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

Rebekka Höch⁵, Ralf F. Schneider⁶,⁷,⁸, Alison Kickuth⁹,¹⁰, Axel Meyer¹¹, and Joost M. Woltering⁵,¹²

*Zoology and Evolutionary Biology, Department of Biology, University of Konstanz, Konstanz 78457, Germany

Edited by Neil H. Shubin, University of Chicago, Chicago, IL, and approved May 19, 2021 (received for review February 4, 2021)

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 alx4ab 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 homeotic 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.

fin spine | acanthomorph | evolutionary key innovation | evo-devo | exaptation

Teleost fishes comprise ~50% of extant vertebrate species and display an astonishing diversity in body plans (1–4). Among the ~30,000 species of teleosts, the spiny-rayed fish—or Acanthomorpha—are evolutionarily the most successful lineage with over 18,000 species, representing approximately one third of all living vertebrates (1, 2, 5). Spiny-rayed fishes evolved relatively recently, during the Early Cretaceous (133 to 150 Mya) (6), and underwent their primary radiation after the Cretaceous–Paleocene mass extinction (ca. 66 Mya) when their lineage came to dominate many aquatic ecosystems (4, 6–10). One of the characteristics that has strongly contributed to the ecological and evolutionary success of the spiny-rayed fishes is fin spines in dorsal and anal median fins (2, 3, 11). Acanthomorph fin spines are mostly present on the anterior part of the dorsal, anal, and sometimes pectoral and pelvic fins and differ from soft rays by increased ossification, lack of segmentation, fusion of lateral half-segments (hemitrichia), and ending in a sharp point instead of bifurcating (11) (Fig. 1I). The main function of fin spines is to serve as a defense mechanism against gape-limited predators (2, 3, 11, 12), and as such, they strongly suggest a causal link to the success of the Acanthomorpha. Interestingly, anterior spines have evolved independently in other successful lineages of teleosts (2, 11–13), such as the Ostariophysians, in particular catfish and carps, underscoring their adaptive significance. However, in none of these lineages has this resulted in such persistent and pronounced individualization and modularization of separate median fin domains as present in acanthomorph fishes (Discussion).

In the acanthomorph dorsal and anal fins, the spiny and soft-rayed parts form distinct morphological and developmental units that behave as separate evolutionary modules (14). Examples of extreme morphological specialization of the spiny fin as compared to the soft rays are the Remora’s suction disk (15, 16), the Frogfishes’ illicium/esca complex (17) and the dorsal part of the Triggerfishes’ “locking mechanism” (18). Species such as the Asian leaf fishes (e.g., Nandus oxyrhynchus) (19) further exemplify the divergence between spiny and soft-rayed fins. As many ambush-hunting fish, they have translucent soft-rayed fins and heavily pigmented spiny fins, whereby the transparency of the unpigmented soft rays enhances camouflage as slight undulations of those fin parts serve to keep the fish stationary. Altogether, this suggests a modularization that is distinct beyond the mere morphological difference between spines and soft rays and also underscores the adaptive significance of individuated spine and soft-ray fin modules.

This individuation that affects a range of phenotypic traits is reminiscent of anatomical modules determined by master control genes that specify different ontogenetic outcomes for serially homologous elements. Examples of such systems are for instance the hox codes in the axial skeleton (20–22) or the hindbrain (23).

Author contributions: A.M. and J.M.W. designed research; R.H., R.F.S., A.K., and J.M.W. performed research; R.H., R.F.S., and J.M.W. analyzed data; J.M.W. wrote the paper; and A.M. and J.M.W. interpreted the research and provided financial support.

The authors declare no competing interest.

This article is a PNAS Direct Submission. Published under the PNAS license.

1Present address: Marine Ecology, Helmholtz Centre for Ocean Research Kiel (Geomar), 24148 Kiel, Germany.
2Present address: Max Planck Institute of Molecular Cell Biology and Genetics, 01307 Dresden, Germany.
3To whom correspondence may be addressed. Email: joost.woltering@uni-konstanz.de.

This article contains supporting information online at https://www.pnas.orglookup/suppl/doi:10.1073/pnas.2101783118/DCSupplemental.

Published July 6, 2021.
radiation as well as provide insight into how spines repeatedly of spine and soft-ray domains will help to elucidate the evolu-
spots). An understanding of the genetic basis for the specification –

–

typical division of spine and soft-ray territories in dorsal and anal 
fins into spine and soft-ray domains. RNA-seq analysis (Fig. 1C) shows transition of spiny and soft-rayed 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 elements (Fig. 2B and SI Appendix, Fig. S2). Treatment during 4 to 6 dpf with the shh antagonist 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

Thus, selector genes act upstream in the hierarchy of differen-
tiation to initiate alternative downstream developmental traject-
ories for meristic structures (24).

In this work, we set out to unravel the developmental basis underly-
ing 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 address 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 evolu-
tionary 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 alx4/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

Fig. 1. (A) Skeleton of adult A. burtoni shows division of the dorsal and anal fins into spine and soft-ray domains. Left inset shows transition of spiny and soft-ray domains for the dorsal fin, Right inset for the anal fin. We investig-
gated the developmental basis for the individuation of the spine and soft-rayed fin domains using RNA-seq analysis (B) and in situ hybridization (C) using 8 to 9 dpf embryos. Tbx2b and alx4a/b define the fin spine territory while hoxa13a/b and evx1 are expressed exclusively in soft rays. Additional transcription factor genes identified in the RNA-seq show an anterior (alx3/pax9) or posterior (hand2/hoxd12) bias but do not segregate with the spine to soft-ray transition. Expression of hoxa9a, hoxa11a, and tbx18 is ubiqui-
tous throughout the fins and underscores shared origins of spines and soft 

ray domains. Anterior is to the left. The skeleton and embryo in A are from ref. 25, 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 mor-
phologically 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, hoxd12, 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. Hoxd12 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 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).
Spiny and soft-rayed fin domains in acanthomorph fish are established through a BMP-gremlin-shh signaling network

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 boundary. The gain of function by mimicking the inhibitory effect of gremlin1b with the small molecule BMP-receptor inhibitor DMH1 (Fig. 2C) and 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.

Downloaded at DKFZ-HGF on September 8, 2021
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, DMH1 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 DMH1 display a similar expansion of hoxa13b and reduction of alx4a and tbx2b domains as treatment with DMH1 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 (Fig. 2C). Therefore, the posterior soft-ray domain is synergistically patterned by shh and gremlin1b, whereby gremlin1b determines the position of the spine to soft-ray boundary in WT fish.

**Interference with BMP Signaling Induces Homoeotic Transformations of Soft Rays 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. DMH1 treatment or loss of gremlin1b is, however, welltolerated 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). 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 (Fig. 2C). Therefore, the posterior soft-ray domain is synergistically patterned by shh and gremlin1b, whereby gremlin1b determines the position of the spine to soft-ray boundary in WT fish.

**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 carp. 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 nonacanthomorph 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
Fig. 3. Interference with BMP signaling induces homeotic transformations in dorsal and anal fins. (A–C) Fin morphology of dorsal (A and B) and anal fins (C) in WT (A, C), gremlin1b−/− (A and C), and DMH1-treated (B) fish at approximately 1 mo postfertilization. Bony structures were visualized using Alizarin red (“AZR”), and fins were imaged using fluorescence microscopy. Insets shown (dashed boxes) were taken using brightfield microscopy (“BF”). In A and B, transversal sections at the level of the spine to soft-ray boundary are shown, which in A was imaged using fluorescence microscopy (Alizarin red fluorescence in white) and in B using fluorescence microscopy (Alizarin red fluorescence in false color red) and differential interference contrast microscopy (“DIC”) (in white). The dorsal fins of gremlin1b−/− fish in A show an expanded spine domain (indicated by red line) and reduced soft-ray domain (indicated by blue line) indicating soft-ray to spine homeotic transformations. Alizarin red staining visualizes the heavier ossification of spines as compared to soft rays. Insets show spine (red arrowhead spine tip) and soft-ray (blue arrowhead segment boundary) characters, at the spine to soft-ray transition. Transversal sections through the spine to soft-ray boundary confirm the presence of fused and unfused hemisegments in spines and soft rays, respectively (section position is indicated with circles and numbers). The DMH1-treated fish shown in B show the opposite transformation displaying spine to soft-ray transformations. The inset shows segments in the most anterior soft ray (blue arrowhead). Transversal sections confirm the presence of unfused soft-ray–like elements in the anterior fin. C shows a comparison of gremlin1b−/− and WT anal fins showing soft-ray to spine transformations. Insets indicate spine and soft-ray characters at the spine to soft-ray boundary. A quantitative analysis of spine and soft-ray counts is provided in SI Appendix, Fig. S5A. (D) Egg spots are present on the soft-ray part of the anal fins of male A. burtoni. In gremlin1b−/−, the egg spots have shifted posterior together with the soft-ray domain. A quantitative analysis of egg spots distribution is provided in SI Appendix, Fig. S5B. AZR: Alizarin red, BF: brightfield, S: spine, SR: soft ray. Anterior is to the left.

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 body plan. Here, we show that the specification of spine and soft-ray domains during embryonic development is the result of a canonical signaling network involved in the patterning of the anterior–posterior fin axis. Intriguingly, the anterior domain can coincide with the development of either a soft ray (as in zebrafish and Clarias) or a spine (as in Ancistrus) (Discussion).

Homologous function of BMP signaling in “specifying discrete identities amongst meristic structures” (quotation from ref. 40).

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 from 4 to 7 dpf results in complete abortion of fin outgrowth, equally affecting spine and soft-ray domains and consistent with the homologous expression of BMP signaling in “specifying discrete identities amongst meristic structures” (quotation from ref. 40).

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 from 4 to 7 dpf results in complete abortion of fin outgrowth, equally affecting spine and soft-ray domains and consistent with the homologous expression of BMP signaling in “specifying discrete identities amongst meristic structures” (quotation from ref. 40).

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 from 4 to 7 dpf results in complete abortion of fin outgrowth, equally affecting spine and soft-ray domains and consistent with the homologous expression of BMP signaling in “specifying discrete identities amongst meristic structures” (quotation from ref. 40).
Fig. 4. Anterior–posterior dorsal fin patterning in acanthomorphs and nonacanthomorphs. (A) African catfish (C. gariepinus) have an extended dorsal fin (blue) comprised of soft rays only. The Alizarin red bone staining on the Left shows the anterior-most fin elements, and arrowheads indicate segment boundaries. In line with its soft-ray identity, hoxa13b and gremlin1b are expressed throughout most of the anterior–posterior fin axis. Alox4a expression is detected in a domain in the anterior fin similar to that in zebrafish (39). (B) Ancistrus catfish have a dorsal fin that is restricted to the anterior part of the trunk. This fin consists of posterior soft rays and a single anterior spine. Hoxa13b is expressed throughout the anterior–posterior extent of the fin, including the first elements, as is gremlin1b. Alox4a expression is confined to the spine and therefore may be involved in the individualization of this element compared to the posterior domain. (C) Model for the signaling network establishing the soft-ray domain in acanthomorph and nonacanthomorph teleosts. In acanthomorphs, the soft-ray domain is established via gremlin1b, which acts posteriorly in synergy with shh to activate hoxa13 through the inhibition of BMP signaling. The absence of these posterior signals results in posterior expansion of alox4 expression and the spine domain either through direct activation by BMP or loss of repression by hoxa13 proteins. In nonacanthomorphs, the soft-ray signature extends throughout the anterior–posterior fin axis, and aiox4 is only expressed in the anterior fin margin, possibly related to the convergent evolution of spiny elements in nonacanthomorphs such as catfish. AZR: Alizarin red. Anterior is to the left.

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 alox4a expression in pectoral fins (SI Appendix, Fig. S8). This therefore might hint at a newly evolved posteriorizing role of gremlin1b in acanthomorph 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 carps 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 alox4 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, hoxa12, and alox4b probes were described before (42, 44). Catfish sequences were identified by BLAST (basic local alignment search tool) against C. gariepinus and Ancistrus sp. embryonal/fetal RNA-seq libraries, and messengerRNA sequences for alox4a, hoxa13a, hoxa13b, gremlin1a, and gremlin1b were deposited in GenBank under accession nos. MWV46856 to MWV46866. Correct identification of “a” and “b” homologs was confirmed by generation of maximum likelihood tree and microsynteny analysis (also reference SI Appendix, Figs. S10 and S11 for gremlin1 and alox4).

Spiny and soft-rayed fin domains in acanthomorph fish are established through a BMP-gremlin-shh signaling network.
Small Molecule Treatment Experiments. Embryos were treated using the following concentrations: 1 μM SA (Selleckchem S7779) (dissolved at 10 mM in DMSO), 5 μM DMH1 (Selleckchem S6356) (dissolved at 50 mM in ethanol), 1 μM DMH1 (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°dH) and were transferred for 24 h and subsequently transferred to normal culturing medium and raised 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 (Illumina Run 122-2001). Samples were sequenced on an Illumina HiSeq2500 125 bp (base pairs) paired ends, and reads were demultiplexed and trimmed using Trimmomatic (version [v.]0.36). The tuxedo pipeline 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. Additional exclusion with extremely low expression (average FPKM (55) < 0.5) and transcripts that were kept in the same medium until the point of analysis. For the phenotypic analysis of DMH1 treatments, embryos were treated in 1 μM DMH1 from mid 4 to mid 5 dpf for 24 h and subsequently transferred to normal culturing medium and raised under standard conditions until the point of analysis. Mock treatments were performed using DMSO and ethanol, which do not result in phenotypic alterations.

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 (S4), SAMN18537261–SAMN18537266 (55–60); GenBank: MW846856–MW846866 (62–72).

ACKNOWLEDGMENTS. We wish to acknowledge S. Nappe, K. Gergen, J. Gervin, C. Dickmanns, and M. Holzem for help in the laboratory; C. Kratochwil for use of equipment; members of the A.M. laboratory for useful discussions; S. Boycheva Woltering for critical reading of the manuscript; and A. Pfeifer and M. Schauber for excellent fish care. This project was funded by Deutsche Forschungsgemeinschaft Grant 20-1W0-2152-1 (to J.M.W.), supported by the Young Scholar Fund of the University of Konstanz (to J.M.W.), and European Research Council (ERC) Advance Grant ("GenAdap" 293700) (to A.M.).