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 axl4a/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 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.

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 ozyrhynchus) (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 determines pigmentation as well as function and further 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).

Telesost 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. 4L). 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 Ostariophysi, 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).

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.

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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 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 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, 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. *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 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 *alx6a* 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* 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 *glf1*, 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 cyclopamine from 4 to 6 dpf fully abolishes *glf1* 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...
at 6 dpf, all chemical treatments were initiated at 4 dpf. Inset in 6 dpf embryo shows the approximate extent of the part of the embryos shown in B and C. (B) Analysis of the expression of gremlin1b, shh, and gli1 (indicated “probe*) in WT embryos treated with cyclopamine, SAG, DMH1, and in gremlin1b−/− embryos. (C) Analysis of the spine to soft-ray transition using the spine marker alx4a and the soft-ray marker hoxa13b in treatment with cyclopamine, SAG, DMH1, and a combination of DMH1 and cyclopamine. Experiments were performed on WT and gremlin1b−/− embryos (except the combination of DMH1 + cyclopamine) and observed on a minimum of 6/6 embryos per probe per treatment/genotype. Anterior and posterior limits of dorsal and anal fin domains become expanded posteriorly, altogether indicating that, as in limbs, BMP and shh signaling (36) are from ref. 25.

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 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 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, DMH1 treatment slightly enhances shh expression in the ZPA but does not increase signaling (as judged by glitl 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, BMP inhibition 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 Homeotic 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, 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 spiney tip, the absence of segmentation, and the anterior fusion of the hemitrichia (Fig. 3A and 3B, 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 3D, 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 hoxa13b expression that arises in a shh-dependent manner in gremlin1b−/− embryos (Fig. 2C). The inhibition of BMP signaling through DMH1 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. 3E and 3F, 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 homeotic 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 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 in 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...
extends along the anterior anal fins of male soft-ray boundary. A quantitative analysis of spine and soft-ray counts is provided in soft ray (as in zebrafish and anterior domain can coincide with the development of either a gremlin pattern (Fig. 3).

Therefore, anteriorly limited expression of gremlin1b, the primary determinant of the spine to soft-ray boundary is BMP inhibition by gremlin1b and shh, the primary determinant of the spine to soft-ray boundary (39, 44). This appears to be a deeply conserved pattern along the fin anterior–posterior axis. In WT A. burtoni, the primary determinant of the spine to soft-ray boundary is BMP inhibition by gremlin1b, and alterations in BMP signaling induce homeotic transformations in fin identity. Interestingly, modulation of BMP levels is capable of inducing homeotic transformations in digits (40) and tooth identity (41). Therefore, spines and soft rays form another example of a deeply homologous function of BMP signaling in “specifying discrete identities amongst meristic structures” (quotation from ref. 40).

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, whereby posterior expression of gremlin1b and shh specify the soft-ray domains (Fig. 4C). In WT A. burtoni, the primary determinant of the spine to soft-ray boundary is BMP inhibition by gremlin1b, and alterations in BMP signaling induce homeotic transformations in fin identity. Interestingly, modulation of BMP levels is capable of inducing homeotic transformations in digits (40) and tooth identity (41). Therefore, spines and soft rays form another example of a deeply 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 by DMH1 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 BJI398 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 and alx4a 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).
It is plausible that convergently evolved spines all rely on the Ancistrus catfish. The tendency for more robustly ossified or acanthodians (spiny sharks) and stem sharks (e.g., hybodonts) anterior fin spine that evolved convergently in chimaeras (50), and robustly ossified anterior fin rays in tetrapodomorphs (49), the posterior fin spine in catfish (47) and sturgeon pectoral fins (48), in both paired and median fins. Additional examples are the an-spiny anterior fin ray elements is a trend present throughout fishes of nonacanthomorphs, which is consistent with the anterior domain of gremlin1b expression pattern in zebrasfish (39). (b) Ancistrus catfish has 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. Alx4a 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 alx4 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 alx4 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 zebrasfish (39) therefore resembles a pectoral fin pattern (although the median fin expression pattern of gremlin1b in zebrasfish 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 zebrasfish 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 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 copyDNA. A primer table is provided in SI Appendix, Table S1. The A. burtoni hoxa11a, hoxa13a, hoxa13b, hoxa12, and alx4b probes were described before (42, 44). Catfish sequences were identified by BLAST (basic local alignment search tool) against C. gariepinus and Acistrus sp. embryonic/larval RNA-seq libraries, and messengerRNA sequences for alx4a, hoxa13a, hoxa13b, gremlin1a, and gremlin1b were deposited in GenBank under accession nos. MW846856 to MW846866. Correct identification of “a” and “b” homologs was confirmed by generation of maximum likelihood 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 at 10 mM in DMEM, 5 μM AMO (Selleckchem S686) (dissolved at 50 mM in ethanol), 1 μM DMH1 (Selleckchem S7146), dissolved at 20 mM in DMEM), and 1 μM BGJ398 (Selleckchem S2183, dissolved at 10 mM in DMSO). Embryos were cultured in 30 μL equilibrated tap water (approximately pH 8, 9°DH) with addition of 0.01 μg/mL Methylene blue and penicillin/streptomycin (Sigma P4333) diluted 1:1,000 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 (with 4% PFA buffered with 1× PBS phosphate buffered saline) overnight at 4 °C, afterward storage in 100% ethanol at 4°C to the dish upon start of treatment, and embryos were kept in the same medium until the point of fixation (with 4% PFA buffered with 1× PBS phosphate buffered saline) overnight at 4 °C, afterward storage in 100% ethanol at 4°C. 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.

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 RS-122-2001). Samples were sequenced on an Illumina HiSeq2500 125 bp (base pairs) paired-ends, and reads were demultiplexed and trimmed using Trimmomatic (v0.39). The trimmed paired-end reads were de novo assembled using Trinity (v2.2.0) 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. Additionally, transcripts with extremely 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 a dissection binocular.

Factors of Interest