Spiny and soft-rayed fin domains in acanthomorph fish are established through a BMP-gremlin-shh signaling network

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With over 18,000 species, the Acanthomorpha, or spiny-rayed fishes, form the largest and arguably most diverse radiation of vertebrates. One of the key novelties that contributed to their evolutionary success are the spiny rays in their fins that serve as a defense mechanism. We investigated the patterning mechanisms underlying the differentiation of median fin Anlagen into discrete spiny and soft-rayed domains during the ontogeny of the direct-developing cichlid fish Astatotilapia burtoni. Distinct transcription factor signatures characterize these two fin domains, whereby mutually exclusive expression of hoxa13a/b with alk4a/b and tbx2b marks the spine to soft-ray boundary. The soft-ray domain is established by BMP inhibition via gremlin1b, which synergizes in the posterior fin with shh secreted from a zone of polarizing activity. Modulation of BMP signaling by chemical inhibition or gremlin1b CRISPR/Cas9 knockout induces homoeotic transformations of spines into soft rays and vice versa. The expression of spine and soft-ray genes in nonacanthomorph fins indicates that a combination of exaptation and posterior expansion of an ancestral developmental program for the anterior fin margin allowed the evolution of robustly individuated spiny and soft-rayed domains. We propose that a repeated exaptation of such pattern might underly the convergent evolution of anterior spiny-fin elements across fishes.

Significance

The “spiny fin,” comprising the anterior part of the dorsal and anal fins, is an evolutionary novelty that contributed to the success of the spiny-rayed fishes. This domain contains heavily ossified spines that serve as defense mechanism and differ from the posterior flexible soft rays. We show that the partitioning of the median fins into spines and soft rays is established through canonical developmental mechanisms responsible for the anterior–posterior patterning of appendages. Furthermore, the coloration of the anal fin in males appears to be genetically linked to soft-ray identity. Comparative analysis including nonacanthomorph fins indicates that the convergent evolution of fin spines across fishes likely involved the repeated exaptation of a deeply conserved developmental program from the anterior fin.

The authors declare no competing interest.

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Thus, selector genes act upstream in the hierarchy of differentiation to initiate alternative downstream developmental trajectories for meristic structures (24).

In this work, we set out to unravel the developmental basis underlying the patterning of discrete spiny and soft-ray domains using the direct-developing cichlid fish Astatotilapia burtoni (25). Cichlids belong to the Acanthomorpha and possess a spiny fin. The established model-system zebrafish and medaka are not suited to answer this question because zebrafish is not an acanthomorph, and medaka has secondarily lost the spiny fin. A. burtoni has the typical division of spine and soft-ray territories in dorsal and anal fins, as well as soft-ray–specific pigment pattern in males (egg spots). An understanding of the genetic basis for the specification of spine and soft-ray domains will help to elucidate the evolutionary origin of these modules at the base of the acanthomorph radiation as well as provide insight into how spines repeatedly emerged across fish clades as a diversity promoting trait.

**Results**

**Mutually Exclusive alx4a/tbx2b and hoxa13a/hoxa13b Expression Marks the Spine to Soft-Ray Boundary.** We previously described the ontogeny of the spiny and soft-rayed domains in the dorsal and anal fins of A. burtoni. Spine and soft-ray territories differentiate simultaneously between 4 to 10 dpf (days postfertilization) from continuous *Anlagen* located along the dorsal and ventral midline (25). The development of fin elements as either soft rays or spines reflects a binary developmental trajectory since intermediate forms do not occur. The partitioning of the fins into two morphologically discrete domains therefore suggests the existence of a code of “master control” genes that direct a developmental choice for the differentiation into soft rays or spines. We performed RNA-sequencing (RNA-seq) on prospective spiny and soft-rayed parts of the dorsal fin of 9 dpf embryos to identify differentially expressed transcription factor genes (Fig. 1A and B). In the soft-rayed posterior part of the fin, hoxa13a, hoxa13b, hand2, and evx1 are strongly up-regulated, while the spiny part of the fin shows strong expression of alx4a, alx4b, alx3, tbx2b, and pax9. To determine their specificity for spiny or soft-rayed fin domains, the expression of these genes was analyzed using whole mount in situ hybridization (Fig. 1C). In both dorsal and anal fins, we find a strong association of hoxa13a/b and evx1, and their anterior limit of expression marks the spine to soft-ray boundary. In line with its function in zebrafish (26), evx1 is expressed in the forming segment boundaries of the soft rays. Hand2 and hand2, however, associate with a more posterior part of the fin, away from the spine to soft-ray boundary. Alx4a, alx4b, and tbx2b associate with the spiny part of the fin and posteriorly demarcate the spine to soft-ray boundary. Pax9 is expressed with an anterior bias but clearly overlaps the soft-ray territory while alx3 is expressed in the anterior-most part of the spiny fin domain. Additional fin patterning genes hoxa9a, hoxa11a, and tbx18 show ubiquitous fin expression and indicate a largely shared developmental program of the two fin domains, consistent with the spiny fin being a relatively young evolutionary modification. Analysis in a time series from 4 to 7 dpf shows that from 5 dpf onwards alx4a and hoxa13a/hoxa13b stably delineate spine and soft-ray domains whereas this is the case for tbx2b from 6 dpf onwards (SI Appendix, Fig. S1).

**BMP Inhibition through gremlin1b Establishes the Soft-Ray Territory in Synergy with shh.** The division of fins into spiny and soft-ray domains reflects an anterior–posterior organization of the median fins. Therefore, we set out to investigate the role of canonical signaling mechanisms used to pattern the anterior–posterior axis of the appendages in the establishment of this division. In limbs and fins, sonic hedgehog (*shh*) secreted from a ZPA (zone of polarizing activity) is essential for correct anterior–posterior patterning (27–32). Shh expression in a posterior ZPA is an ancestral feature of gnathostome paired and median fins (27, 28, 33, 34). In A. burtoni dorsal and anal fins, we observe first shh expression in a ZPA starting at 5 dpf, becoming strongly expressed at 6 dpf, after which shh disappears from the ZPA and becomes expressed in the distal tips of the forming soft-ray and spine fins (Fig. 2B and SI Appendix, Fig. S2). Treatment during 4 to 6 dpf with the shh agonist SAG induces an anterior expansion of hoxa13b in the dorsal and anal fins while the expression of alx4a and tbx2b becomes more anteriorly restricted—indicating an anterior shift of the spine to soft-ray boundary (Fig. 2C and SI Appendix, Fig. S3). Analysis of the expression of gli1, which is a downstream target of *shh* and provides a read out for the range of *shh* signaling (27, 28), suggests that in untreated embryos at 6 dpf *shh* signaling extends anterior of the ZPA for the length of about two to three somites (Fig. 2B). That is, less than half the extent of the forming soft-ray domain, which develops over the width of 6 to 7 somites (SI Appendix, Fig. S1). Furthermore, inhibition of *shh* through treatment with the *shh* antagonist cycloamine from 4 to 6 dpf fully abolishes *gli1* expression but does not lead to a strong displacement of the anterior–posterior position of the spine to soft-ray boundary as indicated by alx4a/tbx2b and hoxa13b expression (Fig. 2C and SI Appendix, Fig. S3) (although the expression levels of hoxa13b are decreased within the prospective soft-ray domain). Thus, this suggests that while *shh* appears capable of expanding the soft-ray
territory, the normal specification of the soft-ray domain occurs at least in part through another, shh-independent, mechanism.

In limbs, shh activates the secreted BMP antagonist gremlin1 (35), which together with BMP4 provides a mechanism downstream of shh to regulate digit identity (36). In the dorsal and anal fins of A. burtoni, gremlin1b becomes expressed at 4 dpf. In the dorsal fin, its expression initially extends anterior of the vent but becomes subsequently restricted to approximately the soft-ray territory at 5 dpf and continues to regress more posteriorly during 6 and 7 dpf (Fig. 2B and SI Appendix, Fig. S2). No expression of the other gremlin homologs gremlin1a, gremlin2, or any other BMP antagonists investigated (noggin1, noggin2, chordin, chordin-like-2) was detected during early fin development (SI Appendix, Fig. S4). BMP4 is expressed throughout the fin, although there may be a bias toward higher expression in the gremlin1b territory (SI Appendix, Fig. S2). We investigated the function of gremlin1b and BMP signaling during fin patterning by generating A. burtoni CRISPR/Cas9 knockout lines (Materials and Methods) and through gain of function by mimicking the inhibitory effect of gremlin1b with the small molecule BMP-receptor inhibitor DMH1. Gremlin1b knockout leads to a more posteriorly restricted hoxa13b domain of approximately the size of the shh signaling zone as inferred from gli1 expression (Fig. 2C). At the same time, alx4a and tbx2b domains become expanded posteriorly, altogether indicating a posterior shift of the spine to soft-ray boundary. The gain of function approach induced the opposite effect with an anteriorly expanded hoxa13b domain and anteriorly shifted alx4a and tbx2b domains (Fig. 2C and SI Appendix, Fig. S3). These anterior shifts are also induced in the gremlin1b knockout treated with DMH1, which therefore rescues the posterior shifts observed in untreated gremlin1b−/− embryos (Fig. 2C). The posteriorized and anteriorized spine to soft-ray boundaries observed in gremlin1b−/− and DMH1-treated embryos are maintained during development (hoxa13b−/−, hoxa13a−, alx4a−, and tbx2b-stained embryos shown at 9 dpf in SI Appendix, Fig. S3). Therefore, BMP inhibition in the posterior fin by gremlin1b is required for the delimitation of the alx4a/tbx2b and hoxa13b domains and influences the anterior–posterior position of the spine to soft-ray boundary.

In tetrapod limbs, gremlin1b is activated by, and acts downstream of, shh signaling (35). In A. burtoni, SAG treatment leads to widespread up-regulation of gremlin1b expression (Fig. 2B). Cyclopamine treatment however, reduces but does not eliminate posterior gremlin1b domain (Fig. 2B). This observation of shh-independent gremlin1b expression is consistent with its early activation at 4 dpf before shh in the ZPA becomes detectable (SI Appendix, Fig. S2). BMP inhibition by DMH1 strongly down-regulates gremlin1b expression, whereas it appears locally up-regulated in the gremlin1b−/− embryos (Fig. 2B). This suggests that, as in limbs, BMP and shh are upstream of gremlin1b (36) but that in median fins these signaling pathways act in part redundantly. In the context of auto- and cross-regulatory interactions of these pathways, we observe that shh in the ZPA is
strongly down-regulated with SAG treatment and up-regulated with cyclopamine treatment, suggesting the presence of an autoregulatory negative feedback loop (Fig. 2B) as has also been observed during limb development (37). Furthermore, DMDH1 treatment slightly enhances shh expression in the ZPA but does not increase signaling (as judged by gli1 expression) to an extent that it explains the far anterior shift of the soft-ray to spine boundary (Fig. 2B). Altogether, these experiments suggest that shh and gremlin1b are acting independently upstream of the specification of the soft-ray domain.

We further tested this hypothesis by combining shh activation and inhibition conditions with gremlin1b knockout and BMP inhibition. Embryos treated with a combination of cyclopamine and DMDH1 display a similar expansion of hoxa13b and reduction of alx4a and tbx2b domains as treatment with DMDH1 alone (Fig. 2C and SI Appendix, Fig. S3), showing that BMP inhibition can posteriorize the fin independently of shh. In gremlin1b−/− embryos treated with cyclopamine, the posterior residual patch of hoxa13b expression disappears completely and alx4a and tbx2b domains now extend throughout the length of the dorsal and anal fin, indicating a complete absence of a soft-ray domain (Fig. 2C and SI Appendix, Fig. S3). Gremlin1b knockout embryos treated with SAG resemble wild-type (WT) embryos treated with SAG (Fig. 2C and SI Appendix, Fig. S3), confirming that hoxa13b expansion and alx4a/tbx2b reduction can occur independent of BMP inhibition. Therefore, BMP signaling determines the position of the spine to soft-ray boundary in WT fish.

Interference with BMP Signaling Induces Homeotic Transformations of Soft Raya into Spines and Vice Versa. Next, we strived to assess the phenotypic consequences of interference with the shh and BMP pathways. Morphological differentiation between spine and soft-ray elements, as indicated by the presence of fin segments and the development of spine tips, first occurs in A. burtoni around 10 dpf (25). Cyclopamine and SAG treatments induced widespread pleiotropic effects outside of the fins and severely compromised embryonic viability beyond 8 dpf, that is, before the morphological differences between spines and soft rays are established and therefore preclude such morphological analyses.

DMDH1 treatment or loss of gremlin1b is, however, well tolerated with phenotypic consequences that appear primarily in the fins and thus allow further morphological analyses of the extent of spine and soft-ray territories. In the dorsal fins of gremlin1b mutants, we observe a posterior shift of the spine to soft-ray boundary caused by a homeotic transformation of the anterior soft rays into spines as indicated by the presence of a spiny tip, the absence of segmentation, and the anterior fusion of the hemitrichia (Fig. 3A and SI Appendix, Fig. S5) (WT/heterozygous (n = 21): 13 to 14 spines, 9 to 10 soft ray; gremlin1b−/− (n = 16): 15 to 20 spines, 3 to 6 soft rays). In the anal fin, a similar posterior expansion of the spine domain is observed whereby only 3 to 4 soft rays are maintained (Fig. 3C and SI Appendix, Fig. S5) (WT/heterozygous (n = 21): 3 spines, 8 to 10 soft ray; gremlin1b−/− (n = 16): 4 to 7 spines, 1 to 6 soft rays). The preservation of soft-ray identity in the posterior fin is consistent with the presence of a posterior patch of alx4a expression that arises in a shh-dependent manner in gremlin1b−/− embryos (Fig. 2C). The inhibition of BMP signaling through DMDH1 treatment for a 24-h window during 4 to 5 dpf results in the opposite phenotype in the dorsal fin with an anterior transformation of spines into soft rays (Fig. 3B and SI Appendix, Fig. S5) (n = 7, spines 3 to 10, soft rays 14 to 21). This treatment induces the same soft-ray expansion in a gremlin1b−/− background (SI Appendix, Fig. S6) (n = 5/5). In the anal fin, no significant shift in number of soft rays and spines is observed (P = 0.06, two-sided t test, n = 6, spines 2 to 3, soft rays 9 to 11) (SI Appendix, Fig. S5), suggesting that additional genetic factors besides BMP signaling determine the presence of the 3 anterior fin spines in the anal fin.

Altogether, the observed homoeotic transformations of spines to soft rays and vice versa underpin that BMP inhibition by gremlin1b is a primary determinant of soft-ray identity as also suggested by the analysis of developmental marker genes.

Gremlin1b Mutants Display Homeotic Transformations in Anal Fin Coloration. The individuation of the soft-rayed and spiny domains of the male anal fin in A. burtoni is also reflected in its coloration. The mouth-brooding African cichlids evolved egg spots, or “egg dummies,” apparently to increase the chances of fertilization during courtship (25, 38). The distribution of egg spots in the anal fin typically shows a bias toward the posterior side of the fin overlapping with the soft rays while being absent from the spiny part. To investigate whether egg spots are in fact part of the same genetic modules that determine soft-ray and spine development, we analyzed the presence of egg spots at 3 mo of age in WT/heterozygous and gremlin1b−/− males derived from two gremlin1b−/− crosses. Comparison of mutant with WT or heterozygous fish (which are WT in appearance with respect to spine and soft-ray distribution) shows an altered distribution of egg spots on the fin. Concomitant with the posterior shift of the soft-ray domain, the egg spots in these fish are present more posteriorly, and egg spots were never observed to overlap with the spiny-fin domain. In the cross analyzed, WT and heterozygous fish have an average of 3.5 egg spots whereas gremlin1b homozygous mutant fish have an average of 2 egg spots (WT/ heterozygous n = 8; gremlin1b−/− n = 9, P = 0.0002, two-sided t test) (Fig. 3D and SI Appendix, Fig. S5). In the same cohort, WT and heterozygous male egg spots are present over 57% of the length of the fin, whereas this is reduced to 28% in homozygous gremlin1b mutant fish (P = 9.6 × 10−6, two-sided t test) (SI Appendix, Fig. S5). Therefore, the distribution of egg spots in the anal fin appears to be determined by the same upstream patterning mechanism as that inducing the soft-ray and spiny-fin domains, whereby the posterior reduction of the soft-ray domain results in a concomitant posterior shift in the presence of egg spots.

Analysis of the Dorsal Fin Pattern in Nonacanthomorph Spiny and Nonspiny Catfish. Anterior spines have convergently evolved in several clades of nonacanthomorph teleosts such as catfish and carps. We wanted to further understand the relationship between dorsal fin patterns and the repeated emergence of fin spines. Furthermore, the dorsal fin pattern of nonacanthomorphs could provide information concerning the evolutionary origin of the acanthomorph fin pattern. We thus compared the anterior–posterior patterning observed in A. burtoni with that in nonacanthomorph species with median fins consisting of soft rays only or in those with convergently evolved fin spines. The nonacanthomorph zebrafish possesses soft rays only, and alx4a is expressed in the anterior-most fin rays of the dorsal and anal fins (39), tentatively suggesting that the spine pattern derives from a domain originally confined to the anterior fin margin. Zebrafish, however, has a narrow dorsal fin that is restricted to the posterior part of the body and that is about the size of the A. burtoni soft-ray domain. This leaves open the possibility that wider and further anteriorly extending non-acanthomorph fins show a similar extended alx4 domain as A. burtoni. We investigated the expression of alx4a, hoxa13b, and gremlin1b expression in embryos of the African catfish (Clarias gariepinus), which has an extended dorsal fin (Fig. 4A) comprised of soft rays only and lacks the typical anterior spine found in many catfish species. Consistent with its soft-ray identity, hoxa13b and gremlin1b expression extends anterior throughout most of the dorsal fin. As in zebrafish, alx4a expression is confined to the anterior fin margin. Analysis in South American Ancistrus catfish whose anterior-most dorsal fin element has convergently evolved...
into a spine, for hoxa13b, gremlin1b, and alx4a, shows a similar pattern (Fig. 4B). In this species, the expression of all three genes overlaps in the first dorsal fin element, which will develop into a spine. Therefore, anteriorly limited expression of alx4a is also apparent in nonacanthomorph fish. This domain is, however, restricted much more anteriorly, and the gremlin1b/hoxa13 domain extends along the anterior–posterior fin axis. Intriguingly, the anterior domain can coincide with the development of either a soft ray (as in zebrafish and Claris) or a spine (as in Ancistrus) (Discussion).

Discussion

Spiny fins can be considered an evolutionary key innovation that arose as a novel module in the spiny-rayed fishes and added to the evolvability and thereby evolutionary success of the teleost fish. During tetrapod limb development, shh and BMP inhibition via gremlin1 are part of a regulatory loop including FGFs expressed in the distal ectoderm, which are required for ZPA survival (27–31, 35). We therefore investigated the potential role of FGFs in the establishment of soft-ray and spiny-fin domains. Fgf16 is expressed along the anterior–posterior extent of the distal edge of the dorsal and anal fins and is slightly up-regulated by DMMH1 and SAG treatment whereas it is somewhat down-regulated by cyclopamine treatment and in gremlin1b−/− embryos (SI Appendix, Fig. S7). Altogether, this potentially indicates a conserved position of ectodermal FGF signaling downstream of shh and gremlin1. Treatment with the FGF antagonist BGG398 from 4 to 7 dpf results in complete abortion of fin outgrowth, equally affecting spine and soft-ray domains and consistent with the relatively homogenous expression along the fin anterior–posterior axis. (SI Appendix, Fig. S7). Therefore, while important for fin outgrowth, ectodermal FGF signaling is not a major factor determining the anterior–posterior division of the dorsal and anal fins into spine and soft-ray territories.

In A. burtoni, the anterior–posterior pattern in dorsal and anal fins differs from that in their pectoral fins. In the latter, hoxa13a/b are expressed throughout the anterior–posterior extent of the fin (42, 43) and alx4a/b remain restricted to the anterior-most fin domain (39, 44). This appears to be a deeply conserved pattern that is for instance also present in shark pectoral fins (32, 45, 46).
expressed in the anterior fin margin, possibly related to the convergent evolution of spiny elements in nonacanthomorphs such as catfish. AZR: Alizarin red.

Also, in *A. burtoni* pectoral fins *gremlin1b* is expressed throughout most of the anterior–posterior axis of the pectoral fin *Anlage* (SI Appendix, Fig. S8) and does not show the posterior bias observed in dorsal and anal fins. Overall, the patterning of the median fins in nonacanthomorph, *Clarias, Ancistrus,* and zebrafish (39) therefore resembles a pectoral fin pattern (although the median fin expression pattern of *gremlin1b* in zebrafish remains to be determined) and may therefore represent a shared ancestral pattern among median and paired fins that became modified in the median fins of spiny-rayed fish. This would have involved an expansion of the anterior pattern and a concomitant reduction of the soft-ray domain (Fig. 4C). Whether in the ancestral fin pattern *gremlin1b* acts to establish the posterior domain remains to be investigated by loss of function approaches in nonacanthomorphs. It is however suggestive that in *A. burtoni* *gremlin1b* loss does not lead to reduction of *hoxa13b* expression or expansion of *alx4a* expression in pectoral fins (SI Appendix, Fig. S8). This therefore might hint at a newly evolved posteriorizing role of *gremlin1b* in acanthomorphs median fins.

It is noteworthy that nonacanthomorphs frequently have a modified first fin element. For instance, in zebrafish and goldfish the first soft ray does not branch distally, and in many catfish species and carp a “spine” develops at this position. This suggests that an individualization of the anterior-most fin exists in nonacanthomorphs, which is consistent with the anterior domain of *alx4* expression in the fin margin of zebrafish (39), *Clarias,* and *Ancistrus* catfish. The tendency for more robustly ossified or spiny anterior fin ray elements is a trend present throughout fishes in both paired and median fins. Additional examples are the anterior fin spine in catfish (47) and sturgeon pectoral fins (48), robustly ossified anterior fin rays in tetrapodomorphs (49), the anterior fin spine that evolved convergently in chimaeras (50), and acanthodians (spiny sharks) and stem sharks (e.g., hybodonts) (51). It is plausible that convergently evolved spines all rely on the same deeply homologous anterior fin individualization. Importantly however, this module appears restricted to the first few anterior-most fin elements only in all lineages except for the *Acanthomorpha,* which show a strong posterior expansion. Furthermore, spines in nonacanthomorph teleosts are different from those in acanthomorphs because the former initially develop as segmented elements that are indistinguishable from soft rays (47) (developing *Ancistrus* catfish dorsal fin shown in SI Appendix, Fig. S9). Therefore, in addition to the expansion of the anterior fin identity, a change in the downstream interpretation of this pattern (in the form of exaptation) was needed for the evolution of true fin spines and the consolidation of a robustly individualized anterior spiny-fin module in the acanthomorphs. Altogether, such changes in fin architecture allowed the emergence of the spiny-rayed fishes and initiated one of the most successful and diverse of vertebrate radiations.

**Materials and Methods.**

**In Situ Hybridization.** In situ hybridization was carried out according to Woltering et al., 2009 (21), 2014 (52), 2015 (20), and 2020 (44). The reported shifts in expression domains in the inhibitor experiments and *gremlin1b*− embryos were observed with complete penetrance.

**Cloning of Probes.** Probes were cloned in pGEMT (Promega A3600) vector using PCR from *A. burtoni, C. gariepinus,* or *Ancistrus* sp. embryonic cDNA. A primer table is provided in SI Appendix, Table S1. The *A. burtoni hoxa11a, hoxa13a, hoxa13b, hoxd12,* and *alx4b* probes were described before (42, 44). Catfish sequences were identified by BLAST (basic local alignment search tool) against *C. gariepinus* and *Ancistrus* sp. embryonal/larval RNA-seq libraries, and messenger RNA sequences for *alx4a, hoxa13a, hoxa13b, gremlin1a,* and *gremlin1b* were deposited in GenBank under accession nos. MW846856 to MW846866. Correct identification of "a" and "b" orthologs was confirmed by generation of maximum likely hood gene trees and microsynteny analysis (also reference SI Appendix, Figs. S10 and S11 for *gremlin1* and *alx4*).
Small Molecule Treatment Experiments. Embryos were treated using the following concentrations: 1 μM SAG (Selleckchem S7779) (dissolved in 10 mM DMSO) (dissolved at 50 mM in ethanol), 1 μM DMM1 (Selleckchem S7146) (dissolved at 20 mM in DMSO), and 1 μM BGJ398 (Selleckchem S2183, dissolved at 10 mM in DMSO). Embryos were cultured in 30 mL equilibrated tap water (approximately pH 8, 9°Hd) with addition of 0.01 μg/mL Methyline blue and penicillin-streptomycin (Sigma P4333) diluted 1:1000 in 0.85% plastic Petri dishes on an orbital shaker at 33 rpm at 28 °C in a heating incubator. Embryos were cultured at a maximum density of 20 embryos per dish (but usually less) and treated from 4 to 6 dpf for ~48 h (with the exception of BGJ398). Chemicals were added to the dish upon start of treatment, and embryos were kept in the same medium until the point of fixation. For the phenotypic analysis of DMM1 treatments, embryos were treated in 1 μM DMM1 from mid to mid 5 dpf for ~24 h and subsequently transferred to normal culturing medium and kept under standard conditions until the point of analysis. Mock treatments were performed using DMSO and ethanol, which do not result in phenotypic alterations.

RNA-Seq Analysis. RNA-seq was performed in triplicate using dissected soft-ray and spine territories of 9 dpf embryos using 10 individuals per sample. RNA was extracted using the ReliaPrep RNA Tissue Miniprep System (Promega Z6111) using the fibrous tissue protocol, and sequencing libraries were generated using TruSeq RNA Library Preparation Kit v2 (Illunina RS-122-2001). Samples were sequenced on an Illumina HiSeq2500 125 bp (base pairs) paired ends, and reads were demultiplexed and trimmed using Trimmmomatic. The trimmed reads were used for transcriptome assembly, and quantification was used (53). Briefly, TopHat and Bowtie2 were used to map reads to the A. burtoni genome (v. 1.0). Cufflinks was used to assemble transcripts, to assemble a merged transcriptome, and to conduct differential gene expression analysis. Data (29,293 transcripts) were then imported into R (v. 3.6.3), and transcripts that showed no expression in at least one out of three replicates in at least one of the two groups (ray or spine) were excluded. Addition of all transcripts with extreme low expression (average FPKM [fragments per kilobase of transcript per million mapped reads] >0.5) were also excluded (20,592 transcripts, 17,733 of which were annotated and 17,597 were unique). Raw P values obtained from Cuffdiff were corrected for multiple testing using the false discovery method for transcripts. Raw sequence data have been deposited in National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under BioProject PRJNA718487 (54) with accession nos. SAMN18537261–SAMN18537266 (55–60).

Phenotype Analysis. Alizarin red staining was performed according to standard protocols and imaged under fluorescence microscopy. Spine and soft-ray counts given in SI Appendix, Fig. S5 were determined by manual inspection under dissection binocular.

Animal Husbandry. A. burtoni were bred and collected at the University of Konstanz as previously described (25). C. gariepinus embryos were kindly provided by Fleuren and Nooyen, Aquaculture ID, Netherlands. Ancistrus sp. embryos were provided by private breeders. Animal experiments were carried out under 35–915/18G1/1832, Tierforschungsanlage (University of Konstanz) Aktenzeige T1807.

Generation of CRISPR/Cas9 gremlin1b Mutant A. burtoni Lines. Guide RNAs (gRNA) were cloned into pT7gRNA and produced according to ref. 61 using oligos. gRNA-1 FW: TAGGACTCAGCAGCTTCGTGCG, gRNA-1 RV: AAAACCGCAGAACGTTGAGGT, gRNA-2 FW: TAGGTTGCTTGGCAGATTGTG, gRNA-2 RV: AAAACACAGTGACAGGACGCA. A mixture of 1 to 2 nL of two gRNAs at 10 ng/μL each including Cas9 protein (NEB M0646T diluted 1/40) was injected at the one to two cell stage. Embryos were cultured individually in 6-well plates on an orbital shaker at 28 °C in the presence of penicillin-streptomycin (Sigma P4333) diluted 1:1,000 and addition of methylene blue. Two independent lines were derived (SI Appendix, Fig. S12): gremlin1b-stopCD38 has an in-frame premature stop codon introduced at codon 38; gremlin1bΔ1740 has a Δ1740 bp deletion including the 5′ 339 bp and start codon. Both lines gave indistinguishable phenotypes. The gremlin1b-stopCD38 was genotyped using fragment mapping on a capillary sequencer (3130xxi Genetic Analyzer, Applied Biosystems) using a 40 cycle PCR with primers M13 tailed FW: CAG-GAAAACGATACGACGACATCTTTCACTTGATG, RV: GTCTGCGTGTCGCTCCGATCC followed by a second one-cycle labeling PCR with a HEX-labeling primer: FW ACCGTACATTGAC. The gremlin1bΔ740 allele was detected using standard PCR and gel electrophoresis using primers FW: CAGTGGCAGACGTCAGCAGATG, RV: GACGACACATTCTTCGCGT.

Data Availability. RNA-seq dataset of A. burtoni 9 dpf fins and catfish gene sequences data have been deposited in NCBI SRA and NCBI GenBank (SRA: BioProject PRJNA718487 (54), SAMN18537261–SAMN18537266 (55–60); GenBank: MW846865–MW846866) (62–72).

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