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Through veiled mirrors: Fish fins giving insight into size regulation

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Abstract

Faithful establishment and maintenance of proportion is seen across biological systems and provides a glimpse at fundamental rules of scaling that underlie development and evolution. Dysregulation of proportion is observed in a range of human diseases and growth disorders, indicating that proper scaling is an essential component of normal anatomy and physiology. However, when viewed through an evolutionary lens, shifts in the regulation of relative proportion are one of the most striking sources of morphological diversity among organisms. To date, the mechanisms via which relative proportion is specified and maintained remain unclear. Through the application of powerful experimental, genetic and molecular approaches, the teleost fin has provided an effective model to investigate the regulation of scaling, size, and relative growth in vertebrate organisms.

This article is categorized under:

Establishment of Spatial and Temporal Patterns > Regulation of Size, Proportion, and Timing

Adult Stem Cells, Tissue Renewal, and Regeneration > Regeneration

Comparative Development and Evolution > Regulation of Organ Diversity

KEYWORDS

allometry, bioelectric signaling, proportion, zebrafish

1 | INTRODUCTION

Proportion is an essential component of form. Although our arms originate from two separate embryonic buds, each with their own innate instructions and environment, they reliably grow to almost precisely the same length (Wolpert, 2010). Such relative proportion in form has long been appreciated by artists, naturalists, and scientists. Beyond mere symmetry, the physical elements that comprise organisms often display recursive patterns that can be used to measure and explain proportions. The Greek sculptor Polykleitos used this principle of recursive pattern to formulate basic principles of proportion, in which the distal phalanges were a fundamental unit upon which to measure and compare all other anatomical structures (Tobin, 1975). By multiplying the distal phalanges by $\sqrt{2}$ (1.4142), the length of the medial phalanges could be derived; continued multiplication of the medial phalanges by $\sqrt{2}$ would then determine the size of the proximal phalanges, and so forth (Figure 1a). Hundreds of years later, Renaissance artists adopted the notion of inherent proportionality as a metric for beauty in human form as well as in architecture, and further extended this principle to detect universal constants of proportion across nature (Livio, 2008). Even today, artists learn to capture variations in form by employing proportional diagrams of the face and body, such as those popularized by Dürer (Figure 1b; Dürer, 1528; Pollio, 1914).

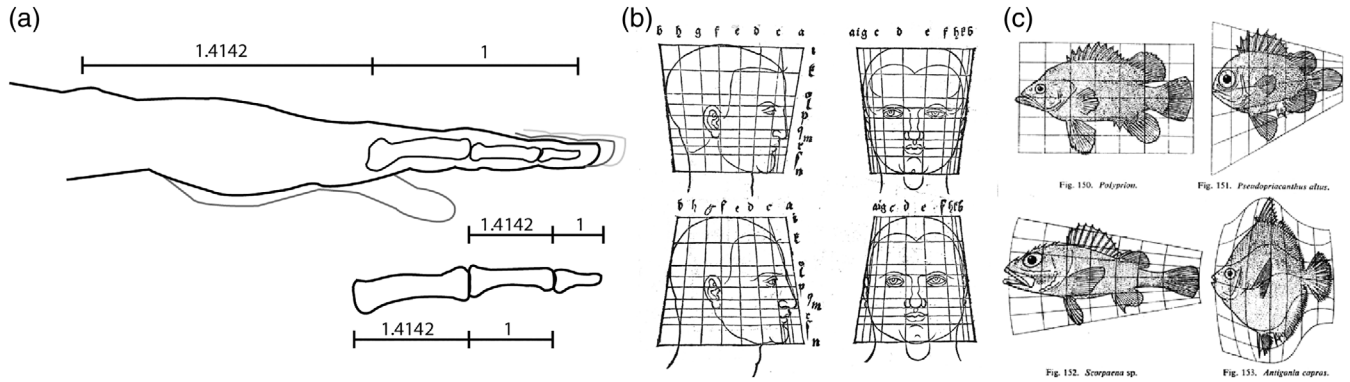

FIGURE 1 Perceptions of proportion. (a) Drawing reprinted with permission from Tobin (1975) illustrating search for mathematical underpinnings of "ideal" proportions by artists such as Polykleitos. (b) Like ancient cultures before him, Dürer used a grid system to reliably capture adult form. He then warped this grid to illustrate shifts in morphology (Dürer, 1528). (c) Inspired by Dürer, D'Arcy Thompson used grid transformations to model relative growth as means for evolutionary variation (Thompson, 1917)

2 | PROBLEMS OF RELATIVE PROPORTION

Inspired by the grid systems used by Dürer, D'Arcy Thompson utilized a coordinate system to map and compare organismal shape (Figure 1c). Simple changes to this grid, such as altering axis proportions, could depict shifts in morphology between species (Thompson, 1917). These geometric transformations illustrate that even complex morphological distinctions between species can be modeled as a simple shift in the relative proportions of ancestrally shared structures. Similar to Thompson's description of changing proportions by mathematical grid transformations, many groups in the late 19th and early 20th centuries attempted to identify biological laws of growth to describe shifts in proportion (Gayon, 2000). These early growth studies were synthesized by Huxley and Teissier with an exponential equation, $y = bx^{\alpha}$, that can be used to characterize the relationships between structures of size y and x (Gayon, 2000; Huxley & Teissier, 1936). This deceptively simple equation permitted complex nonlinear growth of structures or organisms to be compared in a scale-independent manner using log plots, $\log(y) = \alpha(\log(x) + \log(b)$. Shifts in proportionality both during ontogeny and between species can be caused by changes to either the slope or the intercept of this equation (α or b; Newell, 1949). Although mathematically straightforward, the genetic basis of these variables and what developmental processes they represent is not understood.

Deciphering how proportions are established during development and shaped during evolution requires knowledge not only of the determinants of growth rate, but also, of how and why organ and appendage growth ceases at particular sizes. Ultimately, organ and appendage size reflects a combination of cell volume, cell number, and the matrix produced to surround the cells, such as the ossified matrix of bones. How all of these variables interact to reliably produce appropriately sized organs remains a fundamental biological question.

3 | THE ZEBRAFISH AS A MODEL TO ASSESS GROWTH AND FORM

Teleost fishes comprise over 30,000 species and represent approximately half of all vertebrate species (Nelson, 2006). Despite our lack of resemblance, humans are evolutionary descendants of the bony fishes, and thus share common developmental and genetic foundations. Among teleost species, the zebrafish (*Danio rerio*) provides a tractable model for investigation of the genetic and developmental specification of relative proportion. The fins of wild-type zebrafish are extremely regular in their patterning and relative proportions, supporting detailed and reproducible analysis of cell behavior, development and gene function. In addition, the planar structure of fins essentially limits their growth to two dimensions, while their external location places no constraint on outward growth. Thus, alterations in fin proportion can easily be detected and assessed in the living organism, in contrast to internal organs or complex structures such as the skull.

The zebrafish fin itself has a predictable anatomy that has been well-described. Each fin is formed from a series of fin rays comprised of serially partitioned segments of intramembranous bone. Each fin ray segment is composed of two semi-cylindrical hemisegments that surround internal neural, vascular and mesenchymal tissue (Figure 2). Individual bony fin ray segments are regularly spaced and are added sequentially along the proximodistal fin axis during

development, rather than prefigured. Growth of the fin skeleton occurs primarily at the distal fin tip, where dynamic signaling maintains active growth. Similar to arms and legs, fins have a spatially patterned neurovascular network that is organized along each fin ray (Figure 2); as such, each fin ray is a discrete segmental unit, or module, of the fin. As the fin grows, individual rays bifurcate at stereotypical positions along the proximodistal axis and allow for fin widening. These bifurcations are clear anatomical markers of position within the fin, and are hardwired such that their position and overall pattern across the fin reform during regeneration after injury.

In addition to skeletal patterning, fin growth during development and regeneration is also exceptionally regular and reproducible across wild-type zebrafish populations. During juvenile development, fins undergo a period of increased growth relative to body size (allometric growth), followed by a transition to isometric growth in adults in which fin length is maintained relative to overall body size (Parichy, Elizondo, Mills, Gordon, & Engeszer, 2009). The isometric fin growth and growth in total body size continues indefinitely until the end of the lifespan. Given this predictability, zebrafish with alterations in fin patterning and form can be readily identified and isolated either from natural variants or from laboratory-induced mutations. Regardless of their origin, such variant morphologies provide a unique tool to uncover the genetic regulation of pattern and proportion. Although zebrafish have several fins that differ somewhat in how they form, this review will focus on the shared components of patterning that mediate size determination and relative proportion.

4 | ON CATCH-UP GROWTH: REGENERATION AS AN EXTENSION OF DEVELOPMENT AND A WINDOW INTO HOW SIZE IS ENCODED

A key attribute of the teleost fin is its ability to regenerate after injury. Upon amputation, adult zebrafish fins will faithfully grow back to their pre-amputation size (Figure 3). Thus, the regenerating fin provides a unique window into the regulation of size and proportion, which can be leveraged experimentally to ask how proportional information is stored and interpreted. Interestingly, the restoration of fins to their pre-cut size transpires perfectly even at adult stages, when the dynamic signaling of early development has subsided and any gradients establishing patterns have presumably long since dissipated. Because correct scaling of fin regrowth is maintained in adults, even as their overall size continues to increase and in the absence of any developmental morphogens from earlier stages of development of the fins, the tissue remaining in the adult fin after injury must be capable of dynamically integrating innate determinants of proportion with the overall size of the fish. This property implies that there is an intrinsic set point of size that is encoded within fin tissue.

A concept of a "target size" for growth was proposed in analysis of human growth by Tanner (1963). During development, infants and children who have experienced stress, nutrient deprivation, sickness, or injury may show marked growth deficiencies. However, upon improved conditions, their growth rates will rapidly increase. This catch up in growth rate implies compensatory mechanisms and/or knowledge of scaling that modulates relative proportions (Boersma & Wit, 1997; Prader, Tanner, & von Harnack, 1963; Tanner, 1963). An analogous process is observed in regenerating zebrafish fins, in which compensatory growth occurs in proportion to the extent of the deficiency (Lee, Grill, Sanchez, Murphy-Ryan, & Poss, 2005). Foundational studies of Broussonet (1789) and Morgan (1902) noted the propensity of teleost fins to regenerate at greater speeds when cuts were made closer to the fin base. Using cuts at different

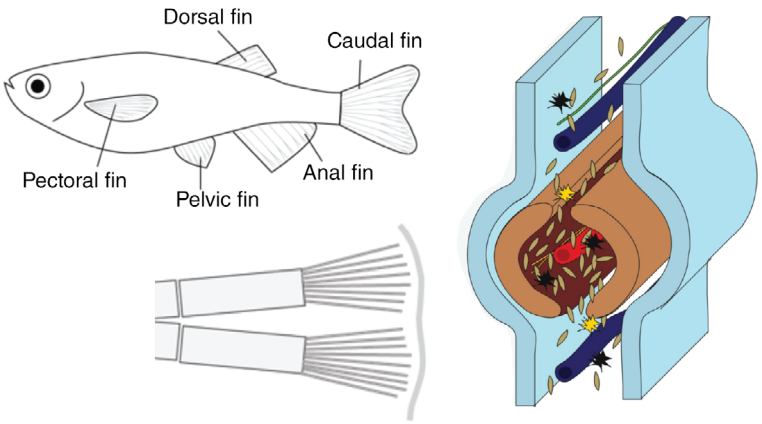

FIGURE 2 Anatomy of a fin. *Left*, Adult zebrafish outward morphology showing position and size of paired and medial fins. Placement and general size of fins are consistent among zebrafish and show little individual variability. Fin rays are comprised of smaller segmented skeletal units formed by intramembraneous ossification. These skeletal units can branch and have filaments extending along length of each ray, actinotrichia, that project outward from the distal-most segments. Reprinted with permission from Daane et al., 2018. *Right*, Schematic of anatomical complexity of a fin ray cross-section (Reprinted with permission from Perathoner, 2012)

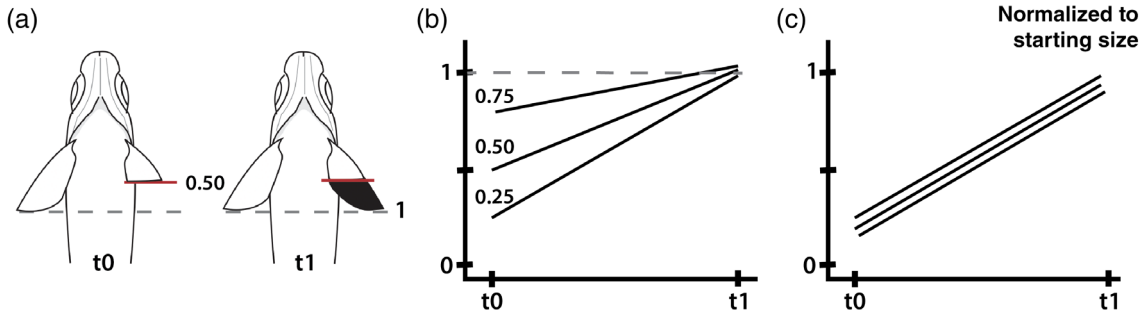

FIGURE 3 Fitting to size: Regeneration and growth rate modeling. (a) Schematic of an idealized fin regeneration experiment using the pectoral fin with an unamputated contralateral fin serving as a size control. Similar experiments performed on caudal fin lobes show comparable data. At time 0, one fin is amputated a set distance along the fin proximodistal axis. Regeneration leads to growth of the appendage until it reaches its starting size (t1); Reprinted with permission from Daane et al. (2018). (b) Schematic of results of classic experiments showing that more proximal cuts have a faster growth rate during regeneration than more distal cuts. The end result is that all fins generally finish regeneration in a comparable time frame. (c) Normalization of growth response relative to starting size of the fin shows that the rate of fin regeneration is scaled by the extent of the remaining fin fragment

positions along the proximodistal axis, Morgan was able to show that the position of the injury affected the rate of growth. Fins cut at 75% of their normal size will grow more slowly than fins cut at 50% of their normal size, the end result being that regardless of the position of the cut, regenerating fins are all restored to their initial size at a comparable time post-injury (Broussonet, 1789; Figure 3). The phenomenon of catch-up growth in the zebrafish fin implies that fin tissue not only retains intrinsic information of position and size, but that the growth response is graded depending on the extent of the difference between "expected" size and detected size (Figure 3). Again, it is important to note that this scaling of growth rate is determined in adult tissues, when the systemic signals of development are no longer present.

5 | GENETIC DETERMINATION OF FIN SIZE

Through forward mutagenesis screens in the zebrafish, mutant strains have been isolated that show distinct, reproducible variations in fin size but retain relatively normal patterning (Figure 4; Table 1). Both short as well as long-finned mutants have been identified (Box 1). Of the genes identified thus far that underlie these scaling phenotypes, regulation of bioelectric signaling is common among the function of their encoded proteins. Mis-regulation of ionic conductance and potential signaling associated with dominant alterations in potassium channel function (*another longfin/kcnk5b (alf)*, *schleier/kcc4a (schl)*) lead to overgrowth phenotypes (Lanni et al., 2019; Perathoner et al., 2014), whereas loss-of-function or dominant-negative mutations in *connexin 43* gap junctional proteins, which mediate ionic connectivity of cells, leads to short fins (Iovine et al., 2005). Importantly, although having different length segments, these fins retain the segmentation and branching patterns seen in wild-type fins despite their different sizes (Figure 4).

Like the *kcnk5b/alf* and *kcc4a/schl* mutants, the *longfin* and *rapunzel* mutants have a long-finned phenotype. However, unlike the first class of mutants, *longfin* and *rapunzel* fins have comparable length of fin ray segments as seen in wild-type fish, albeit more (Goldsmith, Fisher, Waterman, & Johnson, 2003; Iovine & Johnson, 2000). This observation suggests that different mechanisms may be altered among the long-finned mutants. Much work has gone into characterization of the *longfin* mutant by several research groups, and identification of the mutant allele will be insightful in detailing genetic regulation of scaling during development. Although the gene underlying the *rapunzel* mutant phenotype has been cloned, the function of the *Rapunzel* protein in size regulation remains enigmatic (Green et al., 2009). *Rapunzel* is a teleost-specific gene with little structural or functional evidence to integrate it with known pathways of fin development. As Rapunzel mutants have broad skeletal overgrowth phenotypes which are not observed in the other long-finned mutants, the *Rapunzel* protein may act in a more pleiotropic role in growth regulation.

One advantage of the zebrafish system is the ability to assay gene function locally in the fin. This can be done transiently through integration of specific-expression plasmids, or through clonal analysis of injected embryos. This system enables potential dissection of cell type- and allele-specific analysis in injected animals. Through these techniques, it was determined that the genes identified in the mutagenesis screens as affecting fin size act locally within the fins to

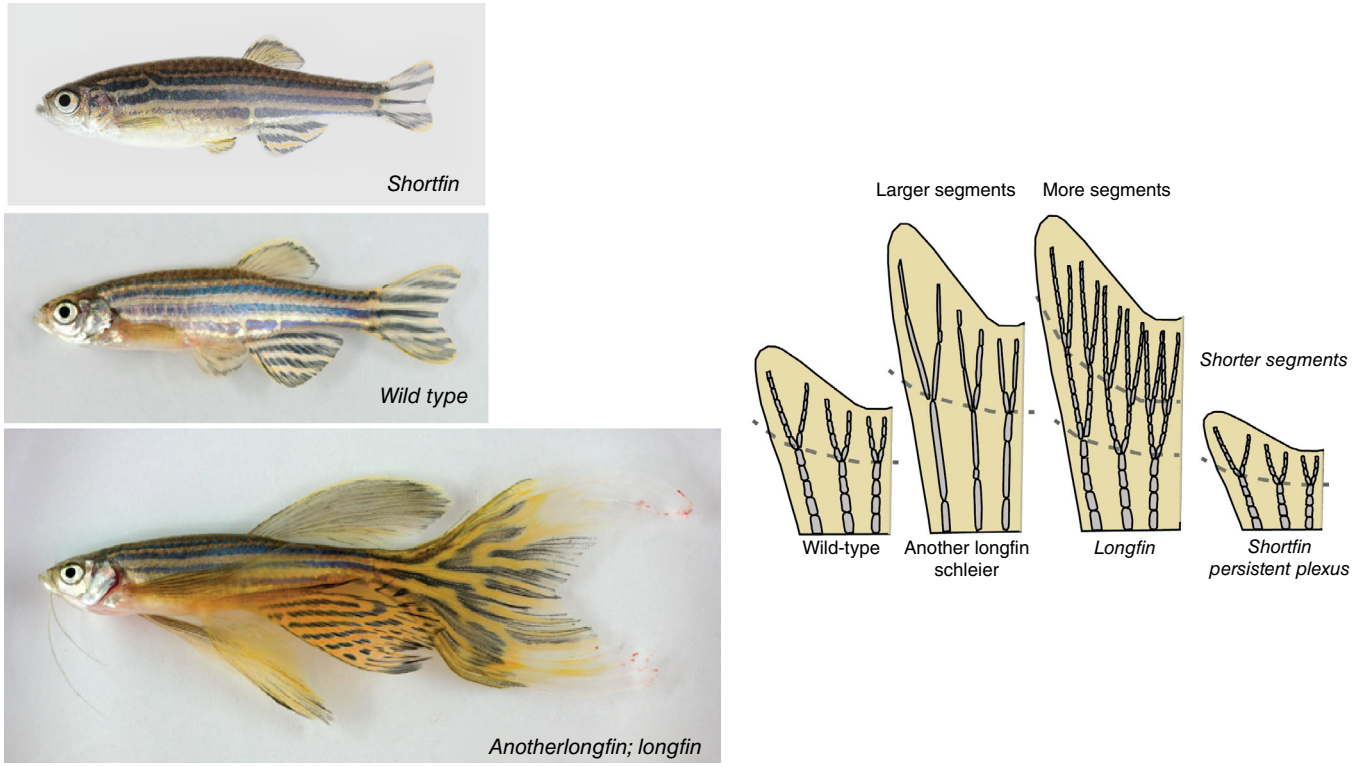

FIGURE 4 Outward scaling phenotypes in the adult zebrafish. *Left*, Zebrafish mutants showing changes in fin size due to mutation. *Right*, Two classes of fin mutants dissociate fin size from segment patterning. One class of mutants affects genes that regulate growth rate (*another longfin* (*alf*), *schleier* (*schl*), and *shortfin* (*sof*)) but maintain normal scaling, whereas another class exhibits relatively normal rates of growth but a greater number of skeletal elements (*longfin* (*lof*)); in the latter case, the mechanism of scaling has been lost or bypassed. Note presence of extended length in barbules in long-finned mutants shown—barbule overgrowth is seen in the class of mutants that show altered growth rate (*alf*, *schl*) but not in *lof* mutants suggesting different mechanisms regulating size between the mutants and between structures

TABLE 1 Zebrafish mutants with altered relative proportion of fins and barbels

Mutant and class	Gene	Encoding	References
Long-finned			
another longfin	*kcnk5b*	Potassium channel	Perathoner et al. (2014), van Eeden et al., (1996))
longfin	*ND*		van Eeden et al. (1996)
schleier	*slc12a7a*	Potassium chloride cotransporter	Lanni et al. (2019)
hiD862	*ND*		Amsterdam et al. (1999)
rapunzel	*rap*	Rapunzel	Green, Taylor, Hindes, Johnson, and Goldsmith (2009)
Short-finned			
shortfin	*cx43*	Gap junction	Iovine, Higgins, Hindes, Coblitz, and Johnson (2005)
persistent plexus	*col9a1*	Collagen	Huang et al. (2009)
mau	*aquaporin 3a*	Aquaporin	Eskova et al. (2017)

regulate size and proportion (*kcnk5b/alf* (Perathoner et al., 2014; Sims Jr., Eble, & Iovine, 2009) and *cx43/sof* (Sims Jr. et al., 2009). In the case of the long-finned mutant *alf/kcnk5b*, local overexpression of either mutant (hypermorphic) or wild-type *kcnk5b* causes local fin overgrowth, suggesting that increased dosage of the protein alone is sufficient to drive growth. Additionally, it was demonstrated that conductance through the potassium channel is necessary for the overgrowth phenotype, supporting a specific role for bioelectricity over other potential aspects of potassium channel function (Perathoner et al., 2014).

BOX 1 BIG VERSUS SMALL IN FINDING MECHANISMS OF SCALING

Change in relative proportions can arise from many causes, some of which directly relate to regulation of proportion and some only peripherally so. In particular, structures with decreased size can arise as a byproduct of general disruption in cellular behavior such as capacity to progress through cell cycle or metabolic/physiological deficiencies leading to inability for growth. Changes that affect the ability of structures to form can result in smaller, yet properly patterned structures. Similarly, changes that cause decreased scaling may reflect overall developmental deficiency, rather than identifying genes that regulate scaling *per se*. In contrast, creation of structures with increased size as a result of mutation, selection, or gene perturbation requires the activation and coordinated patterning of scaling programs. As a result, these supersized structures may be informative in uncovering core processes of how scaling and size are encoded.

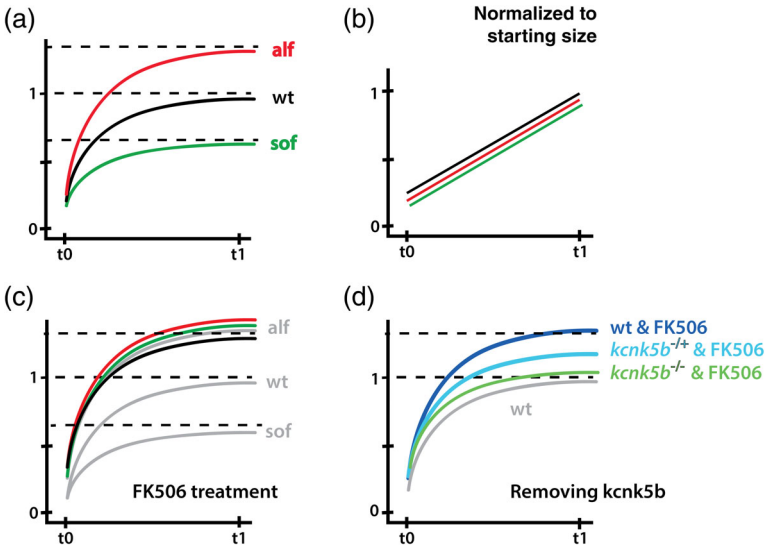

FIGURE 5 Genetic regulation and growth rate. (a) Regeneration profiles of zebrafish long- and short-finned mutants recapitulate growth properties seen in development. (b) However, upon normalization to starting fin size, the regenerative response observed in the scaling mutants is comparable to that of wild-type fish, with amputated fins completing regrowth in the same time frame irrespective of their target length. (c) Treatment of regenerating fins (gray lines) with the drug FK506 (colored as in a) leads to overgrowth of all genotypes similar to that seen in the *alf/kcnk5b* mutant. (d) Deletion of *kcnk5b* leads to loss of FK506-mediated overgrowth. Reprinted with permission from Daane et al. (2018)

Studies on long- and short-finned mutants begin to reveal some of the underlying principles of proportional growth. When *kcnk5b/alf* and *cx43/sof* fins are measured during development and regeneration, they exhibit faster or slower growth rates that ultimately result in changes in segment and fin length (Figures 4 and 5). Interestingly, these mutants still retain the property of catch-up growth observed in wild-type fins, but scaled to reflect their new longer or shorter target fin size (Figure 5). It is unknown if this expression of growth rate differences is retained due to local effects of the genes, or from a more systemic integration of position with growth rate as seen in wild-type fins regeneration. The latter model would suggest a highly robust mechanism by which organs sense their "target size", such that mutations that reset fin size during development automatically adjust the positional regulatory apparatus that guides relative positional growth rate during regeneration. When and how this "target size" is established during development and remembered during regeneration remains poorly understood.

6 | GENETIC MECHANISMS OF SCALING

Evidence indicates that the regulation of scaling in vertebrates is multifaceted, with systemic hormonal regulation as well as local positional information contributing to coordinated growth within and among structures during development. Several different hormones have been associated with regional scaling and allometry, including growth hormone (GH), insulin-like growth factors (IGFs), and thyroid hormone (T3/T4). Growth hormone is secreted by the pituitary gland and subsequently activates production of insulin-like growth factors in other organs. Defects in growth hormone can lead to dwarfism (Eicher & Beamer, 1976; Rimoin, Merimee, & McKusick, 1966), while over-activity of GH can lead

to acromegaly and gigantism in mammals (Lawrence, Goldfine, & Kirsteins, 1970; Palmiter, Norstedt, Gelinas, Hammer, & Brinster, 1983). In endochondral bones, IGF-1 stimulates chondrocyte proliferation and volumetric expansion within epiphyseal growth plates (Guntur & Rosen, 2013). IGF-1 defects can lead to stunted growth in humans and is a locus of major effect associated with body size-reduction in small dog breeds (Sutter et al., 2007). Further, thyroid hormone can also influence organ growth. Excess thyroid hormone (thyrotoxicosis) will lead to increased skeletal growth prior to premature fusion of epiphyseal growth plates (Williams, 2013), while thyroid deficiency is associated with stunted growth (Savoie, 1988). Differential responses of tissues to the same extrinsic factor are likely important in establishing allometric growth relationships. However, despite the clear importance of these extrinsic signals in regulating growth, extensive evidence also exists for the presence of organ-intrinsic signals in regulation of size. Recent analysis in the zebrafish has uncovered a broad role of vitamin D in promoting cell proliferation wherein activation of Vitamin D signaling lead to overgrowth of liver and heart in a local tissue-specific manner (Han et al., 2019). These data support the existence of core organ autonomous signaling factors in regulation of organ size. However, it is unclear what specific role Vitamin D has in regulation of size and patterning over its role in driving cell proliferation leading to hyperplasia and an increase in mass. Interestingly, the genes identified thus far in the zebrafish fin do not appear to affect regulation of systemic hormonal factors, but are sufficient to regulate fin scaling locally.

A recent insight into the mechanisms of fin scaling stemmed from the surprising finding that treatment of zebrafish with the immunophilin FK506 leads to substantive fin overgrowth during both development and regeneration (Kujawski et al., 2014). The resultant elongated fins closely phenocopy those seen in *alf/kcnk5b* or *schl/kcc4a* mutants, which have dominant alterations in potassium channel signaling (Lanni et al., 2019; Perathoner et al., 2014). Although FK506 is known to have many functions, its effect on fin growth appears to be mediated via inhibition of calcineurin and subsequent modulation of calcineurin-dependent signaling pathways (Kujawski et al., 2014). Remarkably, if regenerating fins of different fin-length mutants are treated with FK506, all fins show overgrowth and obtain a final size comparable to that of the *alf/kcnk5b* long-finned mutant, regardless of their starting size or genotype (Figure 5c; Daane et al., 2018). Even *sof/cx43* mutant fins show a greater than 100% increase in size. Thus, treatment with FK506 is sufficient to override intrinsic genetic cues of fin size and to cause fin overgrowth. However, when overgrown FK506-treated fins are amputated and allowed to regenerate in the absence of drug, they grow back to their pre-treatment sizes, indicating that FK506 exposure is not sufficient to alter the positional memory of target fin size (Daane et al., 2018). Based on these results, one might argue that both FK506 treatment and the genes altered in these scaling mutants act by exerting a dominant effect on growth rate, rather than altering the inherent target size of the fin. This hypothesis is supported by the fact that loss of *kcnk5b* or *kcc4*a function leads to wild-type fin phenotypes with no alteration in normal scaling (Lanni et al., 2019; Perathoner et al., 2014). However, further work indicates that this binary division between growth rate and scaling factors may be an overly simplistic model.

The complex nature of intrinsic size regulation becomes apparent when multiple chemical and/or genetic variables affecting fin length are manipulated in tandem. These data point towards a system of integrated bioelectric signals that act in concert to mediate fin size, with the potassium channel Kcnk5b potentially serving as a critical node in this network. Although loss of *kcnk5b* in a normal context does not affect fin size, *kcnk5b−/−* fins that are treated with FK506 fail to undergo overgrowth (Daane et al., 2018; Figure 5). Similarly, Kcnk5b function is a necessary component of the fin overgrowth caused by the *schleier* mutation in Kcc4a (Lanni et al., 2019). These data suggest that Kcnk5b is a common regulator necessary for determining growth of the fin and establishment of size. As Kcc4a and Knck5b both can regulate resting membrane potential (*Vmem*), it is likely that the functions of these two genes are associated, with an additive effect on growth that is manifested as shifts in *Vmem* (Figure 6).

Preliminary data have begun to elucidate how the function of these ion channels may be linked to the extensive canonical intracellular signaling pathways that regulate growth in response to environmental stimuli. Calcineurin may provide a molecular toehold by which to probe these connections, as it appears to bind and regulate at least two different potassium channels. Work by Czirjak, Toth, and Enyedi (2004) presented evidence that calcineurin can bind to the cytoplasmic domain of the Kcnk18 channel (TRESK) and alter channel conductance (Czirjak et al., 2004); mutation of the binding sites or calcineurin inhibition removes this effect. The channel Kcnk5b also contains putative calcineurin binding sites within its cytoplasmic C-terminus, and mutation of these sites results in loss of conductance in cell-based assays (Daane et al., 2018). In addition to calcineurin, recent findings indicate a possible link between mTOR signaling and bioelectric regulation of resting membrane potential. Specifically, the proton pump v-ATPase was found to be required for formation of the blastema during zebrafish caudal fin regeneration (Takayama et al., 2018). These analyses define an unexpected role of bioelectric signaling in integrating growth signals such that coordinated development and scaling of structures arise (McLaughlin & Levin, 2018).

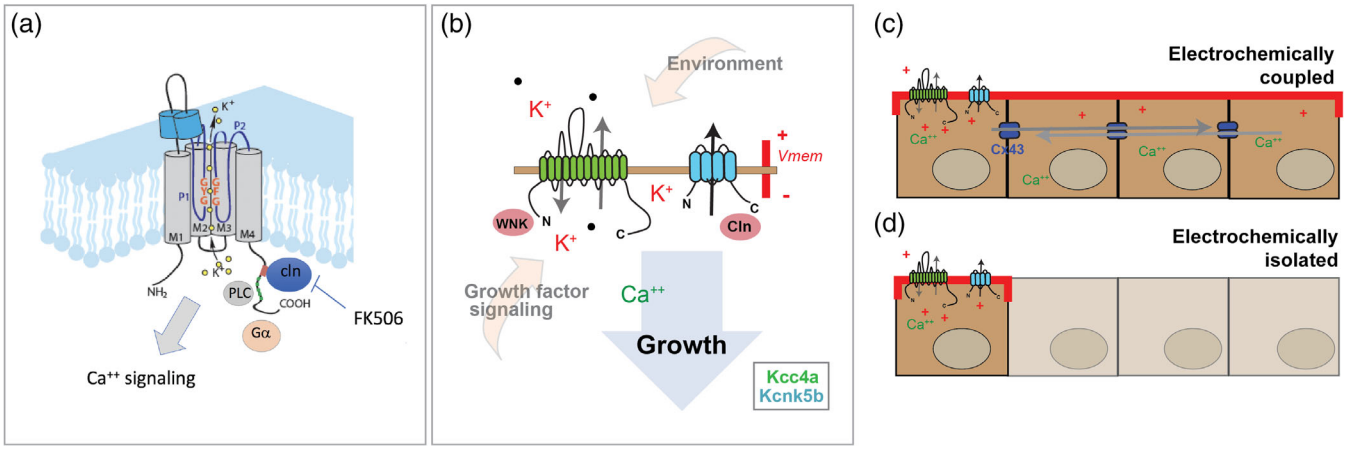

FIGURE 6 Bioelectric algebra as a means for coordinated development and scaling of zebrafish fins. (a) Modulation of *kcnk5b* signaling through conductance and secondary modulation of C-terminus. Cln, calcineurin; Ga, small g-coupled proteins; PLC, Phospholipase C. Reprinted with permission from Perathoner et al. (2014). (b) Additive function of potassium channel function to regulate resting membrane potential (*Vmem*). WNK, WNK kinases. Both *kcc4a* (*green*) and *kcnk5b* (*light blue*) modulate conductance in response to changes in pH, swelling, and other physiological conditions. Conductance can be modulated by internal growth signals, both small G-coupled signaling factors stemming from IGF signaling and Ca^{++} fluxes. Resting membrane potential can also be affected by action of v-ATPase and its regulation of mTOR activity and growth regulation (Takayama, Muto, & Kikuchi, 2018). Following, additive changes in *Vmem* may be a common readout for growth mediated by action of *kcnk5b*. (c, d) Changes in *Vmem* can cause changes in ion potential within and between cells. (c) Connexins electronically couple neighboring cells and allow for dissemination of small ions and molecules, thereby altering *Vmem* of juxtaposed cells. (d) If gap junctions are not present or impaired, such coupling will be limited causing more localized effect of growth and environmental signals (Hoptak-Solga, Klein, DeRosa, White, & Iovine, 2007). (b–d) Reprinted with permission from Lanni et al. (2019)

7 | MOLECULAR SIGNATURES OF POSITION IN THE ADULT ZEBRAFISH FIN

Much work has gone into detailing the gene repertoire across developing, regenerating and adult zebrafish fins with the goal of understanding how polarity is generated and how positional information is stored and recovered. Fins possess asymmetry along both their anterior–posterior and dorsal–ventral axes. Limited focus has been placed on identifying molecules that are differentially regulated between dorsal and ventral aspects of fins, in part because other than male-specific breeding tubercles on the pectoral fins, few morphological landmarks distinguish this axis (Kang, Nachtrab, & Poss, 2013). In contrast, at least two large-scale systematic molecular analyses have assessed changes in molecular expression along the anterior–posterior fin axis. Rabinowicz et al. used differential mRNAseq, proteomics and metabolic profiling in wild-type zebrafish and fin-length mutants to detail molecular differences between resting and regenerating caudal fins along the proximodistal axis (Rabinowitz et al., 2017; Figure 7). Their findings show distinct shifts in expression and metabolic signaling across the fin. In a similar vein, Nachtrab et al. (2013) detailed expression differences across the anterior to posterior axis of the pectoral fins. They identified *alx4* and *dhand* as two regionally expressed factors that are sufficient to change the character and growth of fin rays. In addition to analyses of coding sequences and their respective proteins, other research has investigated the role of noncoding RNAs in fin development and regeneration. Three studies have detailed a role for microRNA asymmetry in resting and regenerating caudal fins and have found that like coding transcripts, specific miRNAs are differentially represented and can affect regeneration (King & Yin, 2016; Thatcher, Paydar, Anderson, & Patton, 2008; Yin et al., 2008). Comparable analysis of long noncoding RNAs in the fin has not been performed. However, recent findings characterizing the role of noncoding RNAs in regulation across species and in the zebrafish heart provides important context for future work in this area (King et al., 2018). Analysis of these complex datasets of molecular signals across organs and species is facilitated by common repositories www.zfregeneration.org (Nieto-Arellano & Sanchez-Iranzo, 2019).

Far less is known about the importance of epigenetic regulation in fin patterning and regeneration and whether regional differences in epigenetic signatures can be detected. Changes in methylation are an attractive focus of study for understanding positional memory in fins and how it is encoded. Upon fin amputation, a general reduction in

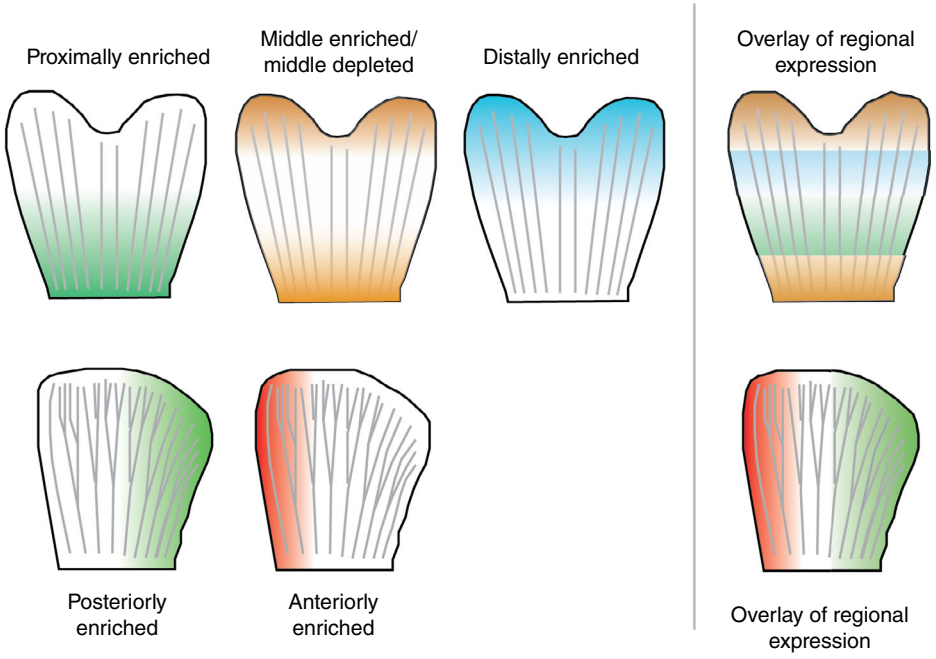

FIGURE 7 Position writ large. Expression profiling of fins reveal distinct PD (caudal fin) and AP (pectoral fin) specification of fin rays. Reprinted with permission from Rabinowitz et al. (2017) and Nachtrab, Kikuchi, Tornini, and Poss (2013)

methylation has been documented, and methylation signatures continue to be altered during the first 3 days of regeneration (Hirose, Shimoda, & Kikuchi, 2013; Stewart, Tsun, & Izpisua Belmonte, 2009; Takayama, Shimoda, Takanaga, Hozumi, & Kikuchi, 2014). The role of these epigenetic modifiers in adult fin regeneration and restoration of positional identity has yet to be systematically resolved. While these findings indicate a broad role for methylation in gene expression during regenerative growth, the specific genes whose expression is altered by changes in methylation status remain unknown. Thus, integrating the specificity of the changes into a comprehensive model of positional memory must await further research.

8 | WHAT ARE THE ANATOMICAL CUES INFORMING SIZE AND SCALING?

Many experiments suggest that growth regulation incorporates whole body size, interaction among specific tissues, and inherent positional identity to establish and maintain relative proportion. However, to achieve and maintain size, cellular growth within organs and tissues must be coordinated among thousands of cells over areas substantially larger than the individual subunits. One common correlate to changes in organ size can be the size and volume of cells. For example, the swelling and increased volume displayed by chondrocytes during development drastically affects the overall size and subsequent length of long bones. Increased cell size can provide a basis for transient increases in organ size, such as seasonal alterations in muscle and liver mass (Bonda-Ostaszewska & Włostowski, 2015), and increased pancreatic and liver mass during pregnancy (reviewed in Ginzberg, Kafri, & Kirschner, 2015). In contrast, the long fins observed in *alf/kcnk5b* mutant zebrafish and FK506 treated wild-type zebrafish are due to an increase in cell number rather than in cell size (Perathoner et al., 2014). Therefore, changes in both cell size and cell number are employed to modulate organ size depending on the specific biological context; however, in the teleost fin, alteration in cell number seems to predominate as a means for differential scaling.

The zebrafish fin provides an accessible model in which to experimentally approach this question. Within the fin, fin rays, segments, hemirays, and blastemas can each be transplanted to heterotopic positions or ablated, and assessed for their effect on positional integration and/or memory of size during regeneration, (for example, Broussonet, 1789; Murciano et al., 2007; Nabrit, 1921; Shibata, Liu, Kawasaki, Sakai, & Kawakami, 2018). Through analysis of zebrafish mutants and mosaics, it is clear that positional information is retained within some tissues and that local cues are essential to its interpretation. Mesenchymal clones, in particular, possess sufficient information to cause overgrowth upon transplant (Perathoner et al., 2014).

Given its central role in the regenerative process, the blastema is a logical starting point for investigation into proportional memory. Tornini et al. (2016) present evidence of potential prepattern of cells in a blastema, suggesting that positional cues are orchestrated from the blastema. These positional cues are consistently reset upon amputation, even following repeated amputation of the same fin. Interestingly, when fin blastemas are transplanted from different proximal positions to a common proximodistal position, the resulting outgrowths are all similar in size, with blastemal donor cells contributing to tissue spanning the proximodistal axis of the regenerate, regardless of their original position (Figure 8; Shibata et al., 2018). Taken together, these experiments suggest that positional information within the blastema can be respecified during regeneration in response to the local environment. Through use of a temperature-sensitive mutant in *pola2* that affects DNA synthesis, recent work by Wang et al. (2019) shows that transient diminution of cell proliferation during formation of the regeneration blastema is sufficient to reduce the size of the blastema, ultimately leading to a shortened fin regenerate. Intriguingly, this perturbation in the blastema is sufficient to cause long-lasting changes in interpretation of scaling of the fin, such that the shortened fins that were re-cut in permissive temperatures with wild-type *pola2* function regenerated to the reprogrammed shorter length. Importantly, this reprogrammed positional information is limited to the local tissue at the site of the original amputation and to a short temporal window when blastema size is established. While the molecular basis of this re-interpretation of scaling and the direct role of *pola2* in this regulation remains enigmatic, these data support the plasticity of scaling through the quality/extent of blastemal formation.

It is tempting to equate segmental patterning with overall specification of size; however, data indicate that skeletal patterning and fin size regulation are distinct processes. For instance, *Evx1* mutant zebrafish lack dermoskeletal joints between fin ray segments and have fins of normal length (Schulte, Allen, England, Juarez-Morales, & Lewis, 2011), while the *longfin* zebrafish mutant retains normal segmentation despite its long fins. Similarly, although segment size and number are variable within *alf/kcnk5b* fins, overall fin size does not reflect this variability (Murciano et al., 2007; Perathoner et al., 2014). These observations suggest that fin size can be genetically separated from segment patterning.

Meticulous experiments that employ transplant of fin rays, whole segments, and even hemiray segments have allowed the assessment of whether donor tissue can establish scale in the regenerate as well as the extent of donor tissue contribution (Birnie, 1947; Murciano et al., 2002; Murciano et al., 2007; Nabrit, 1921; Shibata et al., 2018). With genetically marked tissues, questions concerning contribution of donor tissues to the regenerate can be assessed (Shibata et al., 2018). Using these experimental tools, it has been found that transplanting fin rays with different

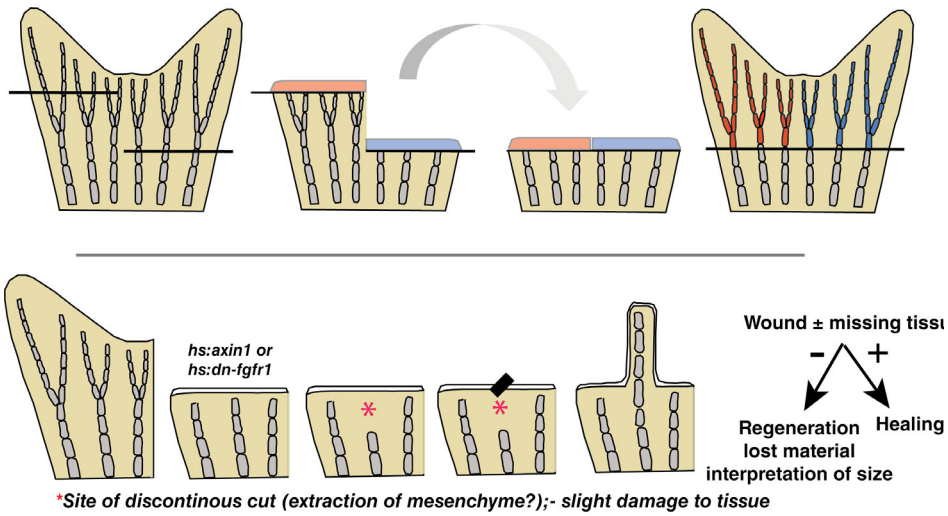

FIGURE 8 Experimental analysis of regeneration and evidence for disparity as a signal for size. *Top*, Blastema transplantations reveal lack of positional determination within blastemal cells. Transplantation of blastemas from different proximal–distal levels to a common proximal site leads to similar contribution and growth from transplants (After Shibata et al., 2018). *Bottom*, Regeneration in context of missing tissue. Fins that undergo amputation in the presence of Wnt or Fgf-signaling inhibitors form wound epidermis but do not initiate regeneration, even if inhibition is subsequently removed after wound healing. If a proximal fin segment is also removed at the time of amputation (*), wound healing (but not regeneration) will still occur, causing a local disparity in tissue architecture. If a superficial injury is applied to the distal epidermis once healing is complete (black bar), regeneration is initiated locally and lost skeletal material reforms. Reprinted with permission from Owlarn et al. (2017)

identities to new positions within a fin leads to a retention of donor size information that is consistent with an autonomous memory of size within fin elements (Birnie, 1947; Shibata et al., 2018). Similar findings were made by Murciano et al. (2002), who observed that if long donor fin segments were grafted to rays with shorter segments, the larger segments integrated their growth to the size of the surrounding elements, and vice versa (Murciano et al., 2002).

The fin hemiray segment provides a smaller physical unit that, like fin rays and segments, can also be removed, transplanted and assessed for retention of positional memory. Hemiray segments normally form in register with their segment pair, leading to the symmetrical joint formation observed in wild-type fin rays. When a single hemiray segment is removed from a fin, the remaining hemiray segment can be amputated and will undergo regeneration, even in the absence of its partner. Thus, a single hemiray stump retains regenerative capacity, although the extent of regeneration is somewhat impaired. Hemiray segments can also be transplanted and grafted to different proximodistal positions in the fin. Heterotypic grafts, in which the proximodistal origin of the graft differs from its position in the host tissue, exhibit growth behavior in accordance with the host tissue location (Murciano et al., 2007). More extensive lineage analysis needs to be done to determine contribution of the donor hemiray cells and effect on the resulting size of heterotypic grafts. Taken as a whole, the data from fin ray, fin segment, and hemiray segment transplantation experiments raise the question of whether positional fate is established not by the cell, but by broader levels of tissue organization such as segment or hemi-segment entities. It is interesting that in cases of altered fin proportion as seen in *alf/kcnk5b*, fin hemirays can form out of register with their complement such that contralateral hemirays are of different sizes (Murciano et al., 2007). Although the cause of this aberrant registration is unknown, altered bioelectric signaling in the *alf/kcnk5b* mutant may underlie the lack of coordination among neighboring segment tissues.

It has been long known that the regeneration of lost structures requires signals present in the ray stump; if rays are locally removed prior to amputation, fins that regenerate will lack these particular rays (Goss & Stagg, 1957; Murciano et al., 2007). Insight into the signals required for integration of positional information may come from similar experimental conditions, such as recent work by Owlarn et al. (2017) that combines suppression of regenerative growth with fin amputation. In this model, fins are subjected to conditional suppression of regenerative growth while undergoing amputation. These regeneration-inhibited fins undergo wound closure and healing, rather than reforming the missing fin (Figure 8). If the conditional inhibition is abated, then injury by either wounding or amputation leads to activation of a normal regenerative response. To test the importance of tissue context in governing the regenerative response, Owlarn et al. created a recessed amputation on a single fin ray within the amputation stump, thus creating a morphological disparity in the healed tissue. When this tissue is injured, it initiates epimorphic regeneration of distal components and reformation of the missing skeletal elements—A process that would not have occurred during normal regeneration in a fin ray with missing segments (Goss & Stagg, 1957). These results suggest that a disparity in signals within a structure may be a driving force in regulating not only position, as has been posited as a means for integration in regeneration, but also for the coordination of outgrowth and size/position within a fin. The molecular nature of such signals has yet to be identified, and may in fact be a broader character state rather than any particular factor. However, the findings of Owlarn et al. (2017) would suggest that to manifest, the establishment of intrinsic size or position during regeneration requires inductive signals that can be caused by wounding.

9 | SIGNALS ACTING TO LIMIT SIZE

It is apparent that driving fin growth through mutation or FK506 treatment can result in a fin larger than normal wild-type proportions. As such, it can be argued that wild-type fin scaling is actively maintained and does not simply reflect a limitation of growth potential. How, then, is fin growth subdued to grow relative to the overall and changing size of the maturing fish? Tantalizing evidence arises from work on the activity of small microRNAs and their potential role in suppression of fin regeneration. Both miR-133 and miR-202 are small regulatory RNAs that can bind and down-regulated gene transcripts in a targeted manner; both miRNAs were also found to be expressed in resting fins and repressed upon initiation of regeneration (Thatcher et al., 2008; Yin et al., 2008). Broad regulators of gene function such as miRNAs may therefore be a key regulatory mechanism responsible for dampening growth and constraining size. Intriguingly, Yin et al. (2008) identified putative miR-133 binding sites in both *kcnk5b* and *cx43*, both of which regulate fin proportion in zebrafish. The predicted regulation of these genes suggests that miR-133 may broadly mediate bioelectric growth-coordinating mechanisms within the fin and provides a roadmap for connecting classical developmental signaling pathways, such as fibroblast growth factor signaling, with bioelectric growth regulation. The protein phosphatase calcineurin has been posited as another molecule that may broadly limit organ size, based in part on the

observation that calcineurin inhibitors such as FK506 cause fin overgrowth (Kujawski et al., 2014; Tornini & Poss, 2014). Such a model is consistent with the known role of Kcnk5b in growth regulation, as *kcnk5b* is necessary for FK506-induced growth (Daane et al., 2018). Regardless of their identity, size-dependent dampeners of fin growth could determine scale by coordinating the decreased function of growth regulators (Figure 9). While this model does not specify how the calculation of size is mediated, it does suggest a possible mechanism for orchestrating the coordinated scaling and tempering of growth.

10 | CHALLENGES IN UNDERSTANDING FIN SCALING AND PROPORTION

Through the use of the zebrafish fin as an experimental platform, the field has made significant advances in understanding how size and proportion are regulated. However, several broad questions that remain to be addressed in our understanding of this phenomenon. Research has established that similar to vertebrate limbs, autonomous signals within the fin can specify size. This intrinsic property is imparted to modules of fin rays and even to individual fin ray segments. Remarkably, this scaling information is correctly reinterpreted during regeneration of adult fins, suggesting that specification of size does not require signals present during development and maturation. Given this evidence for intrinsic size specification, it will be of interest to determine the developmental stage at which tissues receive these instructions, and whether this information can ever be overwritten. If FK506 treatment is performed on regenerating adult tissues, the fins become proportionately larger through overgrowth. However, the memory of size in these fins is not changed following drug treatment, as when the overgrown fins are recut in the absence of the drug, they grow back to wild-type size (Daane et al., 2018). Existing zebrafish mutants with allometric changes in fin length cannot be used to determine the timing of when size is determined, as the genes identified in these mutants act in both developing and regenerating fins. However, conditional and inducible genetic approaches to differentially activate or suppress gene function during fin development and regeneration would provide experimental insight into this question (Box 2). Using such methods, one could ask whether a receptive developmental window exists in which transient increased *kcnk5b* or decreased *cx43* expression results in differential scaling of fin tissue, and if so, whether this new size is maintained when normal gene expression levels are restored.

Another outstanding question is the relevance of tissue composition and identity to overgrowth phenotypes. In zebrafish mutants, coordinated overgrowth is easily recognized by its easily visible effect on fins, barbels, and scales (Figure 2). Fins and barbels are not physically constrained in their growth and can alter their shape and size without obvious deleterious effects on viability. As a result, these classes of overgrowth phenotypes are easier to detect in screens, and therefore may pose a potential bias in the type of genetic mechanisms that have been identified thus far (Box 1). One bias may rest in the shared attributes of affected structures of these mutants. Fins, barbels, and scales have different tissue composition, with barbels possessing a chondrogenic core and fin segments and scales composed of intramembranous bone. However, all three of these structures share substantial regenerative capacity. Conceivably, the proportional changes

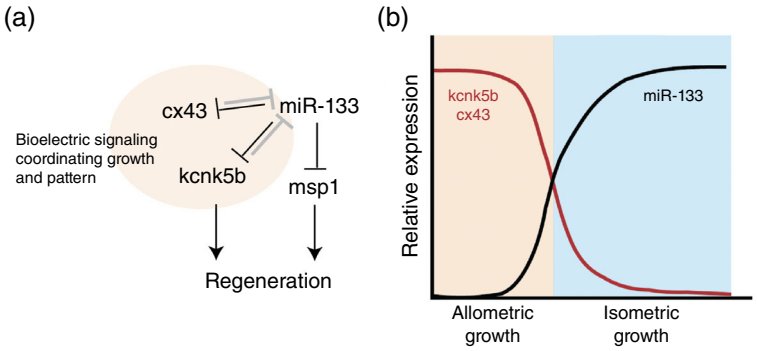

FIGURE 9 Keeping growth in check and means of size regulation. (a) miR-133 controls the progress of fin regeneration through regulation of *msp1* activity. Similarly, miR-133 also was found to regulate both *kcnk5b* and *cx43* in regenerating fins (Yin et al., 2008). These findings raise the hypothesis that size may be regulated by broad dampening of growth-promoting signals, including bioelectric signaling circuits that regulate proportion. (b) A prediction of the relationship between *kcnk5b/cx43* and *miR-133* in establishing allometric growth and maintaining stasis in organ size during development

BOX 2 GENETIC SCREENS FOR SCALING FACTORS

Existing scaling mutants have been identified by examining outward morphology of late juvenile and adult fish following chemical and insertional mutagenesis screens. This approach may bias the types of scaling genes that can be identified to those that (a) affect the fin and (b) support overall viability. Pleiotropy is a well-documented phenomenon, with many important genes reused during development and expressed in multiple organ systems. Thus, relying on detection of viable mutants in *experimental forward genetic screens* may limit the spectrum of genes that can be identified. Few, screens have been carried out to identify *conditional alleles*, or, to look for genes with localized effects, as would be detected in *clonal loss-of-function screens*. Another source of bias may derive from the nature of the genetic changes that can be detected using mutagenesis. Evolutionary variation often arises from changes in gene dosage as well as shifts in gene regulation. Such changes would be difficult to identify using mutagenesis, due to redundancy of gene copy number and enhancer mutational target size. In parallel to experimental mutagenesis, existing reservoirs of *natural phenotypic variation* provide a powerful means with which to reveal genetic mechanisms and explore altered scaling of organ size. Jointly leveraging these types of approaches may prove to be valuable in dissecting regulation of scaling in the fin.

in scaling seen in these structures could be linked to their regenerative capacity, and thus may not represent a universally applicable mechanism for size regulation in other organ systems that lack the ability to regenerate.

A curious finding in teleost fin regeneration is the essential requirement of the fin ray for regeneration of the adult structure: If amputations extend too far proximally into the endochondral base of the fin, regeneration does not proceed, for example, (Goss & Stagg, 1957). One possible explanation for the failure of ray-less fins to regenerate is that specific activities of fin rays are necessary for regeneration to proceed. In this model, the intrinsic morphology of the fin provides a physical foundation for its scaling and growth. Alternatively, the lack of regeneration seen in fins without rays could be due to a discrepancy in proximal positional cues. In this model of positional regulation, scaling is established by detection of graded molecular cues such as those within morphogen gradients (Rolland-Lagan, Paquette, Tweedle, & Akimenko, 2012). In the complete absence of such molecular cues, regeneration and restoration of scale and pattern cannot proceed. Further work using genetic and experimental perturbations in the fin will shed light on these conceptual views. It is evident that signals that regulate fin regeneration, such as mTOR, v-ATPase and miR133, also regulate growth and regeneration in other organs in the zebrafish and during regeneration in other animals (Adams, Masi, & Levin, 2007; Monteiro et al., 2014; Theis et al., 2017; Yin, Lepilina, Smith, & Poss, 2012; Yu et al., 2011). However, it remains unclear how these pathways integrate organ-specific scaling with that of general growth during development.

11 | CONCLUSION

The zebrafish fin provides a powerful model in which to understand how proportion is developmentally and genetically regulated. Through classical tissue extirpation and transplantation experiments and the use of genetically defined mutants with scaling deficiencies, the field has made significant progress. The ability of the fin to regenerate and to retain positional information provides a strong experimental platform for analysis. One critical limitation of this model system is that analysis is done on post-developmental tissue in which intrinsic scale has already been established. Limiting analysis of these positional cues to adult stages may be misleading of the primary signal(s) of how scaling is encoded during development. However, the teleost fin provides a key genetic and experimentally labile substrate for such discovery.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Matthew Harris: Conceptualization; funding acquisition; project administration; supervision; visualization; writing-original draft; writing-review and editing. **Jake Daane:** Validation; visualization; writing-review and editing. **Jennifer Lanni:** Investigation; writing-review and editing.

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