS, MT, Conception and design, Analysis and interpretation of data, Drafting or revising the article

**Ethics**

Animal experimentation: Experiments were performed in accordance with guidelines for animal experiments of Tokyo Tech and CRG, and experiments involved in mice were approved by Animal Ethics Committees of CRS (No. JMC-07-1001P3-JS).

**Additional files**

**Major datasets**

The following datasets were generated:

| Author(s)                     | Year | Dataset title                        | Dataset ID and/or URL | Database, license, and accessibility information |
|-------------------------------|------|--------------------------------------|-----------------------|-------------------------------------------------|
| Onimaru K, Tanaka M           | 2014 | Scyliorhinus canicula Alx4 mRNA, complete cds | http://www.ncbi.nlm.nih.gov/nuccore/KC507187 | Publicly available at the NCBI Nucleotide (Accession no: KC507187). |
| Onimaru K, Tanaka M           | 2014 | Scyliorhinus canicula Pax9 mRNA, partial cds | http://www.ncbi.nlm.nih.gov/nuccore/KC507188 | Publicly available at the NCBI Nucleotide (Accession no: KC507188). |
| Author(s)                          | Year | Dataset title                          | Dataset ID and/or URL                        | Database, license, and accessibility information |
|-----------------------------------|------|----------------------------------------|---------------------------------------------|-------------------------------------------------|
| Onimaru K, Tanaka M               | 2014 | Scyliorhinus canicula Gli3 mRNA, partial cds | http://www.ncbi.nlm.nih.gov/nuccore/KC507189 | Publicly available at the NCBI Nucleotide (Accession no: KC507189). |
| Onimaru K, Kuraku S, Takagi W, Hyodo S, Tanaka M | 2014 | Scyliorhinus canicula element 1586, genomic sequence, a fin/limb enhancer of Gli3 | http://www.ncbi.nlm.nih.gov/nuccore/KF297619 | Publicly available at the NCBI Nucleotide (Accession no: KF297619). |
| Onimaru K, Kuraku S, Takagi W, Hyodo S, Tanaka M | 2014 | Scyliorhinus canicula Hand1 mRNA, partial cds | http://www.ncbi.nlm.nih.gov/nuccore/KF748129 | Publicly available at the NCBI Nucleotide (Accession no: KF748129). |
| Onimaru K, Kuraku S, Takagi W, Hyodo S, Sharpe J, Tanaka M | 2015 | S. canicula Zic3 mRNA, partial cds | http://www.ncbi.nlm.nih.gov/nuccore/KP055652 | Publicly available at the NCBI Nucleotide (Accession no: KP055652). |
| Onimaru K, Kuraku S, Takagi W, Hyodo S, Sharpe J, Tanaka M | 2015 | S. canicula Tbx2 mRNA, partial cds | http://www.ncbi.nlm.nih.gov/nuccore/KP055651 | Publicly available at the NCBI Nucleotide (Accession no: KP055651). |
| Onimaru K, Kuraku S, Takagi W, Hyodo S, Sharpe J, Tanaka M | 2015 | S. canicula Ptch1 mRNA, partial cds | http://www.ncbi.nlm.nih.gov/nuccore/KP055653 | Publicly available at the NCBI Nucleotide (Accession no: KP055653). |
| Onimaru K, Kuraku S, Takagi W, Hyodo S, Tanaka M | 2015 | C. milii Gli3 mRNA, partial cds | http://www.ncbi.nlm.nih.gov/nuccore/KF297620 | Publicly available at the NCBI Nucleotide (Accession no: KF297620). |
| Tanaka M, Munsterberg A, Anderson WG, Prescott AR, Hazon N, Tickle C | 2002 | Scyliorhinus canicula dHand protein mRNA, partial cds | http://www.ncbi.nlm.nih.gov/nuccore/AY057890 | Publicly available at the NCBI Nucleotide (Accession no: AY057890). |
| Sakamoto K, Onimaru K, Munakata K, Suda N, Tamura M, Ochi H, Tanaka M | 2009 | Scyliorhinus canicula HoxA13 mRNA, partial cds | http://www.ncbi.nlm.nih.gov/nuccore/EU005550 | Publicly available at the NCBI Nucleotide (Accession no: EU005550). |
| Muller TS, Ebensperger C, Neubuser A, Koseki H, Balling R, Christ B, Wilting J | 2015 | Gallus gallus paired box 9 (PAX9), mRNA | http://www.ncbi.nlm.nih.gov/nuccore/NM_204912 | Publicly available at the NCBI Nucleotide (Accession no: NM_204912). |
| Takahashi M, Tamura K, Buscher D, Masuya H, Yonei-Tamura S, Matsumoto K, Naitoh-Matuo M, Takeuchi J, Ogura K, Shiroishi T, Ogura T, Izpisua Belmonte JC | 2013 | Gallus gallus ALX homeobox 4 (ALX4), mRNA | http://www.ncbi.nlm.nih.gov/nuccore/NM_204162 | Publicly available at the NCBI Nucleotide (Accession no: NM_204162). |
| Bollag RJ, Siegfried Z, Cebra-Thomas J, Garvey N, Davison EM, Silver LM | 1994 | Mus musculus T-box 2 (Tbx2), mRNA | http://www.ncbi.nlm.nih.gov/nuccore/120407038 | Publicly available at the NCBI Nucleotide (Accession no: NM_009324). |
| Zhang G, Cohn MJ | 2009 | Scyliorhinus canicula Sox9 mRNA, complete cds | http://www.ncbi.nlm.nih.gov/nuccore/EU241880 | Publicly available at the NCBI Nucleotide (Accession no: EU241880). |

The following previously published datasets were used:

| Author(s)                          | Year | Dataset title                          | Dataset ID and/or URL                        | Database, license, and accessibility information |
|-----------------------------------|------|----------------------------------------|---------------------------------------------|-------------------------------------------------|
| Tanaka M, Munsterberg A, Anderson WG, Prescott AR, Hazon N, Tickle C | 2002 | Scyliorhinus canicula dHand protein mRNA, partial cds | http://www.ncbi.nlm.nih.gov/nuccore/AY057890 | Publicly available at the NCBI Nucleotide (Accession no: AY057890). |
| Sakamoto K, Onimaru K, Munakata K, Suda N, Tamura M, Ochi H, Tanaka M | 2009 | Scyliorhinus canicula HoxA13 mRNA, partial cds | http://www.ncbi.nlm.nih.gov/nuccore/EU005550 | Publicly available at the NCBI Nucleotide (Accession no: EU005550). |
| Muller TS, Ebensperger C, Neubuser A, Koseki H, Balling R, Christ B, Wilting J | 2015 | Gallus gallus paired box 9 (PAX9), mRNA | http://www.ncbi.nlm.nih.gov/nuccore/NM_204912 | Publicly available at the NCBI Nucleotide (Accession no: NM_204912). |
| Takahashi M, Tamura K, Buscher D, Masuya H, Yonei-Tamura S, Matsumoto K, Naitoh-Matuo M, Takeuchi J, Ogura K, Shiroishi T, Ogura T, Izpisua Belmonte JC | 2013 | Gallus gallus ALX homeobox 4 (ALX4), mRNA | http://www.ncbi.nlm.nih.gov/nuccore/NM_204162 | Publicly available at the NCBI Nucleotide (Accession no: NM_204162). |
| Bollag RJ, Siegfried Z, Cebra-Thomas J, Garvey N, Davison EM, Silver LM | 1994 | Mus musculus T-box 2 (Tbx2), mRNA | http://www.ncbi.nlm.nih.gov/nuccore/120407038 | Publicly available at the NCBI Nucleotide (Accession no: NM_009324). |
| Zhang G, Cohn MJ | 2009 | Scyliorhinus canicula Sox9 mRNA, complete cds | http://www.ncbi.nlm.nih.gov/nuccore/EU241880 | Publicly available at the NCBI Nucleotide (Accession no: EU241880). |
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