The Shh/Gli3 gene regulatory network precedes the origin of paired fins and reveals the deep homology between distal fins and digits

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One of the central problems of vertebrate evolution is understanding the relationship among the distal portions of fins and limbs. Lacking comparable morphological markers of these regions in fish and tetrapods, these relationships have remained uncertain for the past century and a half. Here we show that Gli3 functions in controlling the proliferative expansion of distal progenitors are shared among dorsal and paired fins as well as tetrapod limbs. Mutant knock out gli3 fins in medaka (Oryzias latipes) form multiple radials and rays, in a pattern reminiscent of the polydactyly observed in Gli3-null mutant mice. In limbs, Gli3 controls both anterior–posterior patterning and cell proliferation, two processes that can be genetically uncoupled. In situ hybridization, quantification of proliferation markers, and analysis of regulatory regions reveal that in paired and dorsal fins, gli3 plays a main role in controlling proliferation but not in patterning. Moreover, gli3 down-regulation in shh mutant fins rescues fin loss in a manner similar to how Gli3 deficiency restores digits in the limbs of Shh mutant mouse embryos. We hypothesize that the Gli3/Shh gene pathway preceded the origin of paired appendages and was originally involved in modulating cell proliferation. Accordingly, the distal regions of dorsal fins, paired fins, and limbs retain a deep regulatory and functional homology that predate the origin of paired appendages.

Significance

In this study, we show that the inactivation of the gli3 gene in medaka fish results in the formation of larger dorsal and paired fins. These mutant fins display multiple radial bones and fin rays which resemble polydactyly in Gli3-deficient mice. Our molecular and genetic analyses indicate that the size of fish fins is controlled by an ancient mechanism mediated by SHH-GLI signaling that appeared prior to the evolutionary appearance of paired fins. We also show that the key regulatory networks that mediate the expansion of digit progenitor cells in tetrapods were already in place in the fins of the last common ancestor between ray and lobe-finned fishes, suggesting an ancient similarity between distal fins and digits.

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this, we generated gli3-knockout (KO) mutants in medaka, a teleost fish with a single copy of shh, to assess gene expression, regulation, and ultimately evolution of the morphoregulatory mechanisms mediated by Gli3 in vertebrate appendages.

**Results**

To explore the role of gli3 in medaka fin patterning, we deployed CRISPR-Cas9 to disrupt its coding region via an 86-bp deletion in exon 5 (Fig. 1A and SI Appendix, Fig. S1). This mutation generates a frameshift that truncates the protein in the middle of the repressor domain. Adult fins could not be analyzed, as fish homozygous for this gli3-inactivated allele die between 2 and 5 wk of age, probably reflecting the pleiotropic functions of gli3 in multiple tissues (SI Appendix, Fig. S1).

By weeks 3 to 5, however, multiple anomalies were observed in the pectoral fin skeleton of gli3 mutant escapers. In particular, these fins had an expanded number of radials and rays (Fig. 1B–G). Alcian blue and Alizarin red staining of pectoral fin skeletons reveals a significantly increased number of proximal radial bones and fin rays in stable (E and F) and transient (G) gli3 mutants, although major anatomical AP asymmetries appear unaltered. Note that, when compared with ones in the anterior margin, the two posterior proximal radials (pr) are larger in both gli3 crispants and WT fish and articulate with the coracoid (cc) bone (arrows in D and G). In the anterior margin of the fin, both the rest of the proximal radials and the anterior-most fin rays (fr) articulate directly with the scapula (sc) in both WT and transient gli3 mutants (arrowheads in D and G). (B-G) n ≥ 14 fins. (E and F) n ≥ 12 fins. (Scale bars, 250 μm.) mo, months-old; wo, weeks-old. (H) WT pectoral fin showing the expression of gli3 in the anterior region of the developing pectoral fin bud (n = 10). (Scale bar, 100 μm.) ed, endochondral disk; ff, fin fold. (I and J) Quantification of skeletal elements in adult (4-mo-old) gli3 crispants. Each point in the graphs represents the measurement of bone number in a single fin (n = 36 WT, n = 32 gli3 KO F0). An unpaired t test was used for the statistical analysis of skeletal element number. ***P = 5.06 × 10^{-15} for the comparison between WT (mean 3.639) and gli3 crispant (mean 4.969) pectoral proximal radial bones. ***P = 2.33 × 10^{-7} for the comparison between WT (mean 11.67) and gli3 crispant (mean 13.13) pectoral fin rays. Bone-staining procedures in juvenile (B, C, E, and F) and adult fish (D and G) were performed in three independent experiments. a, anterior; p, posterior.

**Fig. 1.** Medaka gli3 mutants show an increased number of pectoral fin skeletal elements. (A) Stable lines harboring Δ86 deletions in medaka gli3 exon 5 were generated by CRISPR-Cas9. The diagram shows the position of the deletion relative to the sgRNAs and primers used for screening along with the box of conservation with four fish species (stickleback, fugu, tetraodon, and zebrafish). The Δ86 deletion results in the generation of a premature STOP codon that truncates the predicted protein upstream of the zinc-finger DNA-binding domain of Gli3. (B–G) Alcian blue and Alizarin red staining of pectoral fin skeletons reveals a significantly increased number of proximal radial bones and fin rays in stable (E and F) and transient (G) gli3 mutants, although major anatomical AP asymmetries appear unaltered. Note that, when compared with ones in the anterior margin, the two posterior proximal radials (pr) are larger in both gli3 crispants and WT fish and articulate with the coracoid (cc) bone (arrows in D and G). In the anterior margin of the fin, both the rest of the proximal radials and the anterior-most fin rays (fr) articulate directly with the scapula (sc) in both WT and transient gli3 mutants (arrowheads in D and G). (B and C) n ≥ 14 fins. (E and F) n ≥ 12 fins. (Scale bars, 250 μm.) mo, months-old; wo, weeks-old. (H) WT pectoral fin showing the expression of gli3 in the anterior region of the developing pectoral fin bud (n = 10). (Scale bar, 100 μm.) ed, endochondral disk; ff, fin fold. (I and J) Quantification of skeletal elements in adult (4-mo-old) gli3 crispants. Each point in the graphs represents the measurement of bone number in a single fin (n = 36 WT, n = 32 gli3 KO F0). An unpaired t test was used for the statistical analysis of skeletal element number. ***P = 5.06 × 10^{-15} for the comparison between WT (mean 3.639) and gli3 crispant (mean 4.969) pectoral proximal radial bones. ***P = 2.33 × 10^{-7} for the comparison between WT (mean 11.67) and gli3 crispant (mean 13.13) pectoral fin rays. Bone-staining procedures in juvenile (B, C, E, and F) and adult fish (D and G) were performed in three independent experiments. a, anterior; p, posterior.
observed in \textit{Shh}-null embryos, leading to polydactyly that is identical to that observed upon \textit{Gli3} inactivation alone (12, 13). To examine this relationship in medaka, we produced \textit{gli3/shh} double mutants transiently, as \textit{gli3}-deficient fish are not viable. Transient inactivation of \textit{gli3} in a \textit{ZRS+sZRS shh} mutant background (20) is sufficient to rescue the \textit{shh} loss-of-function phenotype (agenesia of pectoral, pelvic, and dorsal fins). Analogous to the genetic interaction observed in mutant mouse autopods, the fin skeleton of \textit{ZRS+sZRS/gli3 F0} double mutants resembles those of \textit{gli3} crispsants. This effect was seen in both paired and unpaired fins, as supernumerary bones were seen in pectoral, pelvic, and dorsal fins (Fig. 3 and SI Appendix, Fig. S6).

Interestingly, as observed previously in the \textit{ZRS+sZRS shh} mutant (20), the anal fin is not affected by any of these mutant conditions (SI Appendix, Fig. S7).

**Discussion**

Altogether, our results show that the presence of the \textit{shh/gli3} regulatory network in fish fins, so vital for limb formation and digit patterning, is primitive to limbs. Moreover, its functions in unpaired dorsal fins, widely recognized precursors of paired appendages, suggest that the recruitment of this network may have preceded the origin of paired fins themselves.

The correlation of expanded radials and rays in \textit{gli3} fin mutants with the polydactyly in mouse \textit{Gli3} mutants points to a deep homology among the distal tissues of gnathopod appendages. Our analyses suggest that the primitive function of the \textit{Shh/Gli3} module in appendages was to control the proliferative expansion of the distal mesenchyme. Interestingly, the transcriptional control of cell-cycle effectors is an ancient feature of HH signaling, as Ci, the fly ortholog of \textit{Gli3}, also directly regulates several \textit{cyclin} genes (21, 22). In contrast, the fully wired AP patterning systems controlled by \textit{Gli3} likely evolved later during vertebrate evolution, probably in the tetrapod lineage through the appearance of novel far-acting cis-regulatory regions (23, 24). As expected, given their dependence on \textit{SHH} signaling for their posterior up-regulation (25–28), some deeply conserved \textit{Gli}-binding CREs are present in the \textit{Hand} and \textit{Grem1} genomic landscapes. Moreover, our observation that the \textit{grem1b} expression domain is expanded in \textit{gli3}-deficient fin buds at 8 dpf suggests that some aspects of \textit{grem1b} regulation are controlled by \textit{Gli3} at these advanced stages. This is highly reminiscent of the situation in the mouse, as failure to terminate \textit{Grem1} expression in the anterior margin of the handplate leads to a delay in chondrogenic differentiation that contributes to the polydactyly observed in \textit{Gli3}-deficient limb buds (15). The highly conserved \textit{GRS1} enhancer is a good candidate to implement some aspects of \textit{Gli3}-mediated repression of \textit{Grem1/grem1b} at these late stages of appendage development, as its activity is sensitive to \textit{Gli3} gene dosage in the mouse (18).

Finally, another possibility explaining the lack of apparent polarization defects in medaka \textit{gli3} mutants would be that \textit{SHH}-mediated AP patterning in early teleost fins (25, 26) is mediated in a \textit{gli3}-independent manner by a \textit{Gli2} factor(s), capable of being processed into \textit{Gli1} activator and repressor isoforms (29, 30). Interestingly, \textit{Gli2} has been genetically shown to cooperate with \textit{Gli3} in providing posterior identity to the mouse autopod (31).

Overall, our results imply that the distal regions of appendages have a common evolutionary origin and that the \textit{Shh/Gli3} network was modified in fish and tetrapod lineages to produce fin radials and rays in the former and digits in the latter. Interestingly, the only appendage that does not follow these rules, interestingly, the only appendage that does not follow these rules, the anal fin, also has anomalous patterns of \textit{shh} regulation. The absence of these networks in anal fins points to a separate evolutionary origin for anal fins, presumably by independent cooperation of fin patterning networks in a novel site.
Materials and Methods

Animal Experimentation. All experiments involving fish and mice performed in this work conform to European Community standards for the use of animals in experimentation and were approved by the ethical committees from the Universidad Pablo de Olavide, Universidad Mayor, and Consejo Superior de Investigaciones Científicas and the Andalusian government.

Fish Stocks. Medaka WT (iCab) and ZRS+ZRS KO (Δ3.4kb (20)) strains were maintained and bred under standard conditions (32). Embryos were staged in

![Fig. 2. Expression of gli3 target genes in WT and gli3-deficient pectoral fin buds. (A) Hand2, Hoxd12, and Grem1 expression is anteriorly expanded and Pax9 expression is lost in Gli3-deficient E11.5 mouse limb buds (n = 3 per marker and genotype). (Scale bar, 200 μm). (B) ISH assays in WT and gli3Δ86−/− medaka pectoral fins. At 6 dpf (stage 36), the inactivation of gli3 does not greatly affect the expression pattern of hand2, hoxd12a, grem1b, or pax9, all genes involved in limb patterning (n ≥ 4 per marker and genotype). d, distal; pr, proximal. (Scale bar, 100 μm). (C) Schematic representation of the hand2, hoxd12a, grem1b, and pax9 expression domains in 6-dpf WT medaka pectoral fins. Note that the grem1b expression domain (shown in B) runs along the margin of the endochondral disk (ed) and extends into the fin fold. (D) Gene expression quantification by qPCR in WT and gli3Δ86−/− medaka pectoral fins at 11 dpf. The relative expression of the proliferation regulators ccnd1 and ccnd2b is significantly increased in mutant fins. Mutant values (purple bars) are normalized against WT values (green bars), and represented as mean ± SD. Note that the gli3Δ86 allele is still transcribed in homozygous mutant fins, although at lower levels due to nonsense-mediated mRNA decay. Nhand2 = 4, Ngrem1b = 3, Npax9 = 3, Nccnd1 = 2, Nccnd2b = 4, Ncdk6 = 3, Ngli3 = 4; ***Pgli3 = 9.026 × 10−9, **Pccnd1 = 0.0022, *P ≤ 0.05, Pccnd2b = 0.011.](https://doi.org/10.1073/pnas.2100575118)
Fig. 3. Mosaic inactivation of gli3 in fins lacking shh completely rescues the formation of dorsal, pelvic, and pectoral fins. Skeletal staining and fin morphology in adult fish. (A–F) In contrast to WT (A–C), all fin elements are absent in the dorsal, pelvic, and pectoral fins from ZRS+sZRS mutant fish (D–F). (G–I) CRISPR-Cas9 disruption of gli3 significantly increases the number of pectoral and dorsal fin bone elements (black arrowheads in I) compared with WT (A–C). (J–L) gli3 down-regulation in the homozygous ZRS+sZRS shh mutant background totally rescues the dorsal, pelvic, and pectoral fin phenotypes. Note that there is a delay in the ossification of dorsal fin rays in gli3 single and gli3/ZRS+sZRS compound mutants. (Scale bars, 1 mm.) Microinjection of the gli3 CRISPR mixture and bone-staining procedures were performed in three independent experiments.
hours postfertilization as previously described (33). Medaka gil3_A86 and ZBS-Δ14kbs mutant alleles were maintained in heterozygosis due to the higher lethality of the homogenous mutants. gil3_A86 homozygous null larvae died between 2 and 5 wk of age.

Skeletal Staining. Ailican blue and Alizarin red staining experiments were performed as previously described (20, 34). In brief, Skeletal Staining.

following primers were aligned (by PCR) to a universal CRISPR primer:

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times with deionized water and transferred to 0.5% aqueous KOH solution. Temperature. After trypsin enzyme treatment, specimens were rinsed several times with deionized water, cartilage was stained overnight using a 0.1% Alcian blue solution (Alcian blue 8GX; PanReac AppliChem) in 30% acetic acid and 70% ethanol. Fish were then washed with deionized water and changed to a solution containing 1% trypsin from bovine pancreas (PanReac AppliChem) and 30% saturated sodium borate for 8 h (or longer) with gentle shaking at room temperature. After trypsin enzyme treatment, specimens were rinsed several times with deionized water and transferred to 0.5% aqueous KOH solution. Fish bone was finally stained overnight with a solution containing saturated Ailican red S (PanReac AppliChem) in 0.5% KOH. After several washes with a 0.5% KOH solution, fish were gradually transferred to glycerol for documentation. Specimens were visualized with an Olympus SXZ16 binocular microscope and photographed with an Olympus DP71 camera.

CRISPR-Cas9 Design and gil3 Mutant Generation. Two single guide RNAs (sgRNAs) targeting exon 5 of medaka gil3 were designed using the CRISPRscan (35) and CCTop (36) CRISPR design online tools. sgRNAs were generated and purified for injection as previously described (37). For sgRNA generation, the following primers were aligned (by PCR) to a universal CRISPR primer: gil3 exon 5 sgRNA1: 5′-taaatgcacataaGAGCTGTATGCTTGTTAGGCTTAGGCTTTAA-3′ and gil3 exon 5 sgRNA2: 5′-taaatgcacataaGAGCTGTATGCTTGTTAGGCTTAGGCTTTAA-3′. The primer target site is identified by capital letters. Following synthesis, 5 nL of a solution containing both sgRNAs at a concentration of 40 ng/μL and Cas9 protein (Addgene; 47327) at a concentration of 250 ng/μL (38) were injected into one-cell-stage medaka embryos. Oligos used for sequencing of genomic DNA deletions flanking CRISPR target sites were the following: forward primer 5′-CGTGAATTTCAAGAACAATTA-3′ and reverse primer 5′-CGCTCTGATCCTGTTGGCTG-3′. Mutations in gil3 were analyzed by standard PCR and gel electrophoresis as the distance between both sgRNA protospacer adjacent motif (PAM) sequences was 82 bp, long enough to create a deletion easily detected by a shift in the PCR band. Specific deletions in the gil3 gene were further analyzed by Sanger sequencing of the PCR product from F1 embryos (Stab Vida).

Statistical Analyses. The number of pectoral fin proximal radial bones, pectoral fin rays, and dorsal proximal pterygophores was manually counted in adult WT and gil3-crispant fish after a bone-staining procedure. Differences in the number of skeletal elements between both groups were tested by applying an unpaired t test using GraphPad Prism software. Paired two-tailed t tests were used for the statistical analysis of average differences in gene expression levels between mutant and WT samples as measured by qRT-PCR.

Medaka In Situ Hybridization. Depending on the genomic location of the designed primers, antisense digoxigenin-labeled RNA probes were prepared for 4-dpf medaka complementary DNA (cDNA) or gDNA (SI Appendix, Table S1). shh (20), hand2, hoxd12a, grem1b, and pax9 RNA probes were synthesized by cloning the DNA-amplified region using the StrataClone PCR Cloning Kit (240205-5; Agilent Technologies, hoxd12a) or pGem-T Easy Vector (A1360; Promega; pax9, grem1b, and hand2), and these linearized vectors were used as templates for RNA transcription. The gil3 probe was directly transcribed from the amplified DNA since the SP6-RNA polymerase promoter sequence (labeled red in SI Appendix, Table S1) was included in the primers used for ampiclon amplification.

Heterozygous animals were mated in order to collect embryos to perform ISH assays. The embryos were maintained at 28°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM CaC12, 0.16 mM MgSO4, and 0.00003% methylene blue) until 4, 6, 8, and 11 dpf and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 48 h at 4°C. Embryos were manually dechorionated when needed (stages 4 to 8 dpf), dehydrated through an increasing MeOH washing series, and kept at –20°C until the experiments were performed. Overall, the specimens were prepared, hybridized (2 ng probe per microliter), and stained as previously described (39, 40). Embryos at stages older than 4 dpf were permethrinized with 10 μg of Protex for 7 min.

In order to analyze the expression of the different genes in mutant and WT siblings, samples were genotyped after ISH assays. gDNA was extracted using Chelex 100 sodium form (C7901; Sigma) from a piece of tail dissected from each individual larva and standard PCR reactions were performed with primers flanking the deleted region (Fig. 1 and SI Appendix, Table S1). Subsequently, gene expression patterns in homozygous null and WT pectoral fins were analyzed and documented. Both pectoral fins from each gil3 homozygous larva were dissected and transferred to a drop of 3% methyl cellulose (M0387; Sigma-Aldrich) on a slide for documentation using an Olympus SZX16 (model SZX2-ILLB) binocular scope and an Olympus DP71 camera.

Quantitation of Transcript Levels in Medaka Fins by qRT-PCR. Larvae were raised as described above until 11 dpf and deeply anesthetized with 160 μg of tricaine (ethyl 3-aminobenzoate methanesulfonate salt; MS-222, Merck) before dissecting their pectoral fins. The dissection was performed in a drop of embryos' medium and the fins were rapidly moved to an ice-cold drop of PBS. Every pair of dissected pectoral fins coming from a single larva was transferred to a separate 1.5-μL tube containing 50 μL Tissueure (Bio-38032; Bioline) and stored at –20°C until larvae were genotyped (Medaka In Situ Hybridization). In order to obtain sufficient RNA material, each biological replicate consisted of 20 pairs of pectoral fin buds per genotype (WT and gil3-deficient). RNA was extracted following the Tissueure manufacturer's instructions and equivalent amounts of mutant and WT RNA were used to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems-Thermo Fisher Scientific; 4368814). The expression levels of mutually homologous genes, grem1b, grem1b, and pax9, ccn1, ccn2, and cdk6, and gil3 in the developing fins were quantified through qRT-PCR (CFX96 Real-Time C1000 thermalycler; Bio-Rad) and normalized to the expression level of the housekeeping gene ef1α (SI Appendix, Table S1). qPCR reactions were performed in triplicate from two to four biological replicates using iTaq Universal SYBR Green Supermix (Bio-Rad; 172-5124; 25°C). The expression levels in mutant samples were calculated in relation to WT controls (average set to 100%). Assuming a normal distribution of the data, a paired two-tailed t test was performed to test the significance of differences among sample averages. As the gil3Δ86 mutation induces a framshift that results in premature stop codons, gil3 transcription levels are strongly reduced in gil3Δ86 homozygous fins due to nonsense-mediated messenger RNA (mRNA) decay.

Mouse Experiments. Embryonic day 11.5 (E11.5) (44 to 48 somites) gil3-deficient and WT embryos were processed for ISH with riboprobes recognizing Hand2, Hoxd12, Grem1, and Pax9 transcripts (n = 3 per marker and genotype), as previously described (15). Gli3 CHIP-seq and assay for transposase-accessible chromatin with sequencing (ATAC-seq) data generated from E10.5 mouse limb buds have been previously published (41, 42).

Data Availability. All study data are included in the article and/or SI Appendix. Previously published data were used for this work (PMID: 31989924; PMID: 32268095).

Note Added in Proof. During the review of this manuscript, another study (43) reported an additional highly-conserved Grem1 enhancer, termed CRMS, that is, as GR51 (renamed as CRMS2), also regulated by Gli3.

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