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Review

Staying in shape: Planarians as a model for understanding regenerative morphology

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ABSTRACT

A key requirement of tissue/organ regeneration is the ability to induce appropriate shape in situ. Regenerated structures need to be integrated with pre-existing ones, through the combined regulation of new tissue growth and the scaling of surrounding tissues. This requires a tightly coordinated control of individual cell functions such as proliferation and stem cell differentiation. While great strides have been made in elucidating cell growth and differentiation mechanisms, how overall shape is generated during regeneration remains unknown. This is because a significant gap remains in our understanding of how cell behaviors are coordinated at the level of tissues and organs. The highly regenerative planarian flatworm has emerged as an important model for defining and understanding regenerative shape mechanisms. This review provides an overview of the main processes known to regulate tissue and animal shape during planarian regeneration: adult stem cell regulation, the reestablishment of body axes, tissue remodeling in pre-existing structures, organ scaling and the maintenance of body proportion, and the bioelectrical regulation of animal morphology. In order for the field to move forward, it will be necessary to identify shape mutants as a means to uncover the molecular mechanisms that synchronize all these separate processes to produce the worm's final regenerative shape. This knowledge will also aid efforts to define the mechanisms that control the termination of regenerative processes.

Contents

1. Introduction

Planarians have fascinated scientists and non-scientists alike for hundreds of years due to their remarkable regenerative abilities. What could be more intriguing than regrowing a whole new animal from the tiniest of fragments? But these freshwater, non-parasitic flatworms are remarkable not merely because they can replace any and all tissues following injury, but because of the manner in which they do so. The tissue plasticity of planarians is astounding, allowing them to restore body shape almost regardless

Abbreviations: RNAi, RNA interference; hpa, hours post amputation; ASC, adult stem cell; PCG, position control gene; AP, anteroposterior; DV, dorsal-ventral; ML, medial-lateral; BMP, bone morphogenetic protein; dpa, days post amputation; FGF, fibroblast growth factor; CaV, voltage-gated calcium channel; ER, endoplasmic reticulum; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; GJC, gap junction communication; PCP, planar cell polarity.

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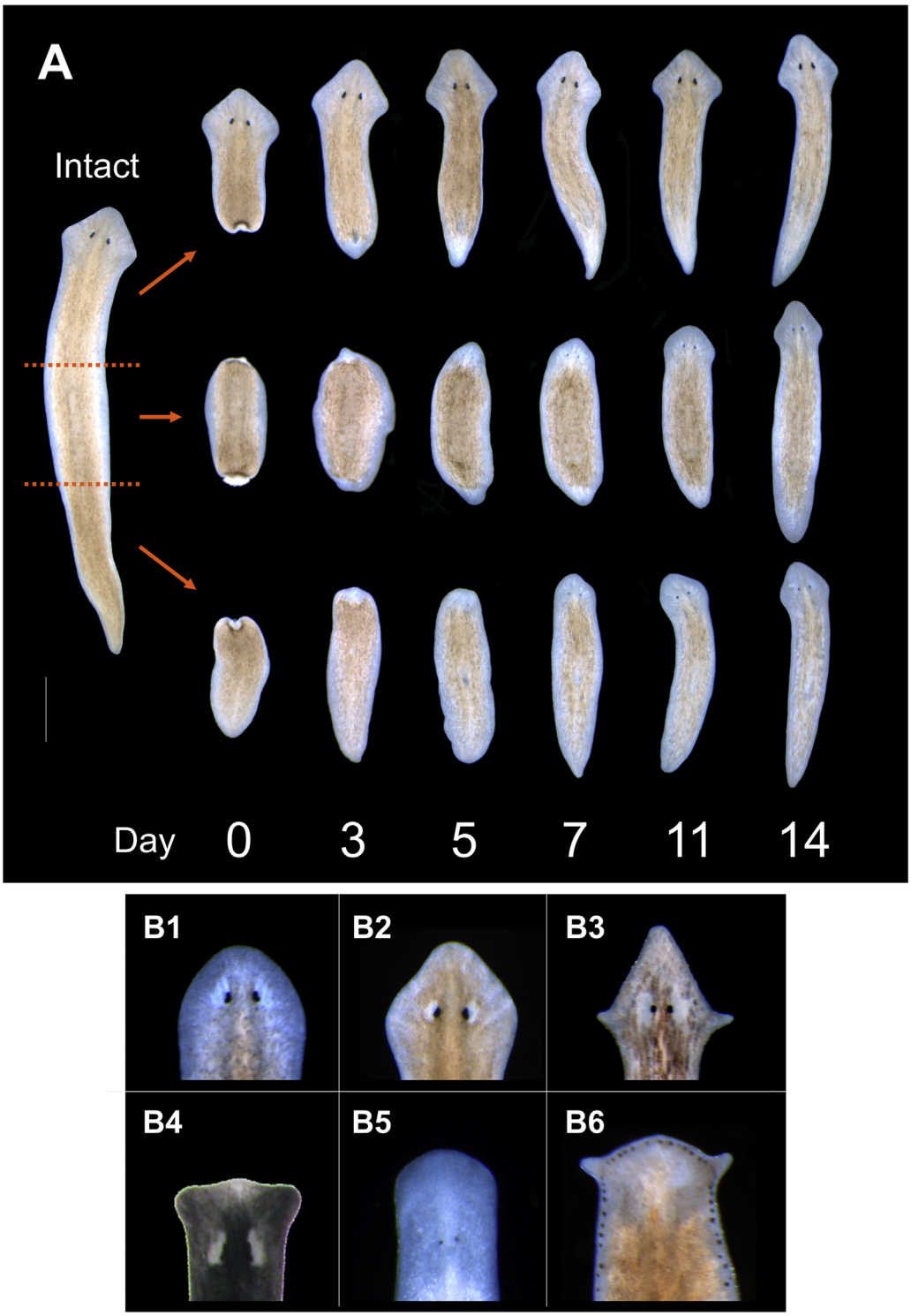

Fig. 1. Planarians as a Model for Regenerative Shape. **(A)** Composite image showing regeneration in a single *Dugesia japonica* worm over 14 days following amputation into head, trunk, and tail fragments. Anterior is up, dotted lines = amputation planes, scale bar = 1 mm. **(B)** Head morphologies of different planaria. **(B1)** *Schmidtea mediterranea*, **(B2)** *Dugesia japonica*, **(B3)** *Girardia dorotocephala*, **(B4)** *Phagocata gracilis*, **(B5)** *Phagocata morgani*, **(B6)** *Polycelis felina*.

of the type of injury, maintaining proper proportions even when the newly regenerated worm is significantly smaller than the original (Fig. 1A). This level of plasticity is important, allowing them to reproduce not only sexually but also asexually by means of transverse fissioning. When worms fission, they literally rip themselves into two, after which head fragments regenerate new tails and tail fragments regenerate new heads. Except the two resulting worms are now much smaller than the original, requiring the scaling of body parts to the new body size. Researchers have co-opted this remarkable ability in the laboratory to study regenerative mechanisms following injury, demonstrating that regeneration on the organismal scale requires not only new tissue production but also reorganization of pre-existing tissues to ensure correct size and proportion of the regenerated animal.

For this reason, planarians make an outstanding model for investigating the mechanisms of regenerative shape, defined here as the

establishment of normal form (or animal-wide morphology) during regrowth. In addition, there are hundreds of known species, many with morphologically distinct features (such as differing head shapes) that facilitate the understanding of organismal shape mechanisms (Fig. 1B). Along with a wealth of historical data, significant progress has been made in understanding the molecular and genetic mechanisms that underlie planarian regenerative capabilities. On a certain level almost all processes associated with regeneration could be considered part of shape establishment; the ultimate goal of regeneration is the restoration of functional tissues and organs, and shape is integral to function. Here we provide an overview of just those mechanisms that have been shown to be most critical for morphology during planarian regeneration. The reader is directed to the many excellent reviews that delve into each subject more thoroughly (see [1–8]). Furthermore, we provide a brief historical context of the study of regenerative shape in planaria, as well as highlight the most important future directions for this field.

2. Historical studies: questions without answers

Planarian regeneration has been studied for centuries (for review see [7]), and their ability to reform into the correct shape following a myriad of injury types was the focus of much of the early historical literature. Rooted in classical embryological methods, the earliest studies of planarians explored changes in shape following virtually every conceivable type of injury and after exposure to a wide range of chemicals, compounds, and environmental conditions. These studies were largely observational, but led to the discovery of many of the characteristics that make planarians a great model for regenerative shape. Harriet Randolph's work in the late 1890′s noted that the amount of new tissue (i.e. the blastema) that was regenerated in planarians was less than what was originally removed [9]. Furthermore, the amount of new tissue produced was proportional to the new smaller worm size (rather the larger original worm) leading to restoration of normal body proportions [9]. Around the same time, Thomas Hunt Morgan noted that planarian heads always regenerated from anterior facing wounds (and tails from posterior facing wounds), and that new pharynges arose not in new tissues but within the old pre-existing tissues [10]. Morgan termed this reorganization of pre-existing tissues "morphallaxis," and suggested it was the result of "an active migration of old tissue" [11,12]. Thus, the study of critical shape processes such as new tissue growth, axial polarity establishment, and tissue remodeling were the focus of planarian research from the beginning.

In his studies, Morgan described how the tiniest of fragments—1/100 or 1/279 of the original worm—were able to regenerate but interestingly not with the correct overall morphology [10]. A few years later (in 1909) Morgan's former graduate student Nettie Stevens concluded, after researching disruptions of regenerative shape (from different amputation schemes that gave rise to ectopic structures such as heads and pharynges), that "an unlimited amount of work on the readjustments in nerve cords and digestive tract" are required to regenerate a symmetrical worm [13]. These kinds of observations led researchers to postulate on the level and mechanisms of regulation that controlled morphogenesis during planarian regeneration.

Morgan hypothesized the existence of a "graded distribution of materials" that determined the polarity of tissues during regeneration, stating that "The phenomena of regeneration are, in part, the outcome of this gradation" [14]. Morgan would eventually discard this line of investigation. But Charles Manning Child built on these ideas to propose his now famous gradient theory for polarity establishment along the anterior-posterior (AP) axis during planarian regeneration [15]. Child's hypothesis was that a morphogenetic gradient (in this case regulated by metabolic factors rather than particles) controlled shape decisions in regenerating tissues [15]. However, subsequent decades failed to identify a definitive gradient. As stated in 1901 by another planarian researcher, Frank Lillie, the "phenomena of regeneration offer many problems, some of which not only appear insoluble in the present state of our knowledge, but actually offer no point of attack" [16]. The question of whether or not the gradient predicted by Morgan and Child regulated planarian shape would be left to modern researchers.

3. Modern era: new "points of attack"

Molecular and genomic advancements have significantly improved our understanding of planarian regenerative processes, uncovering mechanistic explanations for many historical observations. The modern planarian toolkit includes the sequenced genome of the species *Schmidtea mediterranea* [17–19], RNA interference (RNAi) techniques [20,21], and in situ hybridization and immunohistochemistry protocols [22–25]. Not surprisingly, the data reveal that regeneration of functional tissues of the correct size and shape is a rich, complex process requiring communication and feedback at the cellular, tissue and organism levels. Here we highlight the main components that have been identified as key regulators of regenerative shape (Fig. 2). It is likely that these are not the only factors involved in shape establishment, particularly since very little is known about how these disparate processes are coordinated on an animal-wide scale. However, the planarian model system provides one of the best platforms for investigating the natural ability to scale and reorganize the body plan to the correct geometry. And importantly, researchers now have the tools to be able to answer: What do you need to regenerate an animal of the right shape?

3.1. Getting the right number of organs in the right place

To regenerate with proper shape, the correct tissues must be replaced in the correct location following injury. In planarians this process requires: (1) the regulation of stem cells to promote new tissue growth and (2) the establishment of axial polarity in those new tissues. But how does the animal determine what tissues need to be restored and where these new tissues should go? Understanding the signals that regulate these early decisions is essential for regenerative shape and has been the focus of much of the current planarian literature.

In planarians, wounding triggers an increase in apoptosis at the injury site 1–4 h post amputation (hpa) [26] and a body-wide increase in mitosis that peaks between 4–6 hpa [27]. This early proliferation reflects the mobilization of a heterogeneous planarian adult stem cell (ASC) population, also known as neoblasts. In addition to this generic wound response, a regeneration-specific response follows by 12–24 hpa, leading to the upregulation of ASC-associated transcription factors and patterning genes known as position control genes (PCGs) [28–31]. Between 48–72 hpa, a second peak of mitotic activity occurs following ASC migration to the wound site [27]. This gives rise to the regeneration blastema, an unpigmented structure comprised of ASC progeny that will eventually differentiate into many of the missing tissues [27,32,33].

Establishment of axial polarity in the new tissues is controlled by PCGs expressed in muscle cells [31,34,35]. Each of the main planarian body axes is regulated by common developmental signaling pathways. The AP axis is regulated by PCGs involved in Wnt/β-catenin signaling [36–40]. Inhibition of β-catenin signaling causes head regeneration at all wounds (regardless of normal polarity), while upregulation of β-catenin signaling results in regenerates

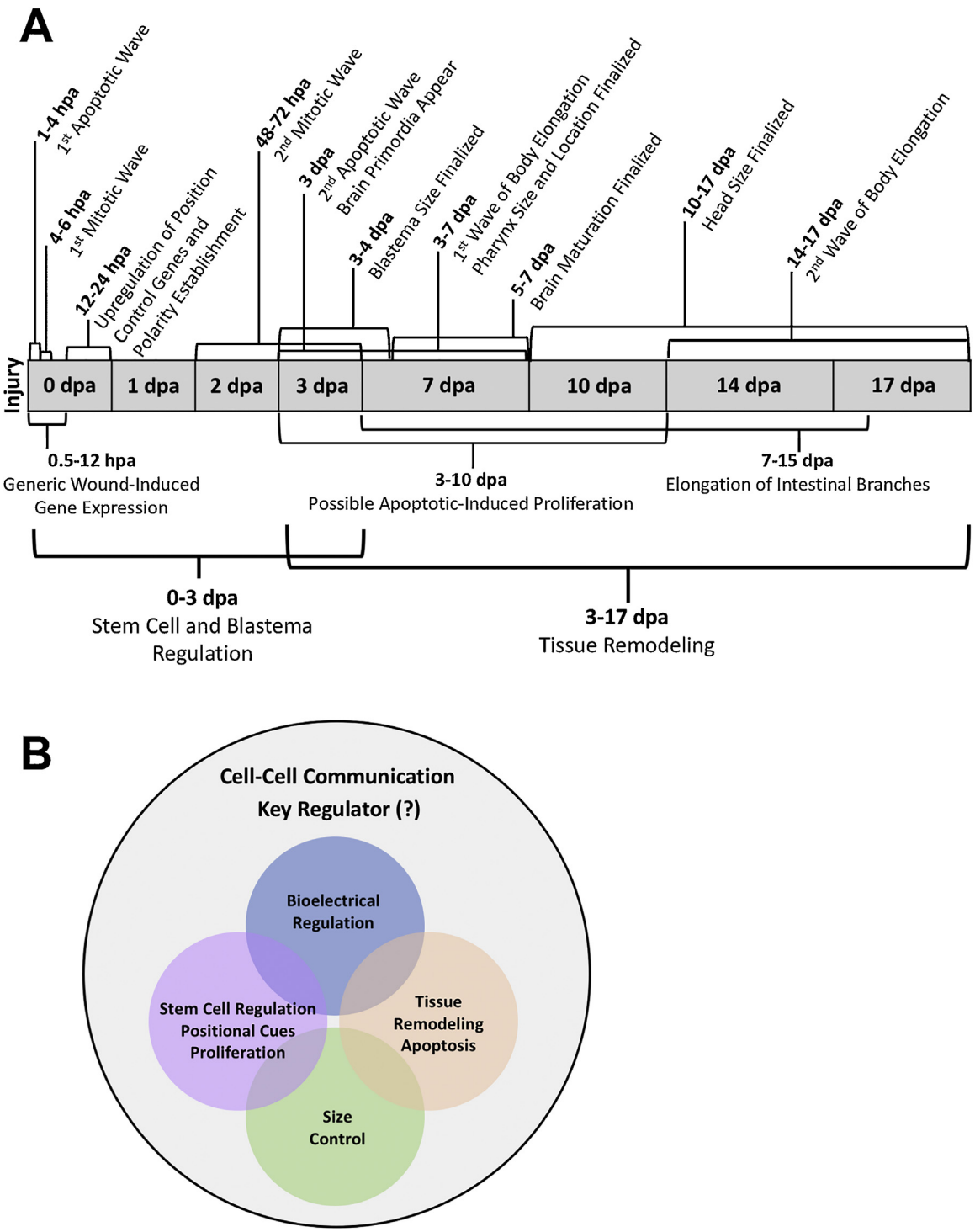

Fig. 2. The Regulation of Regenerative Shape. **(A)** Timeline of shape establishment events during the regeneration of trunk fragments in *S. mediterranea*. Based on data from [26–29,70,78,79]. Note that the timeline will be skewed for other species and/or fragment types. Hpa = hours post amputation; dpa = days post amputation. **(B)** Diagram of potential interactions between the central processes regulating regenerative shape during planarian regeneration.

with multiple tails and no head [37–39,41,42]. Hedgehog signaling is upstream of this β-catenin signaling, and modulation of the Hedgehog pathway leads to similar regenerative AP defects [40]. A growing number of other transcription factors have also been found to regulate the AP axis during planarian regeneration [43–52], suggesting that establishment of the AP axis is a central and early requirement and that many redundancies/parallel mechanisms likely exist to ensure AP polarity is correctly patterned.

The planarian dorsal-ventral (DV) axis is regulated by bone morphogenetic protein (BMP) signaling. BMP is expressed along the dorsal midline, and inhibition of pathway members results in ventral genes and structures being ectopically located on the dorsal side of the animal [53,54]. The BMP pathway also plays an important role in medial-lateral (ML) polarity. Disruption of BMP signaling results in regenerative midline defects, such as duplicated eye tissues [55,56]. Additionally, the Slit family of guidance cues

functions reciprocally with *Wnt5* to pattern the ML axis, and its inhibition during regeneration results in collapse of the midline [57,58]. The involvement of BMP in both DV and ML axial patterning suggests polarity establishment includes crosstalk between each of the individual axial control programs. It is intriguing that AP axis establishment appears to require significantly more regulation than the other axes, perhaps suggesting AP polarity plays a greater initial role in regenerative patterning.

3.2. Ensuring organs are scaled to the right size

An important step in regenerative shape is to ensure that all structures are proportional to the new worm's body size. This requires: (1) tissue remodeling of pre-existing structure, (2) apoptotic pruning of organs that are too large for the smaller regenerate, and (3) size control mechanisms to scale both new and old tissues. The planarian ability to scale tissue and organ size in both regenerative and non-regenerative contexts makes them one of the best model organisms for the study of shape establishment. Intact planaria undergoing starvation will reduce their body size while still maintaining correct body proportions, in an unusual process known as degrowth (Fig. 3). The process of growth and degrowth is thought to be a balance between cell division and cell death, much like size control during vertebrate development [59]. During degrowth, planarians maintain basal levels of proliferation [60] but show an increase in cell death that leads to an overall loss of cells [61]. In contrast, growth as a result of feeding causes more proliferation to occur than cell death, thus resulting in larger animals [61]. This "allometric scaling" of body proportions, where organs have proportionally fewer cells than they previously did, is seen in both degrowth and regeneration [62], suggesting carefully controlled mechanisms specifically scale cell number relative to body size.

As in other regenerative organisms, tissue remodeling during planarian regeneration is mediated largely or in part by apoptosis [63–65]. Also known as programmed cell death, apoptosis is characterized by a defined set of biochemical pathways that activate the caspase family of apoptotic regulators and lead to stereotypical cellular changes including chromatin condensation and membrane blebbing [66]. Following self-destruction, cell remnants are scavenged by phagocytic cells, resulting in a process that disposes of damaged, infected, or unwanted cells [65]. The apoptotic machinery, and the signaling that regulates it, appears to be highly conserved in planarians [26,67–69]. There are two main waves of apoptosis that occur during regeneration. The first apoptotic peak is localized to the wound site at 1–4 hpa and is part of the generic wound response [26], while the second wave of apoptosis occurs 3 days post amputation (dpa) throughout the body of the worm [26]. During the first 3 days post injury (which are associated with blastema formation and polarity establishment), very little to no shape changes in the original tissues are observed. Measurements of trunk regenerates (where both the head and tail were removed) showed no change in pharynx size during the first 3 dpa [70]. It is only on day 3, coincident with the second peak of apoptosis, that regenerative remodeling of pre-existing tissues begins [26].

Signals from apoptotic cells are able to stimulate proliferation non-cell autonomously [64,71], and apoptosis-induced proliferation has been reported in multiple regenerative contexts [72–76]. The first wave of planarian apoptosis does not appear to regulate proliferation, as caspase inhibition does not prevent either the first peak of proliferation [77] nor the formation of the regeneration blastema [70]. There is evidence, however, that the second apoptotic wave does function in apoptosis-induced proliferation, as conditions which blocked this peak reduced proliferation levels at 3 dpa in pre-existing tissues [70]. Additionally, studies of the re-establishment of the planarian intestinal track report that intestinal remodeling in pre-existing tissues was dependent on ASC activity—as irradiation (which kills ASCs) prevented intestinal remodeling from occurring [78]. Together these data implicate apoptosis-induced proliferation as an important possible mechanism during tissue remodeling in planarians and suggest the need for further investigation.

A few studies (including ours) have looked specifically at shape changes and body scaling during planarian tissue remodeling. We tracked organ size during the regeneration of trunk fragments and found that blastema size was finalized between 3–4 dpa, pharynx reduction to its new smaller size occurred from 3 to 7 dpa, and head size was not established until 10–17 dpa [70]. We also observed two waves of body elongation: the first from 3 to 7 dpa, during which the regenerate lengthened from its original square shape (although it was still wider than pre-amputation); and a second round of elongation that was not finalized until 17 dpa, which further thinned the regenerate to its correct proportions [70]. Characterization of neural regeneration has determined that brain primordia arise by 3 dpa [79], with brain maturation continuing through 5–7 dpa (reviewed in [2]). Analyses of regeneration in trunk fragments revealed intestinal branches undergo elongation beginning at 7 dpa [78], concurrent with the second wave of body elongation. It was also shown that along with new intestinal cell proliferation, existing intestinal cells are repurposed during regeneration; for instance anterior enterocytes were found to contribute to the regenerating posterior intestines [78].

We are only just now beginning to elucidate the molecular mechanisms behind tissue remodeling and organ scaling in planarians. For instance, inhibition of insulin signaling in intact worms disrupts proliferation and results in phenotypes that resemble body size reduction during degrowth [80]. A homolog of the death-associated-protein (*Dap-1*) is upregulated upon starvation and regulates autophagy during both degrowth and regeneration [81], while the H^+,K^+-ATPase ion pump is required for both the second apoptotic peak and subsequent tissue remodeling starting at 3 dpa [70]. The Hippo signaling pathway is required for regenerative size control; its inhibition leads to a failure of head fragments to prune the pre-existing brain down to the regenerate's smaller size, while eye removal alone results in disproportionally larger eyes that continue to increase in size beyond the normal regenerative timeframe [82,83].

These data indicate there are rigorous control mechanisms to maintain body proportions during planarian regeneration, many of which have begun to be identified. After fissioning, head and tail fragments use different mechanisms to control size during regeneration [84]. Size control of the brain compartment is regulated in part by Fibroblast Growth Factor (FGF) signaling and its inhibition leads to a dramatic posterior expansion of the brain [85]. A feedback loop of positive Wnt signaling (*Wnt11-6)* in the posterior brain and Wnt inhibition in the anterior, has been shown to modulate the number of brain progenitors needed [86]. Furthermore, regulation of the brain/anterior compartment appears to be linked to regulation of the neighboring trunk compartment through FGF signaling. In this case, FGF combines with additional Wnt signaling (*WntP-2)* to restrict the trunk compartment, and inhibition of this regulation results in expansion of the trunk and ectopic posterior pharynges [87]. These data provide evidence for cooperation between size control mechanisms, PCG regulation, and polarity establishment.

3.3. Making all these tissues talk to each other

The blastema alone does not replace all missing tissues—remodeled pre-existing tissues also contribute to regenerated structures in planarians (for example, see [70]). Following organ loss, proper regenerative shape requires the incorporation of both new blastemal cells and repurposed pre-existing cells, which

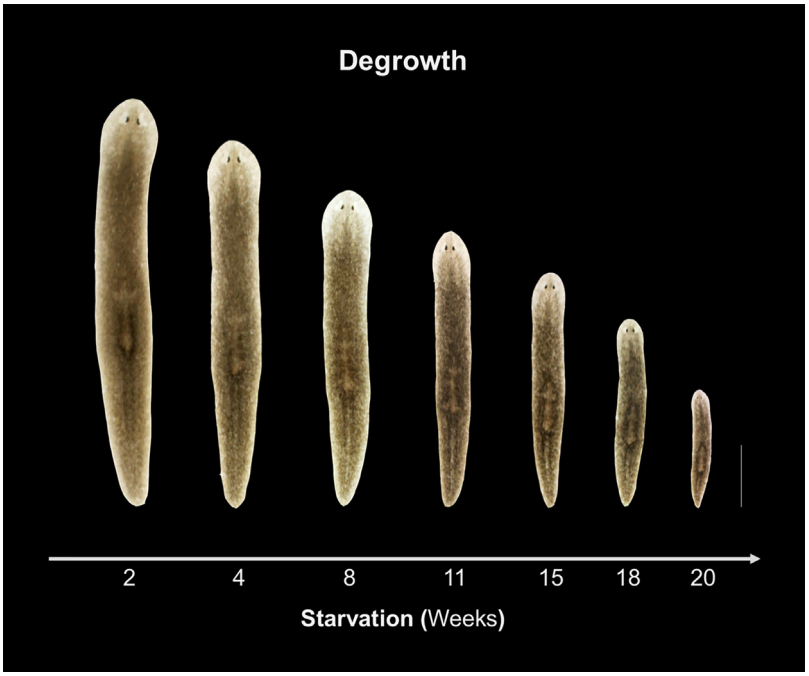

Fig. 3. Starvation-Induced Degrowth in an Intact Planarian. Tissue plasticity in planarians illustrated by a composite image of a single *S. mediterranea* worm over 20 weeks of starvation. Note that as the body size decreases, tissues are scaled proportionally. Anterior is up, scale bar = 1 mm.

combine to produce the final regenerated structure [70,78,88]. For these tissues to be integrated, as well as for polarity information to be conferred, there must be communication between the old pre-existing tissues and the new tissues of the blastema. This is required to functionally connect organ systems across the two tissues. In addition to this inter-tissue communication, there must also be a high level of intra-tissue communication to coordinate tissue remodeling within pre-existing tissues, as well as regulate ASC differentiation within the blastema. Clearly, regulated cell-cell communication is a top priority for regenerative shape.

The exact means by which cells communicate shape information during regeneration is an important area of research that is poorly understood. One mechanism that has been investigated during planarian regeneration is gap junction communication (GJC) [89]. Gap junctions are integral membrane channels that allow the direct passage of ions and small molecules between neighboring cells; in vertebrates these channels are made of connexin proteins, while in invertebrates gap junctions are comprised of functionally homologous innexin proteins [89,90]. Data has shown that planarian innexin genes are expressed in the blastema [91], and disruption of GJC can lead to polarity and patterning defects [92,93]. Importantly, blocking GJC is capable of driving brain and head regeneration at posterior wounds, even when the regenerate still contains the original head [91,94]. These data allude to the possibility that brain/head formation might be a sort of "default" state for regeneration in the absence of other signals and suggest GJC is required for regenerating tissues to determine what tissues are missing and need to be replaced.

Interestingly, the two main organ systems that have been implicated in regulating such communication are the muscles and the nervous system. Some of this cell-cell communication is clearly between ASCs and muscle cells during axial establishment in general and AP polarity in particular [31,34]. The data have also suggested that the brain (like muscle) may function as a signaling center, although the molecular mechanisms remain to be identified. However, simple modulation of neurotransmitter levels such as dopamine and serotonin is sufficient to disrupt regenerative morphology, leading to double-headed or headless phenotypes respectively [95]. And it is inhibition of innexins specifically expressed in the central nervous system that are required for GJC regulation of regenerative polarity [91,94]. As a whole, the existing data overwhelmingly point to a significant amount of cellular communication that must occur both within and between tissues to establish proper regenerative shape.

3.4. Coordinating these mechanisms across the animal

During the restoration of shape in regenerating planarians, there are a lot of distinct processes happening—often at the same time. Some processes are obviously interconnected (for instance expansion of anterior regions requires that posterior regions are decreased), but it is harder to connect-the-dots with others (how do eye number and pharynx size relate to each other). However, in order to establish body proportion across the entire animal, there must be some sort of regulation that coordinates all these disparate processes. A growing body of research from several model systems suggests that bioelectrical signaling mechanisms (such as membrane voltage and ion transport) may serve this function during regeneration in general and the establishment of regenerative shape in particular [70,95–102].

Endogenous bioelectrical signaling relies on ion channels and pumps to mediate ion transport, most frequently across the plasma membrane. One function of this ion flux is to establish a steady-state transmembrane potential, which is found in all non-excitable cells and is in contrast to the rapid changes that occur in neurons [103]. Regulation of membrane voltage has been shown to play a role in many processes during development and growth, including proliferation, apoptosis, and importantly, animal-wide patterning [99,104–107]. Bioelectrical signals can also regulate organ size, such as with K^+ flux during zebrafish fin growth [108]. Studies have found direct links between membrane voltage and downstream transcriptional and epigenetic targets [98,109]. In addition, the flux of individual ions such as Ca^{2+} and Na^+ has been shown to play

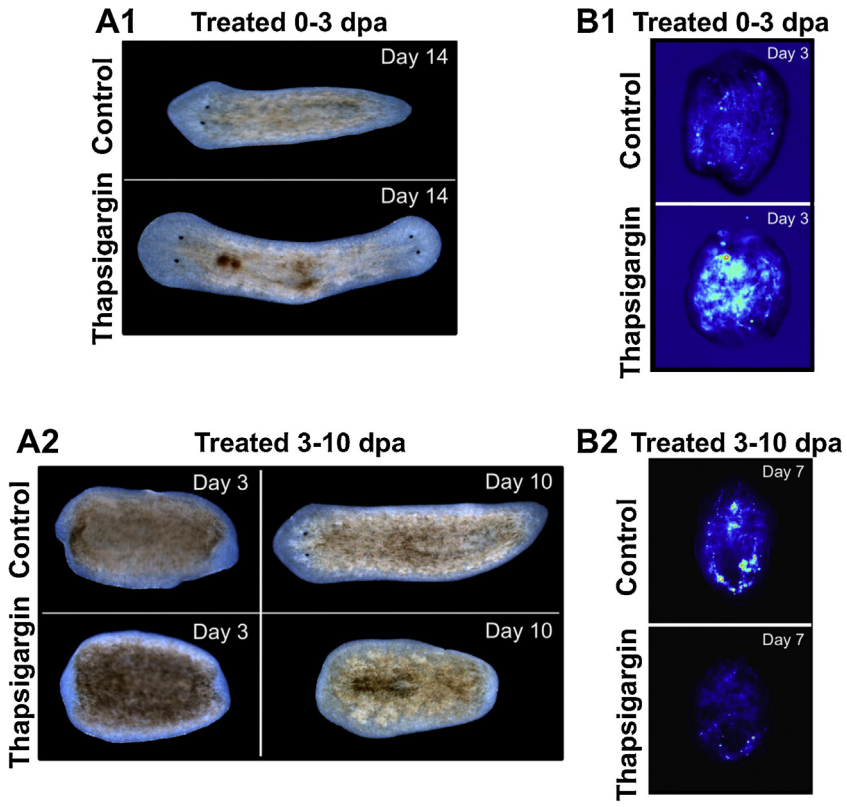

Fig. 4. Ca^{2+} Flux has Distinct Roles in Polarity Establishment vs. Tissue Remodeling. *D. japonica* trunk fragments treated with 1 μM Thapsigargin (Sigma), which inhibits SERCA-mediated ER Ca^{2+} storage, or DMSO as a vehicle control. Inhibition of ER Ca^{2+} storage from 0 to 3 days post amputation (dpa) results in **(A1)** double-headed regenerates due to **(B1)** an increase in intracellular Ca^{2+} levels, as measured by Flou-3 (Molecular Probes). Inhibition from 3 to 10 dpa results instead in **(A2)** a complete lack of tissue remodeling, perhaps due to **(B2)** depleted Ca^{2+} stores. Anterior is left for morphology (n = 10), and up for Flou-3 (n = 5).

signaling roles in regeneration irrespective of membrane voltage [95–97,110].

The field is just beginning to focus on the downstream effectors of identified bioelectrical signals. For example, H^+ flux mediated by the V-ATPase proton pump during zebrafish fin regeneration has been shown to be required for the expression of FGF and retinoic acid pathway members, which in turn are required for blastema cell proliferation and outgrowth [100]. V-ATPase is also necessary for *Xenopus* tail regeneration, where early H^+ flux-dependent membrane voltage changes are required for regenerative proliferation [99]. Specifically, these membrane voltage changes control the later expression of voltage-gated sodium channels in the regeneration bud; and the resulting Na^+ flux is required for the expression of signaling genes such as Notch and BMP, as well as cell proliferation [97]. In both zebrafish fin and *Xenopus* tail regeneration, bioelectrical signaling is also essential for proper innervation during regeneration [97,100].

Planarians are one of the few model systems where the contributions of bioelectrical signaling in regeneration are best characterized [111]. For instance, membrane depolarization of anterior tissues is required for anterior polarity and head regeneration in planarians. H^+,K^+-ATPase plays an endogenous role in maintaining this depolarization, and its inhibition leads to hyperpolarized animals that fail to regenerate heads [70,96]. However, it is the depolarization itself that is important rather than the individual ion channels that achieve it, as ectopic depolarization of the blastema is sufficient to induce head regeneration even at posterior-facing wounds [96]. Anterior membrane depolarization is an early step in planarian regeneration (within the first 24 hpa) and is required for the upregulation of Ca^{2+} into the blastema [96]. Ca^{2+} signaling itself is an important regulator of many planarian regenerative shape processes. Early activation of voltage-gated calcium channels (CaVs) is sufficient to induce head regeneration at posterior wounds, and functions in part to regulate neuronal signaling [95,101,112].

We investigated the effects of inhibition of the sarcoplasmic/endoplasmic reticulum (ER) calcium ATPase (SERCA), which controls Ca^{2+} storage into the ER. Our data showed that SERCA inhibition from 0 to 3 dpa results in double-headed regenerates due to increased intracellular Ca^{2+} levels (Fig. 4A1, B1). In contrast, SERCA inhibition from 3 to 10 dpa (after blastema formation and polarity establishment) resulted in a complete block of tissue remodeling, perhaps due to the eventual depletion of intracellular Ca^{2+} levels (Fig. 4A2, B2). These data demonstrate that tissue remodeling during shape establishment is a distinct process from earlier polarity establishment and initial stem cell regulation. Our preliminary hypothesis is that ER-mediated Ca^{2+} release is required for apoptotic tissue remodeling. Further investigation is needed to determine the actual role of SERCA channels during regeneration.

As in other organisms, bioelectrical signals appear to function during the initiation of regenerative processes in planarians. In some cases, such as the membrane depolarization of the anterior region, bioelectrical signaling appears to be used to define broad anatomical boundaries (*e.g.* regenerate head here). As such, bioelectrical regulation is an important component of regenerative shape and functions to regulate morphology at multiple points during this process. This makes bioelectrical signaling a strong candidate for coordinating communication across multiple tissues. In fact, it is highly likely the signals being transmitted via GJC are bioelectrical in nature (ions)—pointing to the need to integrate stem cell regulation, bioelectrical signaling, and cell-cell com-

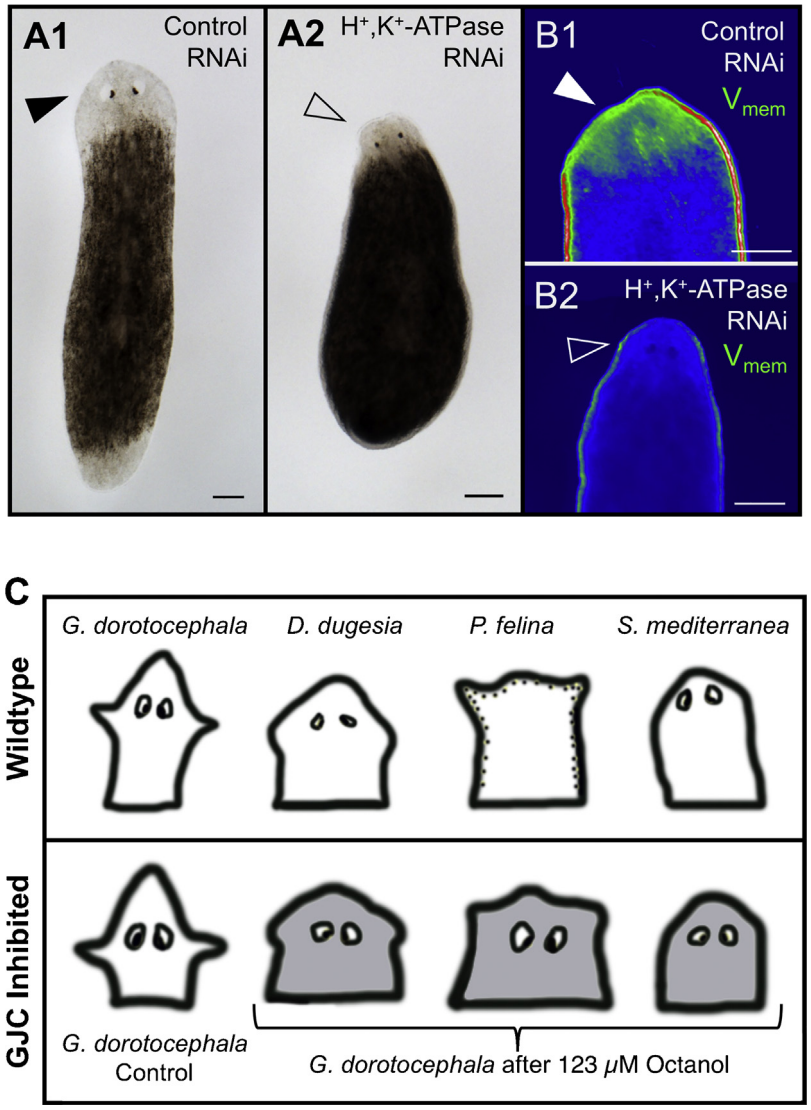

Fig. 5. Example of Shape Mutants for Investigating Regenerative Morphology. **(A,B)** Inhibition of the H^+,K^+-ATPase ion pump via RNAi results in (A) regenerates with shrunken heads, due to (B) ectopic loss of membrane depolarization (green) of the anterior region. V_{mem} = membrane voltage, visualized with $DiBAC_4(3)$ (Invitrogen). Solid arrows = normal, open arrows = abnormal. Control RNAi = VenusGFP. Anterior is up, scale bars = 200 μm. **(C)** Graphic representation of the effects of gap junction inhibition. Octanol treatment causes *G. dorotocephala* to regenerate with head morphologies characteristic of different species. Based on [93].

munication during the establishment of regenerative polarity in planarians.

4. Conclusions and future directions: identifying more shape mutants

The planarian field has made great strides in elucidating the molecular mechanisms behind many of the most important processes that regulate shape during regeneration. It has even provided evidence for the existence of Morgan's gradation of polarity that he proposed back in the early 20th century. It is true that we still have not identified a single regulator that functions as a gradient, either to promote or inhibit anterior fates in planarians. But we have found that there is a gradient of PCG expression along the AP axis that directly correlates with core components of the Wnt pathway [35,113]. This gradient is comprised not of a single molecule but the combined expression of all Wnt/β-catenin signaling regulators taken as a whole, that results in Wnt ligands and positive drivers of β-catenin being expressed more posteriorly, while inhibitors of β-catenin signaling are expressed more anteriorly [37,41,42,57,113,114]. The modern toolkit has enabled us to answer some of the mechanistic questions posed by the earliest planarian researchers.

Despite this, as a field the investigation of regenerative shape is somewhat still in its infancy. We have a wealth of observational and phenomenological data from the historical literature. And we have begun to make inroads on the molecular-genetic pathways regulating stem cells, tissue patterning, apoptotic remodeling, organ scaling, cell-cell signaling, and even bioelectrical regulation. But many important black boxes remain. One of the biggest unknowns, not just for regenerative shape but regeneration in general, is how the termination of regeneration is regulated. This is a particularly important question for planarian regeneration, where unlike development, the growth of tissues is not constrained by outside physical forces (such as by an egg capsule) and there are no outside patterning cues (such as from surrounding yolk cells). Internal mechanisms must exist that function to tightly restrict growth in order to produce tissues of the correct shape and size. Only a few studies have begun to elucidate these control mechanisms. For example, a negative feedback loop (in which *wnt11-6* activates the expression of its

own inhibitor *notum*) appears to regulate brain growth, functioning to restrict brain size [36,86]. In addition, our research has shown that the Planar Cell Polarity (PCP) pathway is required for the termination specifically of neural growth in planarians, as disruption of PCP results in the continual production of new nerves month(s) after controls have stopped proliferating [115]. However, whether this regulation is direct or indirect and the exact mechanisms that are involved are not known, and much research remains to be done.

The most important future direction for the field of regenerative shape is to determine how all of the individual processes involved are coordinated at the macro level—such that the entire animal regenerates with the correct size, number, and placement of organs needed to maintain body proportionality. The data already reveal that there is much crosstalk between the individual processes regulating shape (Fig. 2B). One hypothesis is that there is a "key regulator" of regenerative shape, and it has been postulated that bioelectrical cell-cell communication mechanisms could serve this function; but no one mechanism has been found to date that coordinates all the known processes involved. Therefore a critical direction for the field will be to uncover the "upper" level regulative mechanism(s) that serve to establish overall animal patterning and shape.

One barrier to this research has been the need for a different class of shape mutants. Although examples of dysmorphia result from the disruption of most regenerative processes, to date most of these mutants have consisted of the replacement of one organ with another (e.g. regrowth of a head instead of a tail), the presence of supernumerary organs (such as ectopic eyes or pharynges), or the deletion of organs (as in headless regenerates). While these mutants have been critical in uncovering the mechanisms that regulate individual structures, they mostly do not provide the opportunity to investigate animal-wide regulation of shape. What is needed are mutants in which the correct organ has regenerated in the correct place, but with the incorrect shape. A few such shape mutants exist, for instance the "shrunken" heads we observed following RNAi to H^+,K^+-ATPase, which result from a lack of apoptotic remodeling of pre-existing tissues [70] (Fig. 5A, B). Similarly, inhibition of GJC in *G. dorotocephala* was found to produce regenerates with incorrect head morphologies that more closely resembled head shapes found in other planarian species [93] (Fig. 5C). But there are not enough examples in the literature to resolve the mechanisms involved at the organism level. What is required are large-scale RNAi or chemical screens to identify phenotypes that result in a failure to produce overall regenerative shape. Such investigations will be important for regenerative medicine going forward, so that induction of appropriate organ shapes in situ can serve as a complement to the controlled in vitro regulation of stem cells.

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Declarations of interest

None.

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