

Molecular Mechanisms of Regeneration Initiation and Dorsal-Ventral Patterning in Planarians

by

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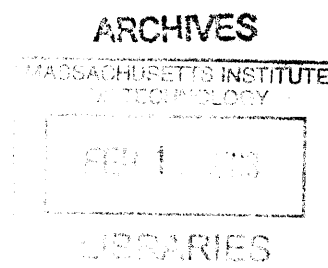
SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF
THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

NOVEMBER 2012

[FEBRUARY 2013]

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Submitted to the Department of Biology on November 27th, 2012 in Partial
Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

Abstract

Regeneration is widespread among animals, yet very little is known about the molecular mechanisms that govern regenerative processes. Planarians have emerged in recent years as a powerful model for studying regeneration and are capable of whole-body regeneration following a limitless variety of injuries. Two major questions in planarian regeneration have been: 1) how are the identities of missing tissues determined?; and 2) how is the decision to mount a regenerative response to injury mediated?

As part of an effort to address question 1), the mechanism by which dorsoventral (DV) pattern is regenerated following amputation was investigated. A planarian homolog of the Bmp family gene *admp* was identified and found to be required for regeneration of lateral tissues as well as the proper regeneration and maintenance of DV polarity. Subsequently, a regulatory relationship between *admp* and a *bmp* homolog was described. In this regulatory circuit, *admp* activates *bmp* expression but *bmp* represses *admp* expression. This arrangement results in a DV regulatory circuit that is buffered against perturbation and able to mediate robust DV and mediolateral regeneration.

Question 2) was investigated by cloning several wound-induced genes and assaying for roles in regeneration initiation. A homolog of the TGF- β inhibitor *follicistatin* was identified in this manner and found to be required for regeneration. Furthermore, *follicistatin* was required for mounting a number of regeneration-specific responses to injury. A suppression screen of candidate planarian TGF- β genes identified an *activin* homolog, *act-1*, as a probable target of Follicistatin inhibition. *act-1* suppressed regeneration-specific responses to injury and was required for terminating some regenerative processes after regeneration was complete. From these data, a model was formulated in which Follicistatin-mediated inhibition of Act-1 is required for regeneration initiation and relief of this inhibition is subsequently required for regeneration termination.

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Acknowledgements:

I would like to acknowledge many many people for making this work possible: scientifically, both Peter Reddien and Danielle Wenemoser contributed significantly to work I present here. In addition, the entirety of the Reddien lab has been enormously helpful throughout my time at MIT. Sylvain Lapan, Chris Petersen, Danielle Wenemoser, Jared Owen and Dan Wagner were invaluable for teaching me experimental protocols and providing reagents. Mansi Srivastava was the force behind all of the phylogenetic experiments I performed. Josien VanWolfswinkel was enormously helpful with computational analysis. Isaac Oderberg and Amber Poirer were wonderful collaborators on work yet to reach fruition. Finally, Irving Wang was helpful on nearly every front, from managing experiments for me when I was away to collaborating on optimising protocols. In addition, Irving provided the beautiful artwork presented in chapters two and three, and taught me the basics that allowed me to create the artwork for chapter one.

I want to thank my committee members Dennis Kim and Terry Orr-Weaver, who provided advice as my research progressed and otherwise helped guide me to the finish line. I am also extremely grateful to Victor Ambros for finding the time to serve on my defense committee.

In addition to his direct contributions to the work presented here, Peter Reddien was an excellent mentor both in terms of experimental advice, my broader development as a scientist, and picking me up when I needed him to. He has taught me so much; I can only hope that moving forward as a scientist I am able to do justice to his herculean efforts.

Danielle Wenemoser's contribution to this work cannot be measured. As mentioned above, her experimental contribution was significant. In addition to this however, she has been the cornerstone to my sanity, my best friend, my babysitter (as in, babysitting me), my running partner, my heart. For having her put up with me on a daily basis, I consider myself extremely extremely lucky.

Bob Goldberg almost singlehandedly convinced me to study Biology while at UCLA, contrary to his intention. He and Tomo Kawashima not only put me in a position to get to grad school in the first place, but taught me the essentials of how science is done and how it can be communicated. For their mentorship I will be forever thankful.

My family also deserve enormous thanks. My parents have been encouraging and loving and there for me when I needed them; without their influence I would not be here. My brother has always been a great friend and I'm extremely grateful that we've stayed in contact during my time out east. My aunt Susie has been a constant presence in my years out here, sending gift packages and reminding me that people care about my future. My cousin Nick deserves double thanks: he was both a friend and an occasional roommate who allowed me to sleep on his couch on my visits to New York.

I am thankful to the friends from California that shelled out the money necessary to visit me while out here and to my countless friends in Boston and Cambridge. All of you have made the last years happy ones for me.

Lastly, I would like to thank my dogs Indie and Juno for keeping me company while composing much of this work, and for providing an excuse to take a break now and then.

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Chapter 1

Introduction

Introduction

a key step forward for understanding developmental processes in general.

I. Canalization and the consistency of development

Development proceeds with remarkable consistency despite enormous variability both in environmental stimuli and in genetic background. A central question in developmental biology is how the vast array of processes that must occur with high precision can be robust to the fluctuations in any number of variables that are encountered on a case-by-case basis. The property of developmental processes being robust to environmental and genetic perturbation was termed “canalization” by C.H. Waddington [1]. Though this property is likely required for the consistency of all developmental processes, the mechanisms by which specific processes are canalized, to what extent these mechanisms are broadly conserved across species, and whether more general features of canalized processes can be inferred by surveying these mechanisms all remain largely unanswered questions. Addressing these questions therefore represents

Within a single species, the sources of variability can be subdivided into those that are of a genetic origin and those that are of an environmental origin. Genetic variability stems from the unique genetic backgrounds that any two individuals, unless clones, possess. While many null alleles can cause embryonic lethality or otherwise compromise the viability of a developing individual, some are tolerable. Moreover, many non-null loss-of-function alleles and gain-of-function alleles are likewise non-lethal. One might expect that vastly decreasing the dose of a gene product would cause the system in which that gene product functions to fail. As a hypothetical example, we can imagine a morphogen that signals at a very specific level to produce a gradient as a result of its acting in concert with both positive and negative regulators. How then is an identical pattern produced in an animal heterozygous for this morphogen but possessing wild-type alleles for the rest of the system? The crux of the issue thus becomes identifying the genetic mechanisms

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that prevent such variance in pathway components from disrupting developmental output.

As mentioned above, environmental variability also affects development between individuals. Environmental variables entail anything external to the genotype of an individual that will impact gene expression or protein function. An obvious example of an environmental variable is temperature. Temperature can alter gene expression and the kinetics of cell division or protein function. In *Drosophila*, temperature affects both the speed of embryonic development as well as the final size of animals [2, 3]. Amazingly, however, animals that develop at different temperatures are otherwise indistinguishable: they all form normal functional flies.

Canalization is a feature that can be observed not only between individuals of a single species, but also evolutionarily. For instance, a single pathway can be utilized in diverse developmental contexts across several species. In this case the perturbations that challenge the system are not variations in the genotype or environment of an individual, but

the accumulation of mutations that ultimately produce a distinct but related species. As even closely related species can vary dramatically in size, it is noteworthy that many pathways can withstand such shifts and remain functional. An example of inter-species canalization is the use of Bmp signaling as a conserved pathway for establishing dorsoventral (DV) polarity across nearly all bilaterians [4]. This is a form of canalization in that a single set of genes comprises the pathway across species, yet these species develop from embryos of vastly different size and shape. In other words, a genetic network that originally evolved to form DV pattern in a single hypothetical bilaterian ancestor has been able to function across embryonic contexts that vary drastically. What are the properties of this network that allows for it to function in this plastic way? It is through this type of interrogation that conserved developmental programs can elucidate mechanisms and features that allow for canalization of developmental processes.

In the following pages, I will discuss examples of a specific biological process which is a particularly dramatic example of

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canalization: patterning of tissue by morphogen gradients. Morphogens are proteins that diffuse from their site of production and that activate specific transcriptional targets in cells depending on the amount of ligand a given cell receives [5]. When a cell receives ligand, the ligand is removed from the pool of extracellular morphogen. The net effect of this is that ligand concentration decreases as a function of the distance a cell is from the source. In this way, a morphogen gradient is formed. This gradient allows for cells in a field to vary their behavior and fate based on their relative distance from a morphogen source. Because tissues can grow during development, and because key morphogenetic programs are often conserved between morphologically diverse species, morphogen gradients must be able to scale and function consistently independent of the size of the tissue being patterned. Because recent work has identified some key mechanisms of how morphogen gradients are made robust, I will review these findings and try to infer some commonalities of robust morphogen-mediated patterning mechanisms. I will then discuss regeneration

as a paradigm for studying canalization and propose that features unique to regeneration make it particularly well suited for this line of inquiry. Finally, I will describe the remarkable regenerative capabilities of planarian flatworms and the use of these animals as a valuable tool for the study of regeneration and canalization.

II. Examples and mechanisms of canalization

As previously stated, nearly every aspect of development must be canalized to some extent due to the inherent variability across individuals. Despite this, descriptions of the mechanisms that make specific developmental processes robust are only beginning to appear. As mentioned above, the canalization of morphogen gradients is an example of one such process. A perturbation that these gradients must respond to and compensate for is variance in tissue size that arises through growth or evolutionary change. In order to respond to this perturbation, morphogen gradients often have the property of being scalable. The

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question of how these gradients scale with size has therefore become an active inroad into identifying the key mechanisms that make patterning processes robust. By reviewing two well-studied examples of the canalization of morphogen gradients I will identify some possible common themes of canalized systems.

Embryonic self-regulation

Though best known for the developmental organizer that bears his name, Hans Spemann carried out another landmark experiment that identified an extreme example of developmental robustness. Building upon similar work that Driesch performed with sea urchin embryos [6], this experiment entailed the bisection of an early stage frog embryo. Spemann used a hair to separate the dorsal and ventral halves of this early embryo and found, amazingly, that dorsal embryo halves not only survived but compensated for the loss of their ventral half and developed into completely normal, albeit half-sized, tadpoles [7]. The ventral halves likewise survived, but

became inappropriately ventralized hunks of tissue that Spemann called a *bauchstück*, or “belly-piece”. The conclusion that was drawn from this experiment was that dorsal embryo halves possessed some mechanism of “self-regulation”; in other words, they were capable of detecting the missing ventral half and of assigning the role of ventral tissue formation to regions that would normally have contributed to dorsal tissues in a full-sized embryo. Therefore, despite removing half of an embryo’s mass, development compensates and continues normally. This process is not unique to amphibians, as related feats are accomplished by other species as well. A prominent example is provided by the existence of identical twins, two individuals that arise from a single fertilized egg that, at some point in early development, became separated into two independent embryos. In addition, a single mosaic mouse can be generated by fusing together two early embryos into one [8]. Much in the way that Spemann observed self-regulation in *Xenopus* embryos, self-regulation must occur in these cases as well to ensure that each half-sized embryo or double-sized embryo recognizes its

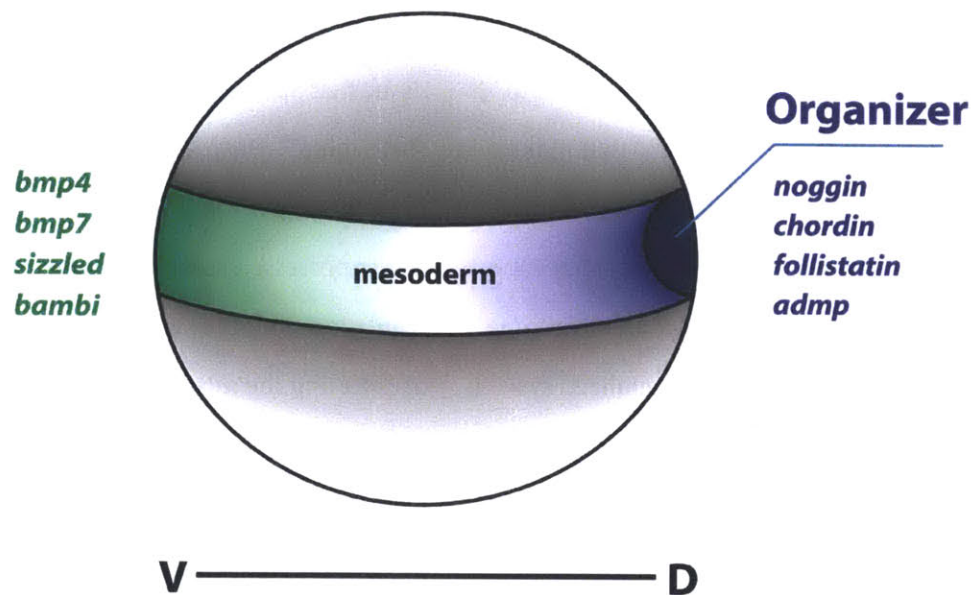


Fig. 1. The *Xenopus* embryo after formation of the organizer. The Organizer secretes Bmp antagonists. Opposite the Organizer, the ventral mesoderm secretes Bmp ligands. This results in a Bmp gradient in which Bmp signaling is high ventrally and low Dorsally.

new size and compensates.

Despite the landmark nature of Spemann's finding, the mechanism by which embryonic self-regulation is carried out remained completely mysterious for over 80 years. To explain how this mystery has been recently addressed, however, it is necessary to first describe early *Xenopus* development.

In the early *Xenopus* embryo, the first act of establishing polarity involves both the localization of maternal mRNAs and the site of sperm entry to the egg [9, 10]. Polarity is

established by the trafficking of Wnt signaling components along microtubules to the side opposite sperm entry, eventually resulting in a local accumulation of nuclear β -catenin [11-13]. In concert with signals from the vegetal pole of the embryo, β -catenin activates transcription of a secreted ligand, *nodal*, and a gradient of this signal is thus produced [14]. The result of this gradient is that the embryo becomes polarized. The region that had nuclear β -catenin will be *nodal*^{hi} and becomes the Spemann-Mangold organizer or simply the "organizer". This structure marks

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the presumptive dorsal side of the embryo. At the other end of the embryo, where there is no nuclear β -catenin, the tissue is nodal^{lo} and becomes the ventral mesoderm. The organizer activates a specific transcriptional program and begins to secrete several extracellular molecules such as Cerberus, Chordin, Noggin, and Follistatin [15-18]. Many of these molecules act as extracellular inhibitors of Bmp ligands that are concurrently being secreted from the ventral mesoderm [19, 20]. Specifically, Bmp4 and Bmp7 are expressed in the ventral mesoderm and, in concert with these dorsally secreted inhibitors, establish a Bmp signaling gradient (Fig 1). Interestingly, there are also Bmp inhibitors that are expressed ventrally. Namely, Bmp signaling activates expression of the Bmp pseudo-receptor *bambi* in the ventral domain, as well as the secreted inhibitor *sizzled* [21, 22] (Fig 1). It is at this stage of development that DV bisecting results in dorsal halves that are capable of self-regulation. Moreover, transplantation of dorsal organizer tissue to a second host embryo can induce a secondary AP axis [23]. This is the “organizing” activity that gives this

tissue its name. Given this organizing ability, and given the vast array of regulatory proteins secreted from the organizer, it was hypothesized that properties of this structure imbued dorsal embryo halves with the ability to self-regulate.

Only recently has it been confirmed that indeed, signals from the organizer do underlie the phenomenon of self-regulation. Importantly, this is consistent with the widespread existence of self-regulation across species, as homologous structures to the organizer are equally widespread among vertebrates investigated [24-26]. As the organizer does not secrete any ventralizing factors, the question of how self-regulation is possible can be reduced to two subquestions: 1) How is ventral identity conferred in a dorsal half embryo that lacks a ventral mesoderm Bmp signaling center? 2) Once a ventral signaling center is re-established, how does the Bmp signaling gradient scale to accommodate the reduced size of the halved embryo? The answer to both of these questions is thought to involve the action of an organizer-secreted Bmp-family ligand called *anti-dorsalizing morphogenetic*

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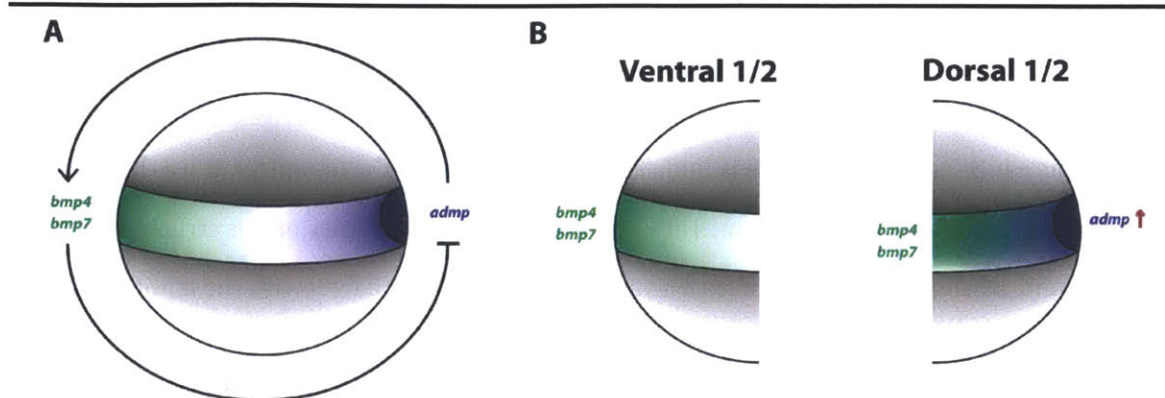


Fig. 2. Oppositely expressed *admp*/*bmp* allows for self-regulation. (A) Dorsally expressed *admp* functions as an activator of the *bmp* pathway, while Bmp signaling inhibits expression of *admp*. (B) Ventral half *Xenopus* embryos express ventral factors but cannot re-express dorsal *bmp* inhibitors, while dorsal half embryos express Bmp inhibitors and are able to activate expression of *bmp* genes through the action of *Admp*.

factor or *admp*.

The unique feature of *admp* that helps to answer the question of embryonic self-regulation is that it is negatively regulated by Bmp signaling and therefore is only transcribed and secreted from cells that receive low Bmp signal [27, 28]. *admp* is therefore produced in the organizer. Given that *Admp* signals through canonical Bmp pathway components, and therefore functions as an activator of the pathway, it was originally mysterious why an activator of a pathway would be repressed by this same pathway.

It was found that inhibition of *admp* through morpholino injection in dorsal-half embryos abrogated the ability of these embry-

os to self-regulate [28]. Dorsal-halves instead retained a uniform dorsal identity. A hypothesis stemming from this observation was that *Admp* signaling in dorsal half embryos is the mechanism by which a new ventral side is established. How does this occur? Through biochemical experiments, it was found that *Admp*, like other Bmp-family ligands, binds to extracellular Bmp inhibitors secreted from the organizer. Therefore, dorsal *Admp* likely exists in a largely inhibitor-bound state. As these inhibitors prevent ligand binding to receptors, *Admp* is unable to signal until relieved of this inhibition. This relief occurs when Xolloid-related (*Xlr*) cleaves Chordin and allows *Admp* to signal. Importantly *xlr* is only expressed ventrally and therefore restricts *Admp* release

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and signaling to the ventral half of the embryo [29]. From these data it was concluded that, though produced dorsally, *Admp* signals ventrally. To further illustrate this point, transplantation of an organizer to a Bmp-null embryo (*bmp2/4/7* morpholino knockdown) is able to rescue and restore DV pattern, suggesting that a Bmp signal must emanate from the organizer [28].

What then happens when an embryo is bisected into dorsal and ventral halves? In the ventral half, Bmp ligands meet no inhibition from organizer molecules and are unable to establish de novo expression of organizer molecules (Fig 2). Therefore these unopposed Bmp signals ventralize the entire half embryo. In the dorsal half, however, expression of inhibitors (*chordin*, *noggin*, etc) is coupled with expression of a Bmp ligand (*admp*). Moreover, as *admp* is a negative target of Bmp signaling, the removal of the ventral embryo half and the Bmp signaling center that exists there leads to derepression of *admp* expression. As *Admp* is secreted it is most likely to be released from Chordin inhibition in regions where Chordin concentration is low-

est, namely the new ventral-most cells of the embryo. The increased ventral Bmp signaling that results from *Admp* activity then catalyzes the beginnings of a new ventral center. This explanation is supported by recent mathematical modeling [30]. These models likewise suggest that the same properties of *admp* regulation, namely the ability of *Admp* to diffuse by Chordin binding and the feedback inhibition of *admp* expression by *Admp* signaling through the Bmp pathway, allow for the subsequent scaling of the DV axis to half of its normal size.

Regulation of organ scaling

Another well-studied example of morphogen-controlled canalization is the ability of organs to scale to the appropriate size. Within an individual, organ size must be carefully controlled during development and able to scale to accommodate organismal growth. Between species, homologous organs of vastly different size exist that share genetic underpinnings. How is organogenesis carried out robustly across such magnitudes of scale and

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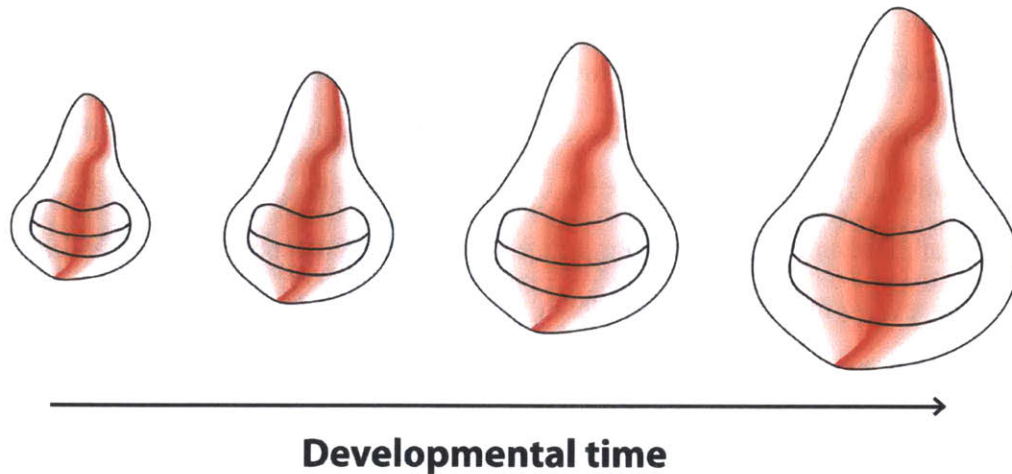


Fig. 3. As development proceeds, the wing disc must grow in size. Consequently, the Dpp gradient (red) must scale accordingly to maintain proper wing pattern

evolutionary distance by conserved genetic pathways?

The *Drosophila* wing is an organ whose size must vary across thousands of related but morphologically distinct species, yet retain a relatively consistent structure. Additionally, the tissue giving rise to the wing grows significantly during development (Fig 3). This organ has therefore become a useful model for investigating the mechanisms of organ size regulation. The wing is formed during metamorphosis from a flat epithelium called the wing imaginal disc, or the wing disc in short [31, 32]. It is during this two-dimensional stage that the size and pattern of the wing are

determined [33]. One factor that contributes to the size of the wing is the nutritional state of the animal. This is, however, the result of body-wide signaling through insulin/TOR and functions to keep the animal's organs growing in concert, rather than controlling scaling or pattern of the wing disc itself [34]. This is supported by the observation that TOR mutant flies are smaller but have normal wings and are of correct body proportions [35]. What then are the mechanisms that pattern the wing?

The wing disc, as mentioned above, is a flat epithelium. It is divided into regions called compartments that represent lineage

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restricted domains; cells in one compartment are prohibited from crossing into an adjacent compartment [36, 37]. The establishment of these compartments and the boundaries that separate them is itself a fascinating and well-studied developmental process but one that will not be discussed in detail here. Once compartments are established, patterning of the wing disc occurs through the secretion of morphogens. In this case, the two major morphogen gradients that are used are *decapentaplegic* (*dpp*), the *Drosophila* homolog of *bmp*, and *wingless* (*wg*), the *Drosophila* homolog of *wnt* [38, 39]. *Dpp* is secreted from a thin stripe at the boundary of the anterior and posterior compartments and acts to pattern the AP axis of the wing disc in a medial to lateral gradient, while secreted *Wg* patterns the DV axis of the wing disc [40, 41](Fig 4).

As alluded to above, control of wing pattern is robust. For example, if cells in one compartment are stimulated to divide more rapidly or more slowly, the total size of the compartment will not change and a normal wing will develop [42]. This demonstrates that compensatory mechanisms exist to maintain

the relative pattern of the wing following severe perturbation. What are these mechanisms? As it has been the subject of recent relevant investigations, I will address this question by discussing specifically the *Dpp* gradient and patterning of the AP axis of the wing disc.

As mentioned, *Dpp* is secreted from a domain at the boundary of the anterior and posterior compartments. As is the case with *Bmp* signaling in early *Xenopus* embryos, this *Dpp* gradient must be capable of scaling with animal growth (Fig 3). However, unlike *Bmp* signaling in early *Xenopus* development, there is no opposing expression of *Dpp* inhibitors such as *chordin* or *noggin*. Instead, *Dpp* is free to diffuse such that expression of *Dpp* receptors limits *Dpp* concentration away from the source as increasing amounts of ligand become receptor-bound and internalized [43, 44]. A gradient of *Dpp* signal is therefore formed such that *Dpp* signaling is high close to the AP compartment boundary and low far from the AP compartment boundary. Consequently, *Dpp* targets are not expressed in cells far from the AP compartment boundary but

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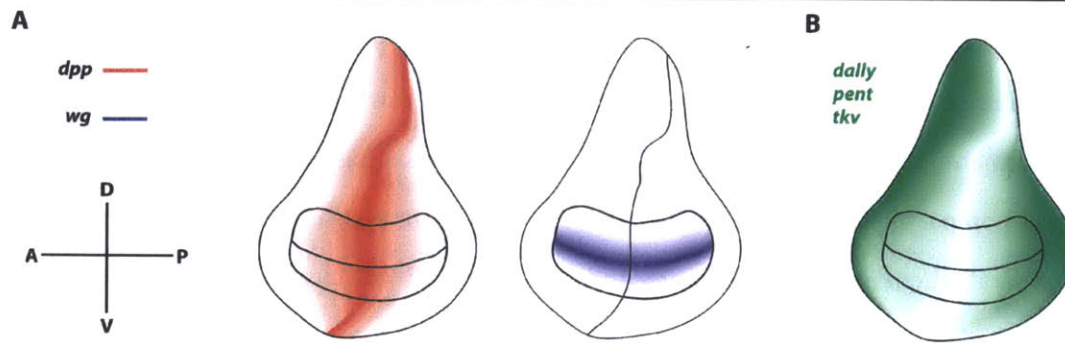


Fig. 4. Expression gradients in the Drosophila wing disc (A) *dpp* is expressed at the AP compartment boundary and forms a medial to lateral gradient, while *wg* is expressed at the DV compartment boundary and forms a medial to lateral gradient. (B) *tkv*, *dally*, and *pent* are pro Dpp factors that are negatively regulated by Dpp and are expressed in areas in which Dpp signaling is low.

are expressed in cells near the AP compartment boundary. At various thresholds of Dpp signaling, different target genes are expressed [39]. Importantly, the action of Dpp in this system can be visualized through an antibody for phosphorylated Mad (pMad) protein, the transcriptional effector of Dpp signaling [45]. The existence of a Dpp gradient along the AP axis of the wing disc can therefore be confirmed through visualization of pMad.

This Dpp gradient is subject to several forms of regulation. Firstly, Dpp negatively regulates expression of one of its receptors, *thickveins* (*tkv*) (Fig 4)[46]. The effect of this regulation is to allow for higher *tkv* expression further away from the Dpp source and thus produce greater sensitivity to Dpp ligand in

those cells. Furthermore, because internalization of Dpp ligand through receptor binding is the mechanism by which extracellular Dpp is depleted, regulation of *tkv* in this way facilitates the diffusion of Dpp further from the source. This occurs because the concentration of Tkv receptor, and therefore the amount of Dpp being bound and removed from the extracellular pool, is lower closer to the Dpp source. Consistent with this, flies overexpressing *tkv* near the AP boundary have a much narrower domain of pMad activation [46].

Another protein that regulates the Dpp gradient is Dally (Fig 4). *dally* encodes a GPI anchored proteoglycan that facilitates Dpp binding to its receptor in a cell-autono-

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mous fashion [43, 47]. Importantly, like *tkv* expression, expression of *dally* is negatively regulated by Dpp signaling [47]. This results in low *dally* expression near the AP boundary and high *dally* expression far from the AP boundary. *dally* is also expressed strongly at the boundary itself, but this is the result of *dpp*-independent regulation [48]. Given the distribution and function of Dally protein, *dally*, like *tkv*, seems to ensure that cells far from the Dpp source are more receptive and sensitive to Dpp signal.

A final regulator of the Dpp gradient is encoded by the recently discovered *pentagone* (*pent*) gene (Fig 4). *pent* encodes a secreted protein whose expression is required for normal spreading of the Dpp gradient [49]. *pent* mutant flies consequently have a narrow domain of pMad activation and develop abnormally proportioned wings. It is not yet known exactly how Pent exerts its effect on the Dpp gradient, but it has been shown to co-immunoprecipitate with Dally, suggesting that it somehow modulates the efficacy of Dpp receptor binding and internalization to allow for Dpp spreading [49]. A proposed func-

tion of Pent is therefore to divert extracellular Dpp from receptor-mediated internalization for the purpose of spreading the ligand. Because *pent* is repressed by Dpp signaling, this spreading activity is chiefly accomplished in regions relatively distant from the Dpp source (Fig 4).

From these examples of Dpp gradient regulation we can conclude that Dpp signaling in the wing disc directly regulates its own distribution through several means. In the cases of *tkv* and *dally* regulation, Dpp effects feedback inhibition of its own signaling. Returning to our original question of scaling, how could these regulatory interactions impact the ability of the Dpp gradient, and therefore AP wing pattern, to scale with size? The central consequence of Dpp negatively regulating both of these factors is that Dpp ligand, paradoxically, is able to move away from its source and be detected far from its source; these regulators allow distant cells to receive and interpret Dpp signal and in doing so prevent nearby cells from becoming saturated with signal. This mechanism of feedback inhibition may partially explain how the

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Dpp gradient scales. As the wing disc grows, some regions will be exposed to less Dpp signal than required to maintain the gradient's shape; the reduction in Dpp received by a cell will in turn stimulate upregulation of *tkv* and *dally*, thereby increasing the efficacy of the signal received and effectively buffering the gradient against the perturbation that growth presents. However, because *tkv* mutants lose most *dpp* signaling and because *dally* is involved in both *wg* and *hedgehog* signaling as well as *dpp* signaling, formally testing the roles of these genes in *dpp* gradient scaling may prove difficult.

pent mutants display a similar Dpp gradient phenotype as *dally* mutants in that they have a narrowed domain of pMad. Moreover, as mentioned above, *pent* is negatively regulated by Dpp signaling. Therefore, *pent* may function in scaling for the same reasons described above for *dally* and *tkv*. Consequently, the requirement of *pent* in Dpp gradient robustness and scaling has recently been tested. Strikingly, it was observed by monitoring pMad signal at several stages during wing disc growth that *pent* mutants completely fail

to scale the Dpp gradient during growth [50]. Instead, the original distribution of pMad remains unchanged despite massive changes in the size of the wing disc. From this we can conclude that *pent* is required for Dpp gradient and AP pattern scaling in the wing disc. This requirement can be conceptualized as discussed with respect to *tkv* and *dally* above: as the wing disc grows and regions along the AP axis become exposed to lower amounts of Dpp, they compensate by upregulating *pent* expression and thereby allowing for Dpp ligand to spread more effectively, restoring signaling to its proper level and expanding the pMad gradient.

Conceptual mechanisms and lessons learned

Both of the mechanisms of establishing robust pattern described above rely on the use of secreted ligands that receive complex feedback from their own signaling. Of particular importance is the use of feedback inhibition in both cases. In the case of embryonic self-regulation, feedback inhibition is observed as Bmp-mediated activation of the

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Bmp inhibitors *bambi* and *sizzled*, as well as *Admp* repressing its own expression through activation of the Bmp pathway. In the case of wing disc scaling, feedback inhibition is embodied by Dpp repressing expression of its receptor *tkv* as well as its co-receptor *dally* and *pent*. Feedback inhibition has the effect of mitigating increases in signaling. Likewise, decreases in signaling in these circuits will lead to derepression of signaling. It is in this way that this regulatory motif lends itself to the maintenance of a homeostatic set point of signaling. Feedback inhibition inherently buffers a regulatory circuit against perturbations in either direction.

Feedback inhibition *per se*, however, is not enough to fully describe the mechanisms at work in these examples. This is because spatial properties of morphogen gradients must also be considered. For example, though Bmp signaling in the *Xenopus* embryo activates *bambi* and *sizzled*, both of these are local factors and inhibition of either has mild but not catastrophic effects on gradient structure and scaling [21, 22]. Rather, it is the long-range communication between Bmp signaling and

dorsal *admp* that is crucial for self-regulation [28]. Likewise, in the *Drosophila* wing disc a factor expressed distantly from the morphogen source, *pent*, is the only factor whose inhibition has been observed to abolish scaling [50]. In both of these cases, the simple motif of feedback inhibition can be more specifically described as inhibition of a morphogen activator by the morphogen itself. In the case of *admp*, Bmp is the morphogen that inhibits *admp* expression, resulting in expression of a Bmp pathway activator distant from the Bmp source. In the case of *pent*, Dpp inhibits *pent* expression, ensuring that *pent* spreads Dpp ligand and increases Dpp signaling distant from the source.

Recent mathematical modeling has demonstrated that such a regulatory motif is indeed capable of regulating scaling. Termed “expansion-repression” feedback control, this model proposes that scaling arises as an inevitable consequence of a morphogen gradient system that has the following properties: 1) the range of the morphogen gradient in question is expanded by the abundance of a second diffusible molecule and 2) expression

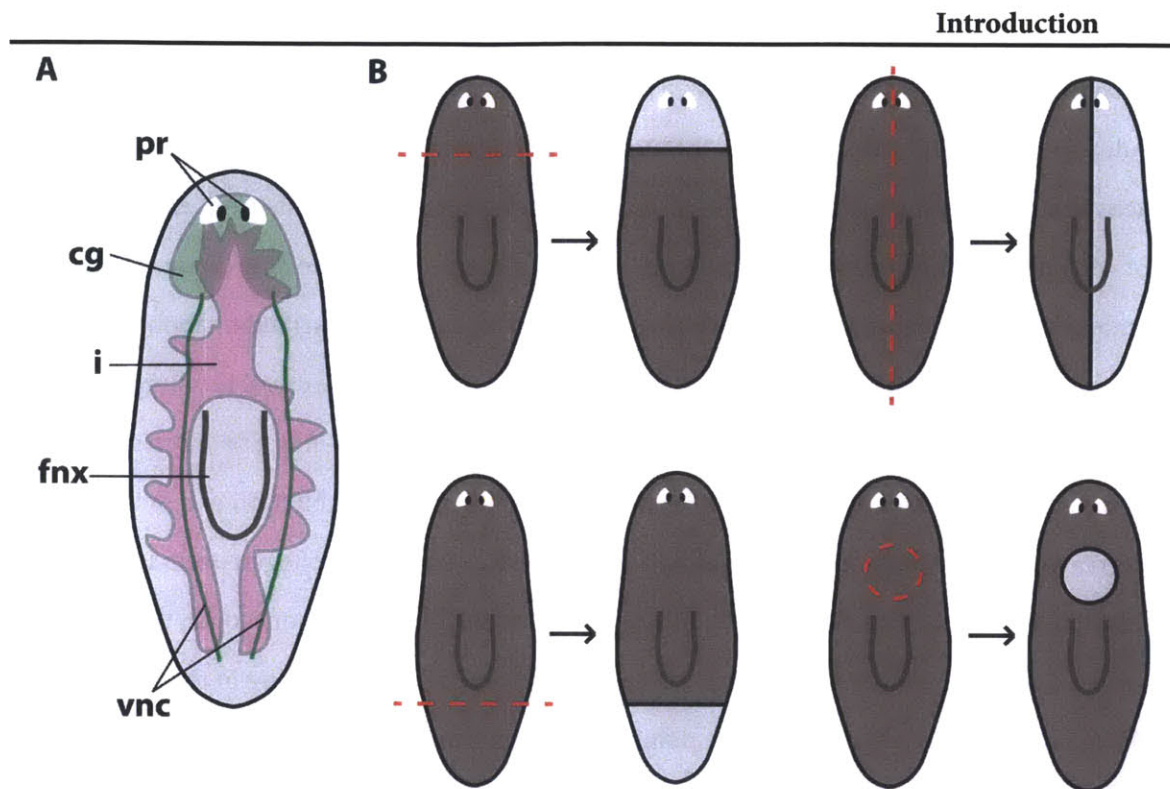


Fig. 5. Planarians as a model system for regeneration. (A) Planarians possess a complex anatomy including photo-receptors (pr), a cephalic ganglia (cg), an intestine (i), a muscular pharynx (fnx), and two ventral nerve cords (vnc). (B) Planarians are capable of regenerating following nearly any type of injury. Depicted are several different inflicted injuries (red dotted lines) after which animals form a regeneration blastema (light gray). This process takes from one to two weeks, depending on the type of injury. Dorsal view, Anterior up for all depictions.

of this “expander” molecule is repressed by morphogen signaling [51]. This description fits the regulatory motif governing *admp/bmp* signaling in *Xenopus* embryos and *dpp/pent* signaling in the *Drosophila* wing disc and further suggests that examples of this motif may allow for scaling of gradients in a number of developmental contexts.

From the findings described above we can conclude firstly that feedback inhibition

can function at several levels to buffer patterning systems to perturbation. Secondly, we can conclude that a specific type of feedback inhibition as described by the “expander-repression” model allows for morphogen gradient scaling in at least two distinct developmental contexts. Though it remains to be seen whether completely different regulatory topologies are used routinely throughout development for conferring robustness, it seems likely that

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the core mechanism described above will be discovered in wide-ranging systems.

III. Regeneration as a form of canalized development

The need for canalization is perhaps no more obvious than when considering the remarkable feats of regeneration that many animals are capable of achieving. In many ways, regeneration resembles a latent form of adult development that is triggered by the disruption of a homeostatic state. What is remarkable however about this form of “development” is that it can produce a consistent output with widely varying starting material. For example, an animal capable of whole-body regeneration must produce a whole animal irrespective of whether it begins with only a head, a posterior, a fragment that contains an overabundance of a specific tissue type, or a fragment that contains none. This property can also be seen in the regeneration of individual organs or body structures: a regenerating limb, for example, may begin with only a small fraction of its original mass, or

it may begin with the majority of its original mass, but in each case it must produce a final structure of the same size [52].

Some properties that become apparent when considering these problems is that mechanisms must exist so that regenerating systems are able to sense: 1) the identity of the tissues that are missing; and 2) the size of the tissues to be regenerated relative to the size of the organism. Therefore, while regenerative systems are subject to many of the same issues that embryos must face, there is the additional problem of the extreme variability inherent in the process of injury. In this sense, regenerative processes represent perhaps the most stringent test of developmental robustness.

This robustness affords unique advantages from an experimental perspective. For example, in a regenerative context it is possible to produce a wide variety of injuries that each present a unique challenge to the system in question. Moreover, these challenges can be combined with gene perturbation; in non-regenerating developmental contexts, experimentally induced challenges are largely

limited to genetic manipulations. Moreover, because a single animal can be subjected to several rounds of injury and regeneration, varied perturbations can be performed in the same animal, thereby removing genetic variability. The study of regeneration therefore represents a powerful tool for investigating mechanisms of robustness against a fixed genetic background.

Moreover, regeneration is widespread among animals. Radially symmetric animals such as hydra and *Nematostella* are capable of whole body regeneration, as are bilaterians such as flatworms [52]. Furthermore, organ regeneration occurs across nearly all species examined: zebrafish can extensively regenerate tail fins, heart and eye; amphibians can regenerate tails and eyes; and mammals can regenerate skin lesions and liver [53, 54]. Therefore by investigating regeneration across diverse species, key conserved features that govern robustness in these processes can be uncovered.

IV. Planarians as a model for the study of regeneration

Among the many examples of regeneration in the animal kingdom, the regenerative capacities of planarian flatworms are possibly the most remarkable. Planarians are capable of regenerating entire animals from nearly any possible injury (Fig 5). Though planarians were first systematically studied by T.H. Morgan in the late 1800s, only recently have molecular tools for the investigation of planarian regeneration become available. Among other methods, *in situ* hybridization can be used to monitor gene expression in whole animals, and RNA interference (RNAi) can be used to perturb gene function [55]. Consequently, planarian regeneration has become a major model for investigating the molecular underpinnings of regenerative processes at large.

Planarians possess a complex anatomy including a central nervous system, two ventral nerve cords, an intestine, an excretory system, a complex musculature, photoreceptors, and other organs [56] (Fig 5). This anatomical

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complexity makes their regenerative capacities all the more remarkable. Planarians regenerate through the production of un-pigmented outgrowths at wound sites called regeneration blastemas. These outgrowths develop the appropriate missing tissue as regeneration proceeds and within a week a functional animal is regenerated [56]. Beyond regeneration, however, planarians also display the remarkable ability to both grow and “de-grow” depending on the level of nutrient intake [56]. Remarkably, small animals that have de-grown otherwise retain the proper proportions of tissues and appear nearly indistinguishable from larger animals.

Where does the tissue produced during regeneration come from? The source of new tissue in planaria is a population of dividing cells called neoblasts. Neoblasts are characterized by scant cytoplasm and are localized to a parenchymal space excluded by the animal’s intestine [56]. The requirement for neoblasts in regeneration is supported by two observations: 1) neoblasts display a potent mitotic response to injury; and 2) animals in which neoblasts are ablated fail to regener-

ate [56-58]. In addition to regenerating, the constant tissue turnover animals experience as adults also requires neoblasts, as animals in which neoblasts are ablated will form lesions and eventually lyse. Within the population of neoblasts exist pluripotent stem cells called “clonogenic neoblasts” or cNeoblasts [59]. Amazingly, a single cNeoblast is capable of replenishing the totality of tissues and the ability to regenerate when transplanted into a neoblast-less host animal [59]. We can conclude therefore that new tissues in planarian regeneration and homeostasis are derived from the proliferation of adult stem cells.

Besides the question of how new tissue is produced, a second major question of planarian regeneration is how this tissue is given pattern. Importantly, planarians utilize conserved signaling pathways to establish polarity along the AP and DV axes [4, 60]. Canonical Wnt signaling patterns the AP axis, with Wnt proteins being secreted from the posterior of the animal and Wnt inhibitors being secreted from the anterior [61]. The necessity of Wnt signaling for establishing proper AP pattern after injury has been demonstrated by the

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observation that inhibition of the Wnt effector β -catenin causes heads to form at non-anterior wound-sites [62, 63]. To regenerate DV polarity, planarians use Bmp signaling [64, 65]. Like in the embryos of other protostomes, such as *Drosophila*, Bmp is secreted from the dorsal side of adult planarians and experimental inhibition of Bmp pathway components leads to ventralized blastemas. Interestingly, inhibition of either of Wnt or Bmp signaling in the absence of injury causes intact animals to gradually lose polarity along the respective axis over a period of weeks. In the case of β -catenin inhibition, animals become radialized, with heads present all along the AP axis [62, 66]. In the case of Bmp pathway inhibition, animals gradually become ventralized and lose dorsal-specific gene expression [65]. These results suggest that patterning mechanisms are not only active during regeneration in planaria, but are also continuously active during normal adult tissue turnover.

The robust ability of planaria to accommodate the diverse perturbations that trigger regeneration makes them an ideal system in which to study intra-species ca-

nalization. Furthermore, because of their use of conserved developmental pathways, this model system also presents an inroad into understanding the mechanisms by which developmental systems have been canalized to function across broad evolutionary spans. In the following chapters I will present work that identifies a key conserved mechanism of DV pattern regulation during planarian regeneration and adult tissue turnover, and work that examines a mechanism of missing tissue measurement following injury. These findings represent early steps toward describing comprehensively how animals detect the nature and magnitude of perturbations produced by injury and therefore how animals modulate regenerative mechanisms to accommodate these perturbations.

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Introduction

Chapter 2

A Bmp/Admp regulatory circuit controls maintenance and regeneration

of dorsal-ventral polarity in planarians

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Published as:

Gavino, M.A., and Reddien, P.W. (2011). A Bmp/Admp regulatory circuit controls maintenance and regeneration of dorsal-ventral polarity in planarians. *Curr Biol* 21, 294-299.

Abstract

Animal embryos have diverse anatomy and vary greatly in size. It is therefore remarkable that a common signaling pathway – BMP signaling – controls development of the dorsoventral (DV) axis throughout the Bilateria [1-8]. In vertebrates, spatially opposed expression of the BMP-family signaling proteins Bmp4 and Admp (anti-dorsalizing morphogenetic protein) can promote restoration of DV pattern following tissue removal [9-11]. *bmp4* orthologs have been identified in all three groups of the Bilateria (deuterostomes, ecdysozoans, and lophotrochozoans) [12]. By contrast, the absence of *admp* orthologs in ecdysozoans such as *Drosophila* and *C. elegans* has suggested that a DV regulatory circuit of oppositely expressed *bmp4* and *admp* genes represents an innovation specific to deuterostomes. Here we describe the existence of spatially opposed *bmp* and *admp* expression in a protostome. An *admp* ortholog (*Smed-admp*) is expressed at the ventral pole and laterally in adult *Schmidtea mediterranea* planarians, spatially opposing the dorsal-pole domain of *Smed-bmp4* expression. *Smed-admp* is required for planarian regeneration following parasagittal amputation. Furthermore, *Smed-admp* promotes *Smed-bmp4* expression and *Smed-bmp4* inhibits *Smed-admp* expression, generating a regulatory circuit that buffers against perturbations of Bmp signaling. These results suggest that a Bmp/Admp regulatory circuit is a central feature of the Bilateria, used broadly for the establishment, maintenance, and regeneration of the DV axis.

Results and Discussion

Spatially opposed expression of *bmp* and *admp* genes in adult planarians

Planarians are flatworms famous for their regenerative capacities. The ability of planarians to regenerate entire adult animals from small tissue fragments makes them well suited for study of body axis polarization and patterning [13]. Furthermore, their phylogenetic position as a member of the protostome superphylum the Lophotrochozoa makes them ideal for identifying features that are conserved across the Bilateria.

Planarians utilize a dorsally expressed *bmp4* ortholog, *Smed-bmp4* (in short, *bmp4*), to maintain and regenerate the DV axis [2-4]. We isolated a putative *admp* ortholog in the planarian *Schmidtea mediterranea* that is to our knowledge the first characterized in a protostome [14-16] (Figure S1 and see functional data below). We cloned two highly similar *admp* sequences (*Smed-admp-1a* and *Smed-admp-1b*, see Figure S1 and

experimental procedures for details); it is unknown whether these sequences reflect the existence of distinct *admp* alleles or of highly similar *admp* paralogs. We refer to a single gene in this text as *Smed-admp* (in short, *admp*).

admp expression was detected in sub-epidermal cells on the ventral animal midline and around lateral animal edges at the dorsal/ventral boundary (Figures 1A and 1B). These ventral and lateral domains spatially oppose the *bmp4* expression domain on the dorsal midline [2, 3]. Double-labeling with *admp* and *bmp4* RNA probes revealed that expression of these genes does not detectably overlap (Figure 1C). *admp* expression opposing *bmp4* expression in planarians is noteworthy, as it provides the first example of spatially opposed *bmp* and *admp* expression outside of the deuterostome lineage. Following head and tail amputation, lateral *admp* expression first appeared at wound sites by 48h whereas ventral *admp* expression decreased at 24h, 48h, and 60h before increasing again at 72h (Figure S2A). These data indicate that ventral *admp* expression is regulated

A *bmp/admp* regulatory circuit in planaria

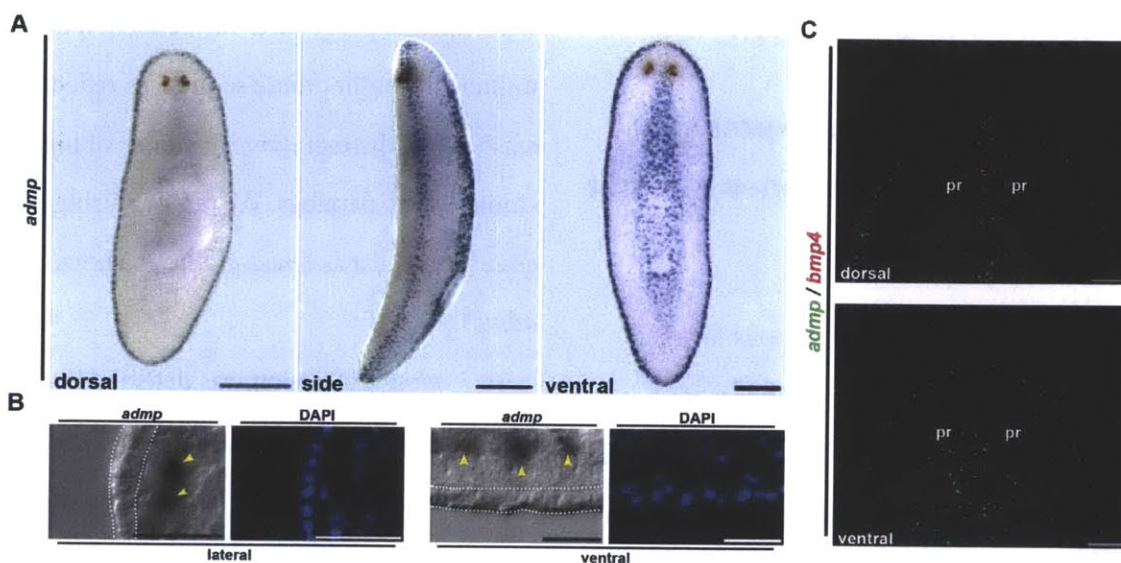


Figure 1. *Smed-admp* is expressed ventrally and laterally. (A) *in situ* hybridization with *Smed-admp* RNA probe displayed ventral and lateral expression. (B) Transverse sections (20 micron), differential interference contrast (DIC) images: *admp*-expressing cells were subepidermal (yellow arrowheads). White lines: epidermis. (C) Wild-type animals double-labeled with *Smed-admp* (green) and *Smed-bmp4* (red) RNA probes. Pr: photoreceptors. Bars: 200 microns for (A), (C); 20 microns for (B). Anterior, up.

following transverse amputation, possibly by wound-induced factors. *admp* expression was not detected dorsally at any point during regeneration (Figure S2A). Together, *admp* and *bmp4* form complementary expression domains that identify the dorsal and ventral midlines as well as the lateral, dorsal-ventral boundary of animals.

Smed-admp is required for lateral planarian regeneration

To investigate the role of *admp* in regeneration, we inhibited *admp* expression with RNA interference (RNAi) and amputated animal heads and tails. Planarian regeneration involves new tissue outgrowth at wound sites called a blastema [13]. *admp*(RNAi) fragments displayed regeneration defects including indented head and tail blastemas, a hallmark phenotype of planarian Bmp-pathway

A *bmp/admp* regulatory circuit in planaria

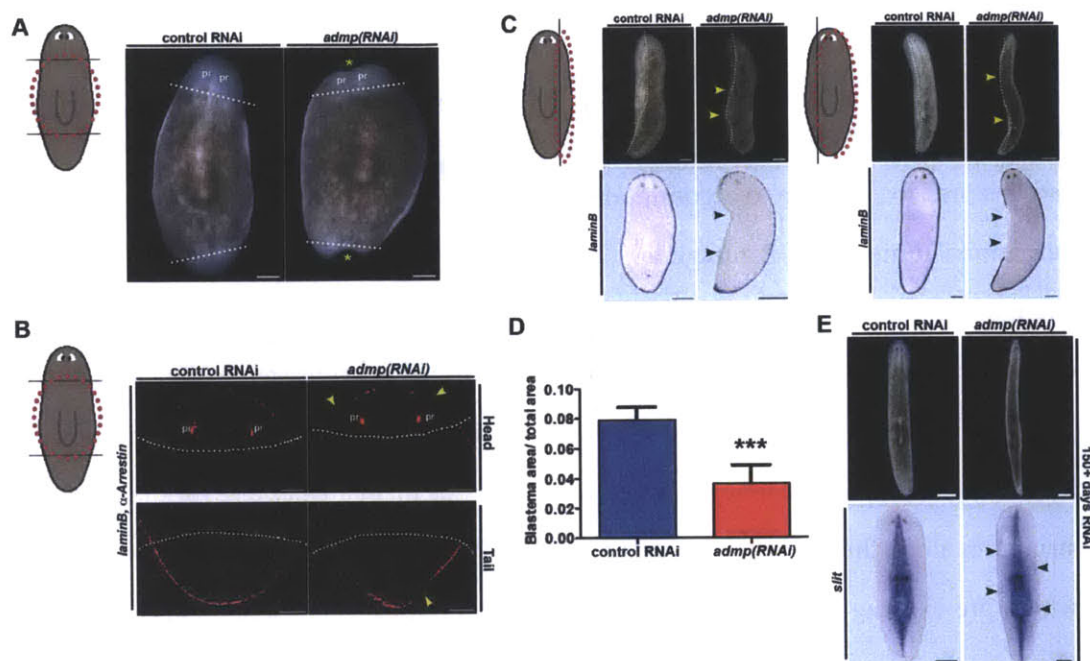


Figure 2. *Smed-admp* is required for planarian regeneration. (A) Transversely amputated *admp*(RNAi) animals displayed aberrant midline regeneration (yellow asterisks, n= 14/20). Pr: photoreceptors. Bars: 200 microns. (B) Transversely amputated *Smed-admp*(RNAi) animals failed to regenerate DV boundary, *laminB*⁺ cells (yellow arrowheads, n=7/7). Scale bars, 200 microns. (C) *admp*(RNAi) animals failed to regenerate a missing side (yellow arrowheads, n=22/26 thin and 19/19 thick fragments) and lateral *laminB*⁺ tissue (black arrowhead, n=13/16 thin and 19/19 thick fragments) following parasagittal amputation. Bars: top, 500 microns; bottom, 200 microns. (D) Blastema size: unpigmented tissue at amputated sides (thick fragments), from anterior pharynx tip to tail, divided by worm area (difference with control was significant, p=0.0006, unpaired t-test). (E) Non-amputated *admp*(RNAi) animals became thinner than control animals following 155 days of RNAi and aberrantly expressed the ventral midline marker *slit* [31] at lateral positions following 163 days of RNAi (n=7/7, black arrowheads). RNAi of *admp* was shown to be effective and specific (see Figures S2B and S2C). White lines: approximate blastema boundary. Dorsal view, anterior up.

dysfunction [2, 3], as well as uncoordinated movement (Figure 2A, and Movies S1 and S2). *in situ* hybridization with a marker for lateral-edge cells identified defects in regeneration of lateral DV boundary tissue (Figure 2B). We conclude that *admp* is required for the regeneration of tissues at lateral animal edges,

at the midpoint between dorsal and ventral poles, following transverse amputation.

Bmp signaling is crucial for lateral planarian regeneration following sagittal amputations [2, 3]. Parasagittal amputation produces two left-right asymmetric fragments: a thin fragment that must

regenerate an appropriately sized *bmp4* expression domain *de novo*, and a thick fragment that must reposition and rescale its *bmp4* expression domain to accommodate new animal dimensions. Parasagittal amputations therefore present a stringent test of establishment and scaling of DV as well as medial-lateral (ML) pattern. *admp(RNAi)* thin fragments were able to regenerate some structures along the anteroposterior (AP) axis within pre-existing tissue (photoreceptors and pharynx); however, they failed to regenerate a new side and corresponding lateral marker expression (Figure 2C). *admp(RNAi)* thick fragments also failed to regenerate a side (Figures 2C, 2D, and S2D). Furthermore, non-amputated *admp(RNAi)* animals displayed aberrant body dimensions and ML marker expression following several months of *admp* inhibition (Figures 2E, S3, and Movie S3), suggesting that *admp* is crucial for maintaining body form and proper ML pattern during animal homeostasis. We conclude that *admp* is required for lateral planarian regeneration and ML pattern maintenance.

***Smed-admp* promotes *Smed-bmp4* expression**

The indented head and tail blastemas and the failed lateral regeneration in *admp(RNAi)* animals are consistent with a defect in Bmp signaling [2, 3]. *bmp4* promotes dorsal tissue maintenance and regeneration; we therefore investigated the role of *admp* in DV patterning. Whereas animals inhibited for *admp* expression alone did not show dorsal expansion of ventral markers, *admp(RNAi)* animals exposed to a low dose of *bmp4* dsRNA became more ventralized near wound sites than did control animals exposed to the same *bmp4* dsRNA dose (Figure 3A). These results indicate that animals depleted of *admp* activity become hypersensitive to small decreases in Bmp signaling level during DV axis regeneration.

We next assessed whether *admp* influences *bmp4* expression. Thin fragments produced by parasagittal amputation must re-express *bmp4* during regeneration.

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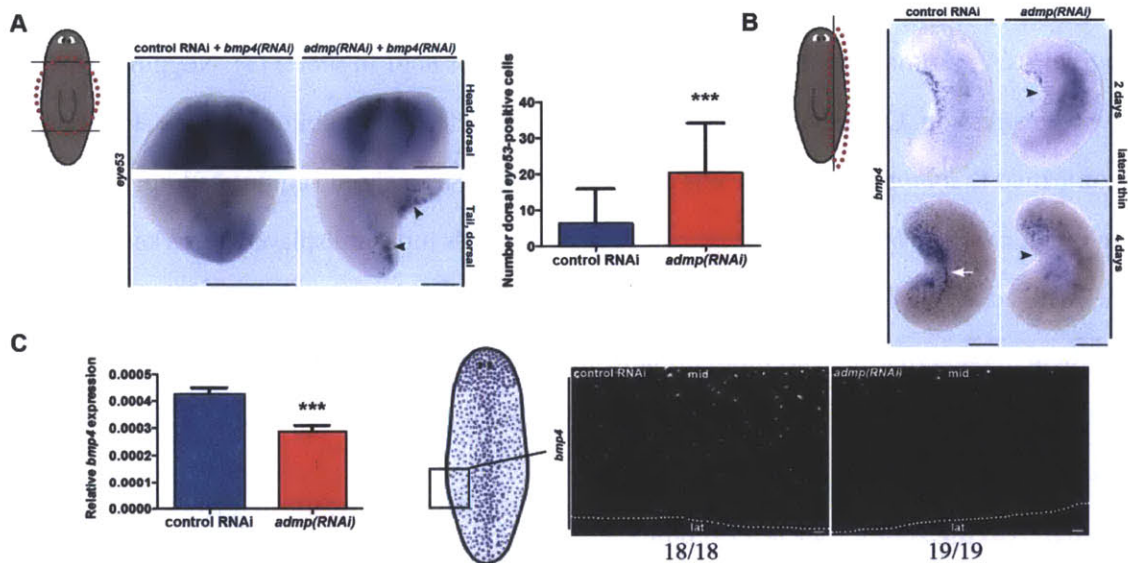


Figure 3. *Smed-admp* inhibits ventral fates and is required for normal *Smed-bmp4* expression. (A) *Smed-bmp4* dsRNA addition in *Smed-admp(RNAi)* animals caused ectopic dorsal *eye53* expression (black arrowheads). Out of focus signal in control animals is from ventral cells. Difference in dorsal *eye53*-expressing cell numbers was significant, $p < 0.0001$, unpaired t-test. (B) *admp(RNAi)* thin fragments had reduced *Smed-bmp4* expression (black arrowheads, $n=5/6$ and $n=9/15$ at 2 and 4 days, respectively) and failed to reposition *Smed-bmp4* expression as in control animals (white arrow). (C) Left, *bmp4* expression depiction. Eight micron optical sections (identical exposures) from left, post-pharyngeal regions of intact *admp(RNAi)* and control RNAi animals. *admp(RNAi)* animals had reduced *bmp4* expression (37/37 animals blindly scored correctly, three independent experiments). mid: medial dorsal, lat: lateral dorsal. Right, *bmp4* expression was reduced in intact *admp(RNAi)* animals (by quantitative RT-PCR). Difference was significant, $p < 0.0001$, paired t-test. RNAi of *bmp4* was shown to be effective (see Figure S4B). White lines: approximate lateral animal edge. Bars: 200 microns for (A), (B); 20 microns for (C). Dorsal view, anterior up.

admp(RNAi) thin fragments displayed reduced *bmp4* expression and this expression domain did not reposition to reflect a new dorsal midline (Figure 3B). These results indicate that *admp* promotes *bmp4* expression and controls the positioning of *bmp4* expression during regeneration of left-right asymmetric fragments. Whether *Admp*

signaling regulates *bmp4* expression by direct action or through some other mechanism is unknown.

In addition to regenerating, planarians undergo extensive tissue turnover and growth as adults - processes that also require patterning genes for instructing new cell identities [17-19]. Consequently, if *admp*

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promotes *bmp4* expression, non-amputated *admp(RNAi)* animals should display reduced *bmp4* expression. Quantitative RT-PCR confirmed that *bmp4* expression was reduced in intact *admp(RNAi)* animals (Figure 3C). This decrease in *bmp4* expression was particularly apparent in cells more distal from the dorsal midline (Figure 3C). Together these data indicate that Admp signaling is required to maintain the appropriate level and broad spatial distribution of *bmp4* expression during adult tissue maintenance and growth.

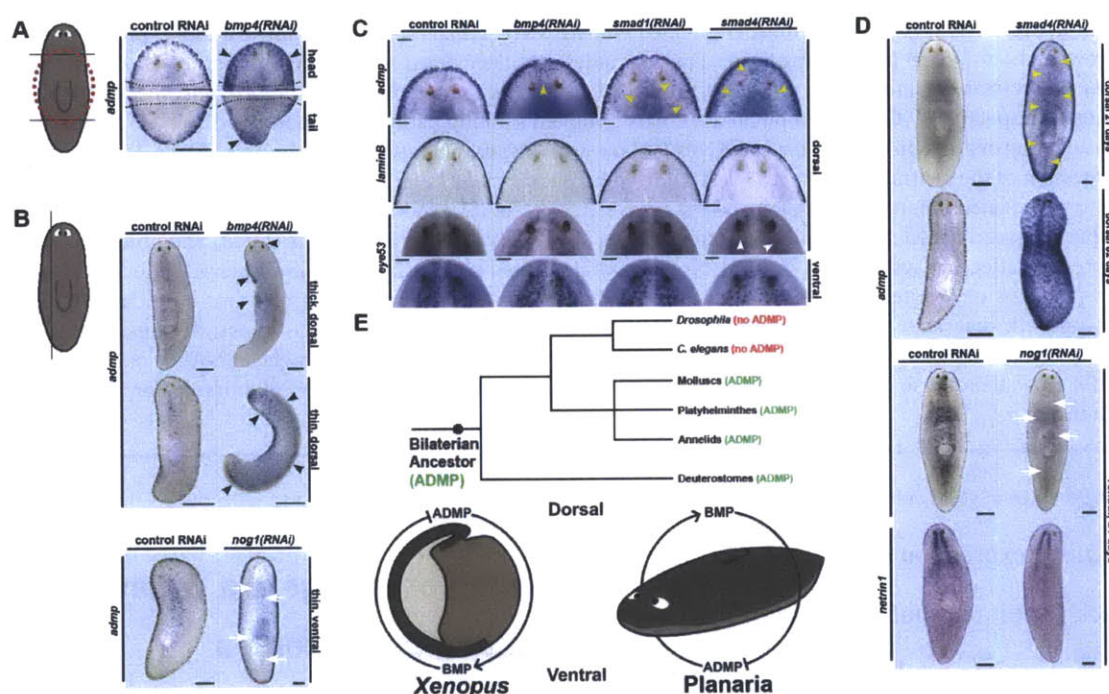
***Smed-bmp4* inhibits *Smed-admp* expression**

To determine whether *admp* is regulated by Bmp4 signaling, we examined the effect of Bmp pathway inhibition on *admp* expression. In both transversely and parasagittally amputated *bmp4(RNAi)* animals, *admp* expression was increased and expanded dorsally (Figures 4A and B). To conversely test whether an increase in Bmp signaling leads to a reduction in *admp* expression, we inhibited a ventrally

expressed planarian *noggin* homolog (*Smed-nog1* or *nog1* in short) [3]. Noggins are well-characterized inhibitors of Bmp signaling [20]. Parasagittally amputated *nog1(RNAi)* animals indeed displayed a marked decrease in ventral *admp* expression (Figure 4B). Together these results indicate that *admp* expression is negatively regulated by Bmp signaling.

We next investigated whether the change in *admp* expression observed in *bmp4(RNAi)* animals reflected failure of specific regulation of *admp* or was the simple consequence of ventralization. Following Bmp pathway inhibition, we compared expression of *admp* to genes with similar ventral or lateral expression domains. Whereas regeneration occurs quickly (within days), intact non-amputated animals inhibited for *bmp4* or the Bmp effectors *smad1* or *smad4* gradually become ventralized over a period of weeks [2, 3]. This slow transformation allows for greater temporal resolution in assessing the changes in gene expression that occur following Bmp signaling loss. After three weeks of RNAi, intact *bmp4(RNAi)*, *smad1(RNAi)*,

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and *smad4(RNAi)* animals all displayed dorsal expression of *admp*, despite little to no expansion in the expression of other tested genes (Figure 4C). Strikingly, *smad4* inhibition resulted in broad dorsal expansion of *admp* expression after three weeks of RNAi and ubiquitous DV expression of *admp* after 82 days of RNAi (Figure 4D). In both of these cases, inhibition of *smad4* resulted in more extensive dorsal expression of *admp* than did inhibition of either *smad1* or *bmp4* (Figure 4C and Figure S4C). Because Smad4 proteins are

required for all forms of TGF β superfamily signaling [21], these results suggest that *admp* expression may also be regulated by non-Bmp TGF β signaling. In contrast to Bmp pathway RNAi, three weeks of *nog1* RNAi in intact animals caused a potent reduction in the ventral *admp* expression domain without affecting other ventral markers (Figure 4D and Figure S4A). These data indicate that *admp* expression is negatively regulated by Bmp signaling during adult homeostasis and growth. Together with the observation that *admp* promotes

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Figure 4. *admp* expression is negatively regulated by Bmp signaling. (A) Transversely amputated *bmp4(RNAi)* animals had ectopic, dorsal *admp* expression (black arrowheads, n=14/14) in pre-existing tissue. 19 days of regeneration, dorsal view. (B) Parasagittally amputated *bmp4(RNAi)* animals had ectopic dorsal *admp* expression (Black arrowheads, n=5/5) in pre-existing tissue; parasagittally amputated *Smed-nog1(RNAi)* animals had reduced ventral *admp* expression (White arrows, n=20/20). 14 and 19 days of regeneration for *bmp4(RNAi)* and *Smed-nog1(RNAi)* animals, respectively. (C) Non-amputated animals inhibited for Bmp pathway components displayed ectopic dorsal *admp* expression after 21 days of RNAi (yellow arrowheads, n > 9/9 for each condition). Weak dorsal expression of the ventral marker *eye53* was detected in *Smed-smad4(RNAi)* animals (white arrowheads, n=5/11). Ventral and lateral marker expression was otherwise unaffected. (D) Non-amputated *Smed-smad4(RNAi)* animals displayed broad dorsal *admp* expression after 21 days of RNAi (yellow arrowheads, n=23/23) and ubiquitous *admp* expression after 82 days of RNAi (n=5/5). Non-amputated *Smed-nog1(RNAi)* animals displayed reduced ventral *admp* expression (white arrows, n=8/8) but normal *netrin1* expression. Dotted lines: blastema boundary. (E) Top, phylogenetic diagram of bilaterians annotated with the existence of putative *admp* orthologs. Bottom, schematic of proposed conserved Bmp-Admp circuit in *Xenopus* embryos (left) and planarians (right). RNAi of *smad1*, *smad4*, and *nog1* was shown to be effective (See Figure S4B). Bars: 100 microns for (A), (C); 200 microns for (B), (D). Anterior, up.

bmp4 expression, we propose that inhibition of *admp* expression by Bmp4 produces a feedback circuit that buffers against fluctuations in Bmp signaling levels, conferring robustness in DV and ML patterning. This model is supported by the observation that *admp* depletion results in animals that are hypersensitive to *bmp4* inhibition. Although planarians lack identified orthologs of the Bmp modulators *chordin* [22] and *sizzled* [23], the presence of a homolog of the Bmp pseudoreceptor *bambi* [24] (Figure S4D), as well as a greatly expanded family of *noggin* genes [25] and the ability of Admp to regulate *nog1* expression (Figures S2D and S3D), suggests that additional regulatory mechanisms likely function to fine tune the activity of this central Bmp/Admp circuit.

Admp orthologs are widespread among protostomes

Because *Smed-admp* represents the first *admp* ortholog characterized in a protostome, we searched the genomes of other lophotrochozoans to determine whether *admp* orthologs are widespread in protostomes. Indeed, predicted *admp* orthologs were identified in the genomes of the snail *Lottia gigantea*, the leech *Helobdella robusta*, and the polychaete annelid *Capitella teleta* (Figure S1C). The presence of putative *admp* orthologs in these species, coupled with the expression pattern and functional properties of *Smed-admp*, suggest that a Bmp/Admp regulatory circuit

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is an ancestral and central feature of the DV axis (Figure 4E). This model predicts that an *admp* gene was present in the ancestor of *C. elegans* and *Drosophila* but subsequently lost in the evolution of these species; consequently the potential widespread significance of *admp* genes for the DV axis of Bilaterians was previously unknown.

Conclusions

Development proceeds in a remarkably reliable fashion despite the myriad forms that embryos assume and widely varying conditions they encounter [26]. Robust patterning of the DV axis is a crucial component of this process. The DV axis can scale during growth and, in some cases, is capable of restoration following surgical manipulation [9, 27-29]. How Bmp signaling is able to generate consistent DV pattern in diverse species and respond appropriately to perturbation has been a central mystery in developmental biology. Our data demonstrate that a molecular circuit of spatially opposed *bmp4* and *admp*

expression is crucial for the regeneration and maintenance of both DV and ML pattern in planarians. This circuit may function to buffer against changes in Bmp level that naturally arise from differences in patterned tissue size, the genotype of individuals, or environmental influences encountered. The requirement of a Bmp/Admp circuit for both planarian regeneration and deuterostome self-regulation [10, 11, 16] suggests that restoration of the DV axis in embryonic regulation and adult axial regeneration share mechanistic features. The presence of spatially opposed expression of *bmp* and *admp* in planarians (lophotrochozoans) and in several deuterostomes [15, 16, 30], suggests that a Bmp/Admp circuit is a widespread feature of the DV axis that emerged concurrent with the first bilaterally symmetric animals.

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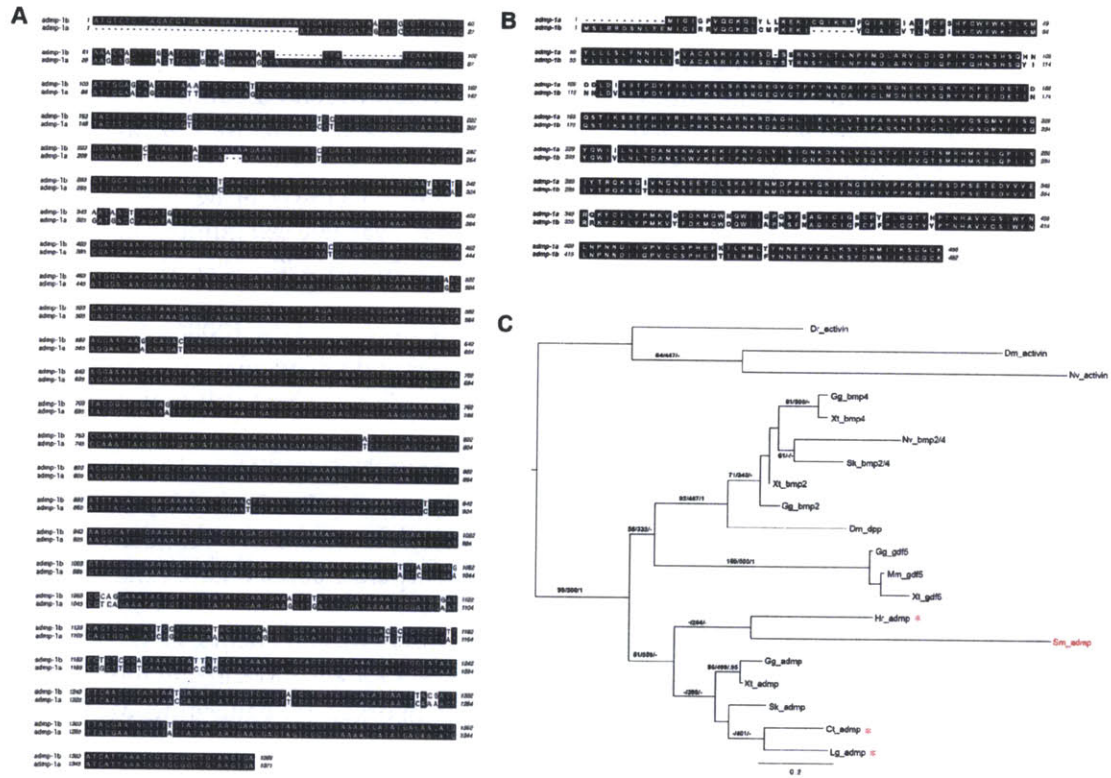
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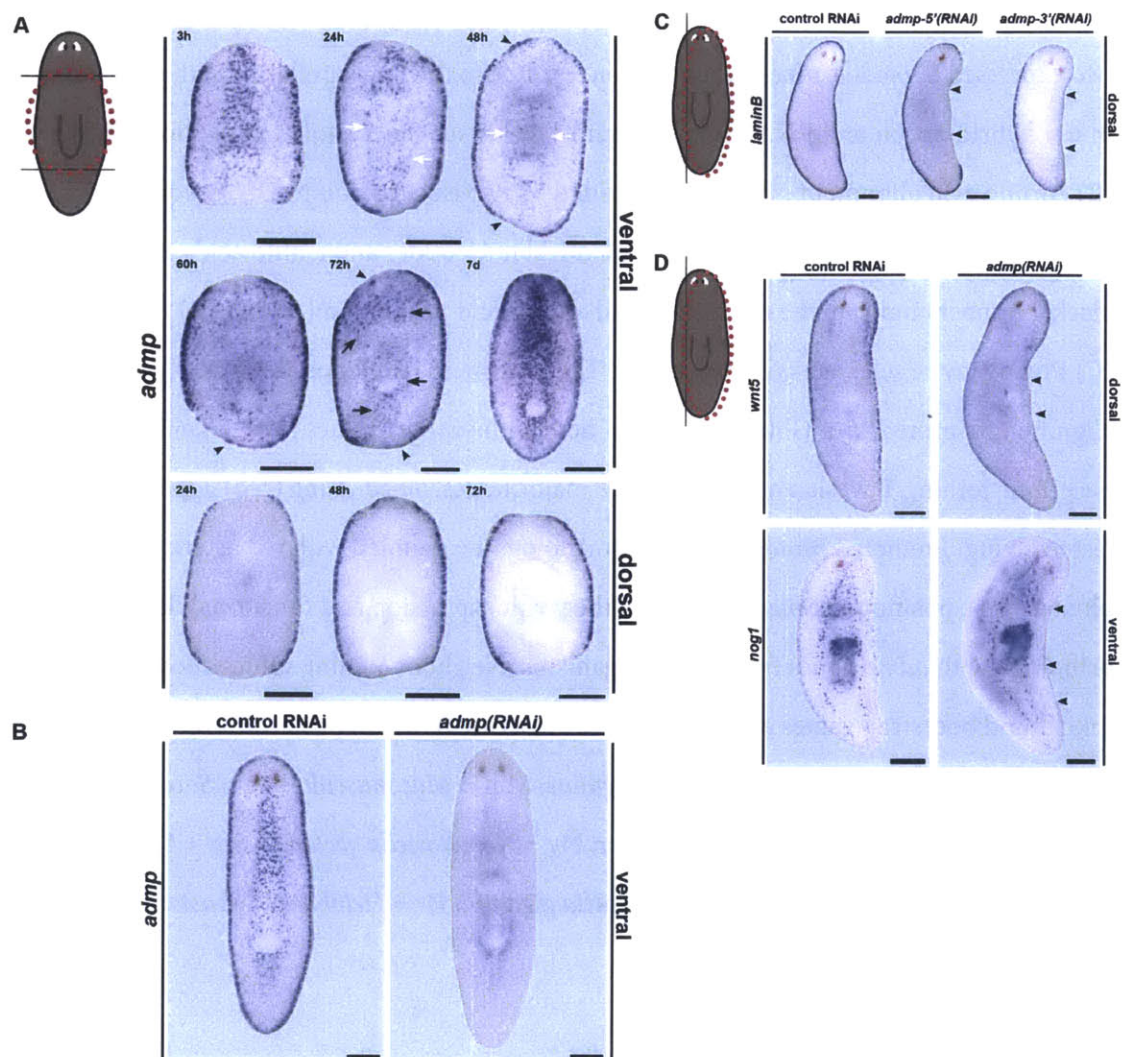
Supplemental Figures and Legends



A *bmp/admp* regulatory circuit in planaria

Figure S1. Sequence and phylogenetic analysis of *Smed-admp* (A) Nucleotide alignment of two expressed *S. mediterranea admp* sequences. Due to the high similarity of these sequences, *in situ* hybridization and RNAi directed against either sequence should target both sequences. (B) Amino acid alignment of proteins encoded by two isolated *admp* cDNA sequences. Sequences were aligned using ClustalW. Identical nucleotides and amino acids are boxed in black. Numbers indicate the nucleotide and amino acid position in (A) and (B) respectively. (C) Phylogeny of selected TGF beta genes. The maximum likelihood tree based on a ClustalW alignment trimmed with Gblocks is shown here with support values from Likelihood/Neighbor-Joining/Bayesian analyses for the major nodes. *Smed-admp* (red) appears to be fast evolving. Predicted protostome *admp* orthologs are denoted with red asterisks. The phylogenetic position of *Smed-admp*, together with expression and functional data, support orthology with *admp* genes from other organisms. Neighbor-joining values above 250 and Likelihood bootstrap values above 50 are shown, as are Bayesian posterior probabilities above 0.95. Xt = *Xenopus tropicalis*; Gg = *Gallus gallus*; Mm = *Mus musculus*; Sk = *Saccoglossus kowalevskii*; Dm = *Drosophila melanogaster*; Nv = *Nematostella vectensis*; Sm = *Schmidtea mediterranea*; Ct = *Capitella teleta*; Lg = *Lottia gigantea*; Hr = *Helobdella robusta*; Dr = *Danio rerio*.

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Figure S2. Additional analyses of *admp* expression and knockdown. **(A)** *admp* expression following head and tail amputation. Expression of *admp* in the ventral domain decreased between 24h and 60h (white arrows) before increasing again at 72h (black arrows). Expression of *admp* in the lateral domain began to return to wound sites at 48h, 60h, and 72h (black arrowheads). At no point during regeneration was dorsal *admp* expression observed. **(B)** *admp(RNAi)* animals displayed greatly reduced *admp* expression (n = 6/6) **(C)** Animals inhibited for *admp* expression using dsRNAs complementary to either the 5' half or the 3' half of the *admp* gene (*admp-5'* or *admp-3'*, respectively) recapitulated the *admp(RNAi)* lateral regeneration phenotype observed in animals treated with full length dsRNA (n = 6/9 for *admp-5'* and n = 4/8 for *admp-3'*). **(D)** *admp(RNAi)* thick fragments failed to regenerate the lateral marker *wnt5* (n = 7/9, black arrowheads) or normal lateral expression of *nog1* (n = 9/9, black arrowheads) 10 days following parasagittal amputation. Bars: 200 microns. Anterior, up in all pictures.

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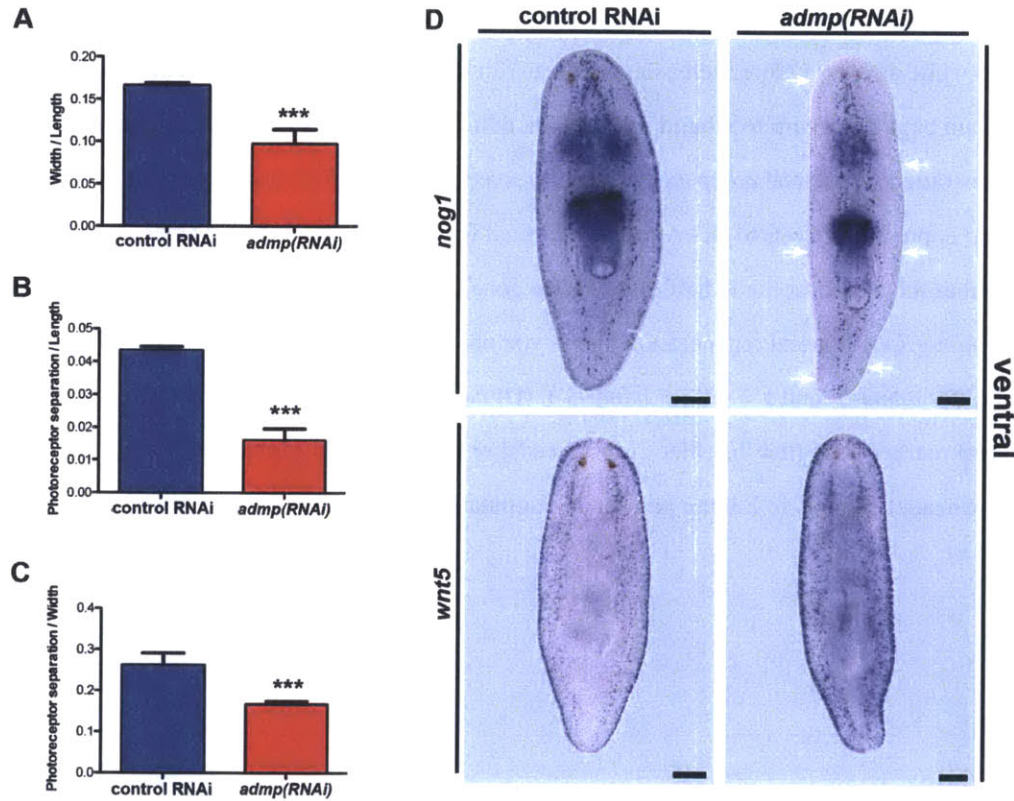
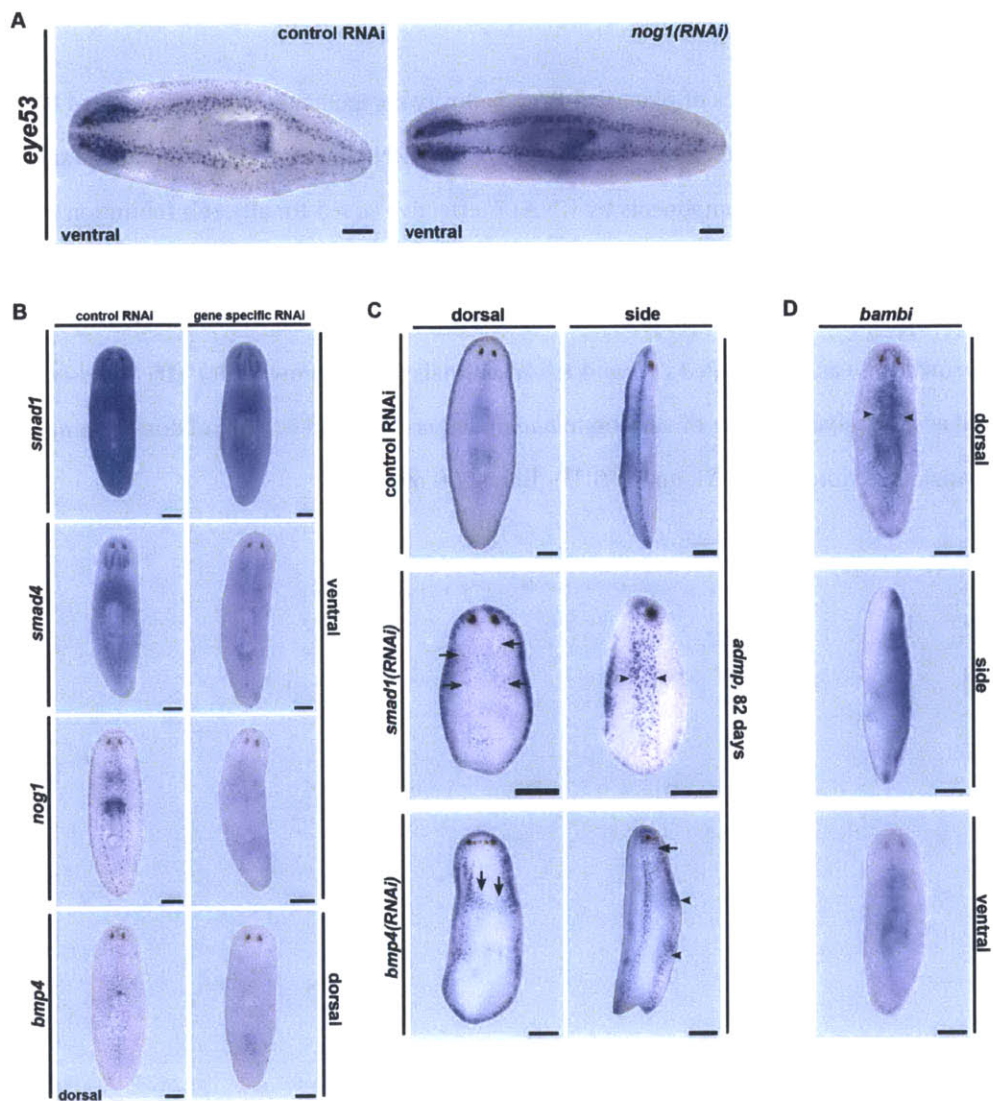


Figure S3. *admp* is required for proper body proportion and ML pattern in intact, non-amputated animals. (A) Intact *admp*(RNAi) animals were thinner than control animals. Difference was significant, $p < 0.0001$, unpaired t-test. (B) Intact *admp*(RNAi) animals had more closely positioned photoreceptors than control animals. Difference was significant, $p < 0.0001$, unpaired t-test. (C) Photoreceptor separation was more greatly affected than total body width as measured at the pharynx in intact *admp*(RNAi) animals. Difference was significant, $p < 0.0001$, unpaired t-test. (D) Intact *admp*(RNAi) animals displayed no decrease in lateral *wnt5* expression, yet had reduced lateral *nog1* expression ($n = 10/10$, white arrows) suggesting that the observed effect of *admp* RNAi on *nog1* expression was specific. Measurements were calculated as a ratio with total animal body length in (A) and (B) and of total animal body width in (C). Error bars represent standard deviation in (A-C). Anterior, up in all pictures. Bars: 200 microns.

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Figure S4. Additional analyses of planarian Bmp pathway components. **(A)** Expression of the ventral marker *eye53* is unaffected after 21 days of *Smed-noggin1* RNAi in intact non-amputated animals. **(B)** Inhibition of Bmp pathway components by RNAi is effective ($n > 5$ for all). **(C)** Inhibition of *smad1* or *bmp4* for 82 days results in dorsally expanded *admp* expression ($n = 4/5$ and $5/5$, respectively, black arrows). Additionally, the lateral domain of *admp* expression is expanded in *smad1(RNAi)* animals (black arrowheads) and duplicated in *bmp4(RNAi)* animals (black arrowheads). **(D)** *Smed-bambi* was identified as a putative ortholog of vertebrate *bambi* and is expressed in a broad dorsal domain (black arrowheads). Anterior, left in (A), up in (B-D). Bars: 200 microns.

Materials and Methods

Isolation of *Smed-admp*

A BLAST search was performed on an assembly of the *S. mediterranea* genome (<http://genome.wustl.edu>) to identify putative *admp* orthologs. Two highly similar *admp* sequences were amplified by PCR from asexual *S. mediterranea* cDNA: *admp-1a* (5'- GATTGGGATAGGACCCGTTC -3' and 5'- TCCCAAGCTAAATACGATTAAAAG -3') and *admp-1b* (5'- TTGGCATTGGAATAAATTC -3' and 5'- TCCCAAGCTAAATACGATTAAAAG -3'). Complete gene sequence was determined using 5' and 3' RACE PCR (Ambion). An additional highly similar but variant *admp* sequence was identified in sexual *S. mediterranea* genomic sequence. All *admp* experiments were carried out using the *admp-1b* sequence.

RNAi experiments

PCR was used to amplify the *bmp4* (5'- TTGATGCCAAAGATTCGTTC -3' and 5'- TCAAAATCCCAAGCTAAATACG -3'), *smad1* (5'- TCGTGTTAATTACCATATTGTTGC -3' and 5'- TGAAGTTAGATTCCACAAGAATAAAGC -3'), *smad4* (5'- GAATTCCTCCAATGGACCAG -3' and 5'- TCCCAAGCTAAATACGATTAAAAG -3'), and *nog1* (5'- GAAAGATTTGAGGTGATTTTCC -3' and 5'- AGATAAAAATCTCAGAACCTTGAATC -3') genes, in addition to *Smed-admp*, from asexual cDNA. Gene sequences were determined using 5' and 3' RACE PCR (Ambion) for all genes. PCR products from all genes and the control gene *unc-22* from *C. elegans* were cloned into the pPR244 RNAi expression vector using Gateway recombination reactions as previously described (1). RNAi experiments were performed by feeding the animals a mixture of liver and bacteria expressing dsRNA (1). Twenty milliliters of bacterial culture was pelleted and resuspended in 60 µl of liver. Animals were fed on day 0, day 4, and day 7 and amputated on day 8 for *bmp4*, *smad1*, *smad4* and *noggin1* RNAi regeneration experiments. For *bmp4*, *smad1*, *smad4*, and *noggin1* RNAi homeostasis experiments, animals were fed on day 0, day 4, day 7, day 14, and fixed on day 21. In all *admp* RNAi experiments, animals were fed on day 0, day 4, day 7, and fed at least

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five more times, once weekly. For *admp* RNAi regeneration experiments, animals were amputated one day after the final feeding. For *admp* RNAi homeostasis experiments, animals were fed weekly for 3+ months and fixed one week after the final feeding.

Phylogenetic analyses

BLAST searches were performed on assemblies of the *L. gigantis* (<http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html>), *H. robusta* (<http://genome.jgi-psf.org/Helro1/Helro1.home.html>), and *C. teleta* (<http://genome.jgi-psf.org/Capca1/Capca1.home.html>) genomes to identify putative *admp* gene sequences in these species. These sequences, along with *Smed-admp* and several deuterostome TGF β genes, were then aligned using CLUSTALW (2, 3). The alignments were trimmed using GBlocks (4) allowing for smaller final blocks, gap positions within the final blocks, and less strict flanking positions. Neighbor joining analyses were performed using Phylip (5) with default parameters and 500 bootstrap replicates. Maximum likelihoods were calculated using PhyML (6) with the WAG model of amino acid evolution, 4 substitution rate categories, proportion of invariable sites and γ distribution parameter estimated from the dataset, and 100 bootstrap replicates. Bayesian analyses were performed using MrBayes (7, 8). Two chains were started and allowed to run for 10 million generations, 1 tree was sampled every 100 generations, and the first 7,500 trees were discarded as burn-in.

in situ hybridizations

Whole-mount *in situ* hybridizations and fluorescence *in situ* hybridizations (FISH) were performed as described (9).

qPCR

Total RNA was isolated from control and *admp*(RNAi) animals. cDNA was prepared using oligo-dT primer and quantitative PCR (qPCR) was performed using SYBR Green (Applied Biosystems).

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Data were normalized to the expression of GAPDH as previously described (10). *bmp4* specific primers were used to evaluate gene expression (5'- AAATGTACGGATTTTGGAGGAATA -3' and 5'- GTAGGCAAAGGAGCTTTATTACCA -3'). Samples without reverse transcriptase were used as the negative control template.

Immunostaining

Immunostainings were performed as previously described (11) using tyramide signal enhancement.

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Chapter 3

Tissue absence initiates regeneration through Follistatin-mediated inhibition of Activin signaling

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Manuscript currently under review for publication

Abstract

Regeneration is widespread, but mechanisms activating regeneration remain mysterious. Planarians are capable of whole-body regeneration and mount distinct molecular responses to wounds that result in tissue absence and those that do not. A major question has become how these distinct responses are activated. We describe a *folliculin* homolog (*Smed-folliculin*) required for regeneration initiation in planarians. *Smed-folliculin* inhibition blocks responses to tissue absence, but does not prevent homeostatic tissue replacement. Conversely, an *activin* homolog (*Smed-activin-1*) inhibits responses to tissue absence, and is required for the *Smed-folliculin* phenotype. Strikingly, inhibition of *Smed-activin-1* causes faster than normal regeneration. Finally, *Smed-folliculin* and *Smed-activin-1* are induced by injury, with *Smed-folliculin* being expressed at higher levels following injuries that cause tissue absence. These data suggest that wound-induced *Smed-folliculin* inhibits *Smed-Activin-1* to trigger regeneration specifically following injuries involving tissue absence. This identifies a mechanism that regulates the decision to initiate regeneration, a process important across the animal kingdom.

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Introduction

Regeneration is a widespread phenomenon observed in diverse contexts and species. Invertebrates such as *Hydra* and *Nematostella* are capable of whole-animal regeneration from tissue fragments, and vertebrates such as zebrafish, amphibians, and mammals are capable of regenerating damaged or missing organs [1, 2]. Despite this widespread relevance, the central mechanisms that drive regeneration, and to what extent these mechanisms are conserved, are poorly understood. How is regeneration initiated? How is the regenerative state terminated? These are fundamental questions that are only beginning to be explored.

Planarians are flatworms capable of robust body-wide regeneration in response to an almost limitless variety of injuries and, with the recent development of molecular tools, have emerged as a powerful model for exploring the underpinnings of regeneration [3]. The bulk of regeneration in planarians occurs over a period of about a week. During this period, new tissues are formed at wound

sites in a process called blastema formation, and pre-existing tissues are reorganized to accommodate reduced animal size and further generate missing tissues [4, 5]. The source of regenerated tissue in planarians is a population of adult dividing cells called neoblasts [5], which include pluripotent stem cells called clonogenic neoblasts (cNeoblasts) [6]. Importantly, neoblasts are the only cycling cells in adult animals and can be specifically ablated by gamma irradiation, allowing for dissection of the requirements for neoblasts in regenerative processes [5]. Recent work has described the earliest molecular and cellular events that occur following injury, both within the neoblasts and in differentiated tissues [7-10]. One finding to emerge from this work is that animals initiate distinct cellular and molecular responses to “major injuries” that remove significant tissue and require regeneration, such as head or tail amputation, and to “simple injuries” that require only minimal healing for repair (i.e., wounds that do not elicit blastema formation), such as punctures or incisions. Following simple injury, for example, animals

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display an increase in mitotic numbers 6h after injury before returning to baseline levels [7]. In addition, hundreds of genes are transiently induced at wound sites before becoming undetectable by 24h after injury [8]. Following major injury, these same responses are observed, but a second set of unique responses are also activated: the 6h increase in mitotic numbers is followed by a second increase 48h after amputation [7], and wound-induced gene expression persists beyond 24h and is refined over the course of several days [8]. These responses are referred to as the “missing-tissue response” [7, 8]. How animals distinguish between injuries involving varying amounts of tissue loss and regulate these distinct wound response programs remain unknown.

We identified two wound-induced genes, *Smed-follistatin* and *Smed-activin-1*, that function together to regulate the magnitude and duration of the molecular and cellular “missing-tissue” responses required for regeneration. We demonstrate that planarian regeneration is inhibited by Activin

signaling and that Follistatin-mediated inhibition of Activin signaling is required for regeneration to occur. Furthermore, the wound-induced expression of *Smed-follistatin* is regulated by the amount of missing tissue after injury, suggesting a mechanism by which regenerative responses can be specifically initiated when necessary.

Results

***Smed-follistatin* is a wound-induced gene required for regeneration**

To identify genes mediating regeneration-specific wound responses, we inhibited several genes recently identified as being wound-induced [8]. This was accomplished using RNA interference (RNAi) followed by amputation of the heads and tails of animals. Within days of amputation, control animals produce unpigmented regeneration blastemas at wound sites, which contain newly differentiated tissues. By contrast, inhibition of *Smed-follistatin* (*follistatin* or *fst*), a gene encoding a homolog of the TGF- β

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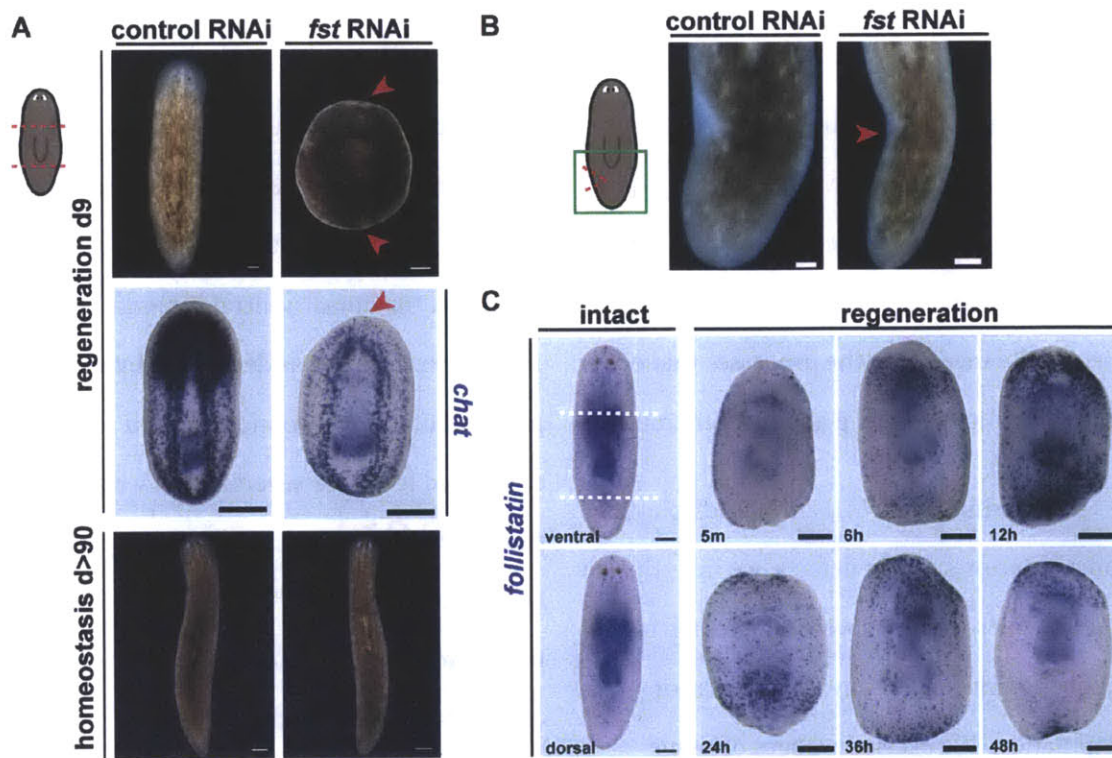


Figure 1. *fst* is wound induced and required for regeneration (A) *fst*(RNAi) animals did not form blastemas after amputation (top, arrowheads, n>100) and did not regenerate a brain as assayed by *in situ* hybridization with an RNA probe for *choline-acetyltransferase* (middle, arrowhead, n=9/9), but displayed no phenotype in the absence of amputation (bottom, n=30/30, 123d RNAi). (B) *fst*(RNAi) animals did not form blastemas following excision of a wedge of lateral tissue (arrowhead n=14/14). (C) *fst* was expressed in sparse cells throughout the intact animal as assayed by *in situ* hybridization. Following head and tail amputation, robust *fst* expression was observed at both anterior and posterior wound sites, with peak expression observed at 12h post-amputation. Scale bars = 100µm for live pictures, 200µm for *in situ* pictures. Anterior up.

superfamily inhibitor Follistatin, resulted in animals that did not form regeneration blastemas (Fig 1A). *fst*(RNAi) animals also failed to regenerate a brain following head amputation, instead displaying fused ventral nerve cords at anterior wound sites, and did not regenerate anterior markers (Fig 1A, Fig 1 – supplement 1). Planarians

constantly maintain their adult tissue through cell turnover involving neoblasts [5]. Consequently, most genes required for regeneration are also required for tissue turnover because of an involvement of the gene in neoblast biology [11]. Strikingly, *fst*(RNAi) animals did not shrink or lose structures in the absence of amputation,

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even after several months of RNAi (Fig 1A), suggesting that *fst* has a regeneration-specific function and is not required for neoblast-mediated tissue turnover. Because of the rarity of genes required for regeneration but not tissue turnover, *fst* was a good candidate for specifically mediating the processes that occur following injury to bring about regeneration.

Given that *fst(RNAi)* animals displayed some anterior-specific defects, we investigated whether these animals were able to regenerate following injuries not involving anterior amputation. We excised wedges of tissue from the lateral midbody of animals, leaving anterior and posterior poles intact. *fst(RNAi)* animals injured in this manner failed to produce a blastema at the site of tissue excision (Fig. 1B), indicating that *fst* is required for regeneration in general, rather than just regeneration of heads and tails.

fst expression was found to be induced at wounds by six hours following amputation [8]. To expand upon these findings, we assessed *fst* expression at several time points following amputation, as well as in

unamputated animals. In unamputated animals, *fst* was expressed in sparse cells broadly throughout the animal (Fig 1C). Expression was enriched ventrally and in a thin domain around the periphery of the animal, at the dorsal-ventral boundary (Fig 1C). *fst* expression was detectable by six hours post amputation at wound sites and persisted at reduced levels for several days, with a peak in expression level around 12h post amputation (Fig. 1C, Fig 1 – supplement 1). Injection of *fst* dsRNA only after amputation caused poor blastema formation and regeneration defects, such as brains that were reduced in size or absent (Fig 1 – supplement 1), consistent with a requirement for wound-induced *fst* expression in regeneration. We conclude that *fst* is a wound-induced factor required for regeneration.

***follistatin* is required for the regeneration-specific neoblast response**

To characterize the defects underlying regeneration failure in *fst(RNAi)* animals,

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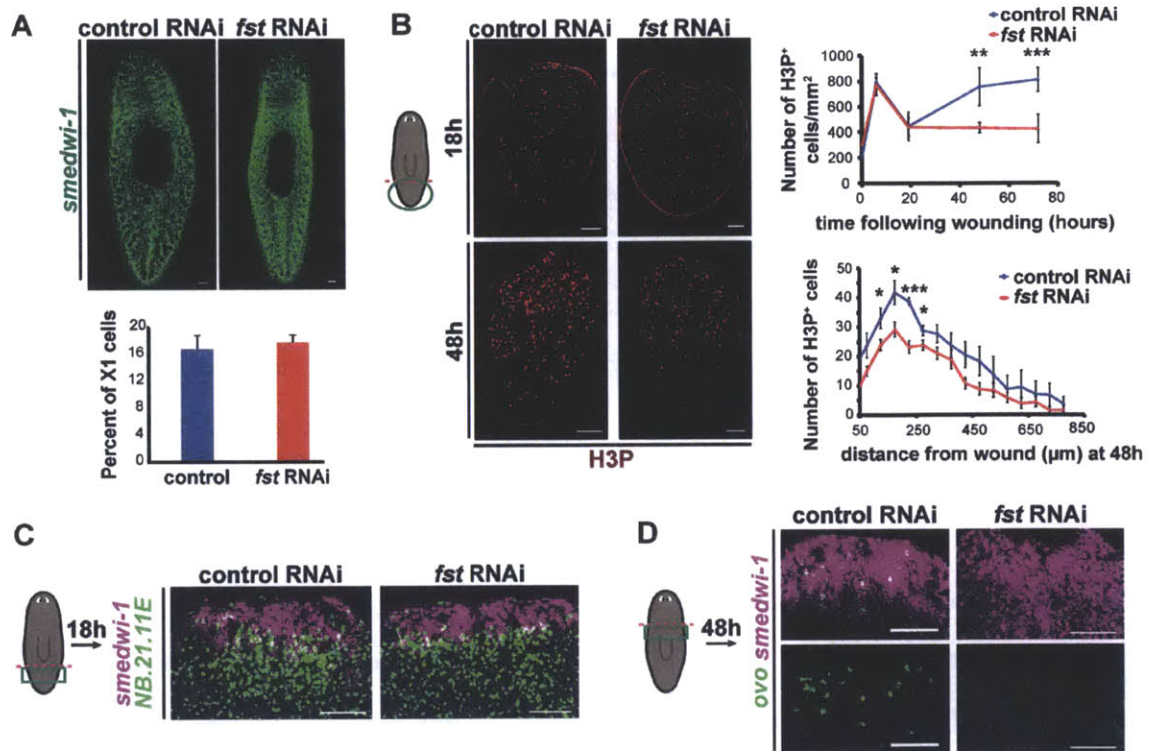


Figure 2. *fst* is required for the neoblast response to missing tissue (A) *fst* RNAi did not affect total neoblast number or distribution as assayed by *in situ* hybridization for the neoblast marker *smedwi-1* (top, n=5/5) and by flow cytometry (bottom, X1 cells as percent of live cells) (B) Labeling of mitoses with H3P antibody in amputated tail fragments (left). *fst*(RNAi) animals displayed reduced mitoses 48h and 72h after amputation (top right, p<.01 and p<.001, two-tailed t test). Mitoses were enriched toward wound sites 48h after amputation in *fst*(RNAi) animals but were fewer in number (bottom right, p<.001 at 200μm, two-tailed t test). (C) Neoblasts migrated to wounds in *fst*(RNAi) animals as assayed for the presence of *smedwi-1*⁺ cells at wounds (NB.21.11E⁺ cells mark pre-existing tissue) (n=10/10). (D) *fst*(RNAi) animals lacked photoreceptor progenitors following head amputation as assayed by *ovo*⁺/*smedwi-1*⁺ cells (p<.001, two-tailed t test). Scale bars = 100μm. Anterior up.

we first investigated whether *fst* regulates neoblast function in regeneration. Neoblasts can be visualized by detecting neoblast-specific transcripts through whole-mount *in situ* hybridization [12]. In addition, because neoblasts are the only planarian cells with >2N DNA content, they can be quantified using flow cytometry [13]. *fst*(RNAi) animals

displayed normal neoblast numbers prior to amputation, as determined both by *in situ* hybridization and by flow cytometry, indicating that the failure of *fst*(RNAi) animals to regenerate is not caused by neoblast loss (Fig. 2A). We next assessed whether neoblasts fail to respond to injury in *fst*(RNAi) animals. The neoblast response to injury involves two

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peaks in mitotic numbers, separated by a mitotic minimum at a time at which neoblasts migrate to wounds [7]. The first increase in mitotic numbers peaks 6h after injury, is generically induced by all injury types, and is spatially widespread. The second mitotic increase peaks 48h after injury, specifically following major injuries, and is biased toward wound sites. We tested whether these mitotic responses to injuries require *fst* by using an antibody for the mitotic marker phosphorylated histone H3 (H3P), and an established wound response assay [7]. *fst(RNAi)* animals displayed a normal mitotic peak 6h after amputation, indicating a normal generic injury response was present (Fig 2B). By contrast, these animals failed to display a second, 48h peak in mitotic numbers (Fig 2B). Despite this absence of a regeneration-specific proliferative response, *fst(RNAi)* animals did display localization of mitoses toward the wound site 48h after amputation (Fig 2B), and an enrichment of neoblasts at wound sites 18h after injury (Fig 2C), indicating that neoblast migration occurred normally.

Given that *fst(RNAi)* animals displayed a defective proliferative response to missing tissue, we next tested whether these animals produced normal numbers of known progenitor cell types following amputation. In control animals, amputations that remove the head induce the formation of photoreceptor progenitors that express the *ovo* gene [14]. These progenitors are normally produced by neoblasts in tissue proximal to the wound, but *fst(RNAi)* animals failed to produce *ovo*⁺ progenitors following amputation (Fig. 2D). From these data we conclude that *fst* is required for induction of several aspects of the regeneration-specific neoblast response to injury.

follistatin is required for responding to tissue absence following injury

The abnormal missing-tissue-specific mitotic response of *fst(RNAi)* animals raised the possibility that other responses to missing tissue could also require *fst*. Animals display an increase in apoptotic cell numbers in

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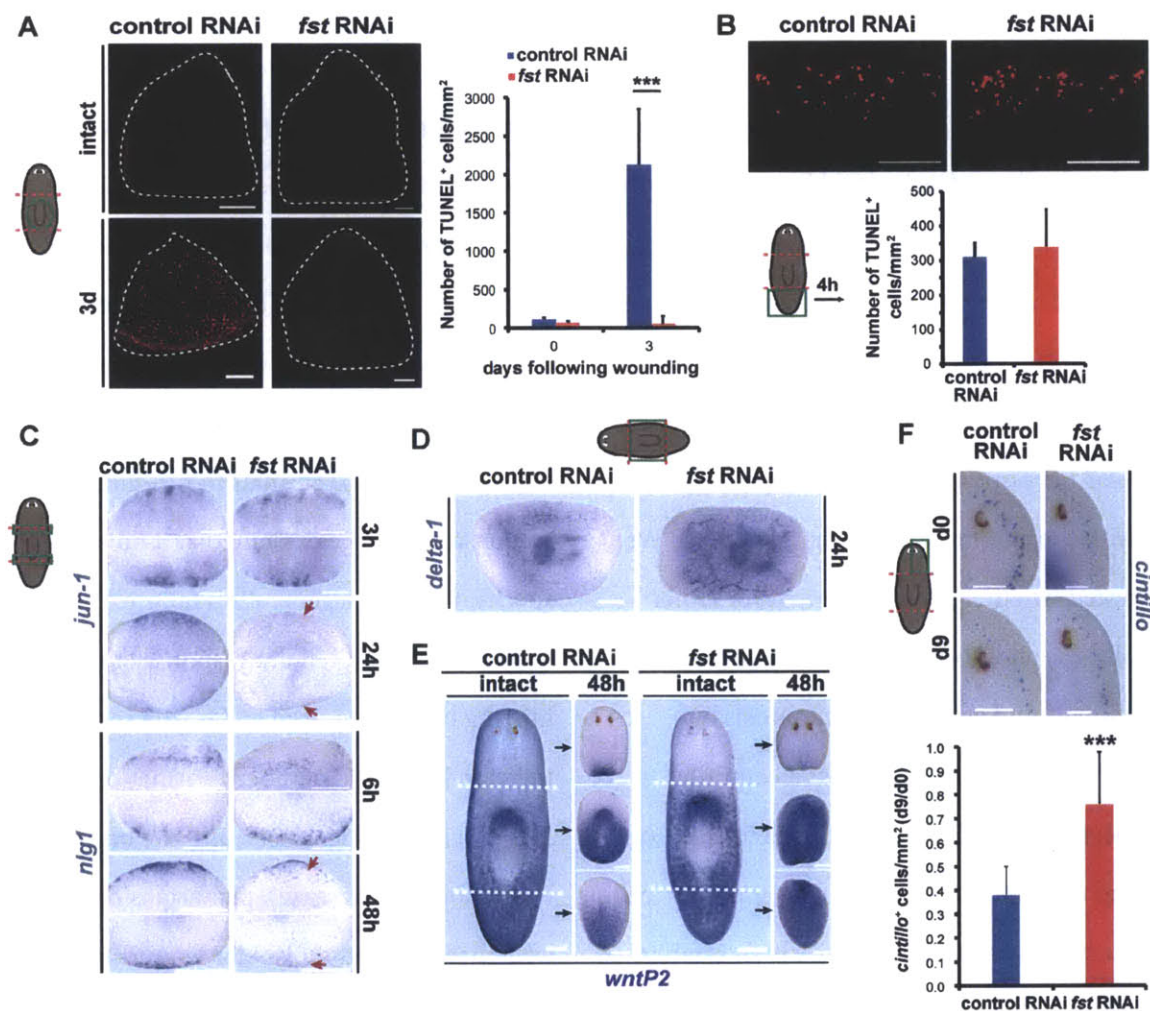


Figure 3. *fst* is required for missing-tissue responses and morphallaxis (A) *fst(RNAi)* animals did not display an increase in apoptosis 3d after amputation as assayed by quantification of pharyngeal TUNEL⁺ cells ($p < .001$, two-tailed t-test). Dotted white line = pharynx outline. (B) *fst(RNAi)* tail fragments displayed a normal apoptotic response at wound sites 4h after amputation as assayed by total TUNEL⁺ cells in tail fragments ($n = 6/6$). (C) *fst(RNAi)* animals displayed normal wound-induced gene expression 3h and 6h after amputation (*jun-1*: $n = 20/20$, *nlg1*: $n = 5/5$), but expression was greatly reduced compared to controls at 24–48h after amputation (arrows; *jun-1*: 17/19 correctly scored blindly, $p < .01$ Fisher's exact test, *nlg1*: 22/27 correctly scored blindly, $p < .01$, Fisher's exact test). (D) *fst(RNAi)* animals had increased wound induced expression of *delta-1* 24h after amputation ($n = 12/12$) (E) *fst(RNAi)* animals did not rescale expression of *wntP-2* along the AP axis 48h after amputation ($n = 18/21$). (F) *fst(RNAi)* animals failed to reduce the number of *cintillo*⁺ cells in head fragments to accommodate reduced animal size following amputation ($p < .001$, two-tailed t test). Scale bars = 100 μ m. Anterior up in (A–C),(E),(F). Anterior left in (D).

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response to injury [10]. Like the mitotic response, this apoptotic response consists of a generic injury phase and a missing-tissue specific phase: first, a local burst in apoptosis occurs at the wound site 4h following any type of injury; second, a body-wide burst in apoptosis occurs 72h after injury, but only in cases involving missing tissue [10]. The level of apoptosis in this latter phase scales with the amount of missing tissue [10]. We tested whether *fst* was required for either of these apoptotic responses by amputating animals and assaying for apoptosis by TUNEL. Planarians possess a centrally located muscular pharynx used for feeding and defecation [5]; measuring apoptotic cell numbers within the pharynx is an established assay for quantifying the body-wide increase in apoptosis that occurs 72h post-amputation [10]. Strikingly, *fst(RNAi)* pharynges displayed no increase in apoptotic numbers 72h post amputation, whereas roughly 2,000 apoptotic cells per mm² were observed in the pharynges of control animals, a roughly 20-fold increase from pre-amputation levels (Fig 3A). Importantly, *fst(RNAi)* animals

had a normal 4h apoptotic peak, indicating that the apoptosis phenotype of *fst(RNAi)* animals is not a consequence of a general requirement for *fst* in apoptosis (Fig 3B). Furthermore, the 72h apoptotic response is neoblast- and regeneration-independent, occurring even in animals that have had their neoblasts ablated [10]. Therefore, the failure of *fst(RNAi)* animals to produce this response cannot be explained as a non-specific result of regeneration failure. In addition to the cellular responses to missing tissue discussed so far (the second increase in mitotic numbers, formation of photoreceptor progenitors, and the body-wide apoptotic increase), planarians display a well-characterized molecular wound-response program with features specific to major injuries [8]. The wound response program involves the induction of hundreds of genes, including patterning factors, chromatin-remodeling factors, and transcription factors. In response to simple injury, the induction of most of these factors is transient, occurring within 0.5h to 6h of injury and becoming largely undetectable by 24h post-injury.

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Importantly, however, this expression program persists and is refined following major injuries. Therefore, the persistence of wound-induced gene expression represents another aspect of the planarian missing-tissue response. To address whether *fst* is required for persistent wound-induced gene expression, we assayed the expression of two wound response genes by *in situ* hybridization at several time points after amputation. We observed decreased expression of these genes in *fst(RNAi)* animals as compared to control animals when assayed at 24h-48h post-amputation or later, even though expression levels were indistinguishable at earlier timepoints (Fig. 3C). Notably, some wound-induced genes display expression that inversely scales with missing tissue amount; for example, *Smed-delta-1* is wound-induced and displays higher expression after an incision or puncture (simple injuries) than after amputation (a major injury) [8]. Amputated *fst(RNAi)* animals displayed a higher, rather than lower, level of *Smed-delta-1* expression than did control animals 24h after amputation (Fig 3D). This result

indicates that the lower expression levels observed for other wound-induced genes in *fst(RNAi)* animals does not reflect generically lower gene expression at wounds, but instead a specific requirement for *fst* in mediating missing-tissue-specific gene expression.

To rule out the possibility that the observed failures in missing-tissue gene expression responses were a non-specific result of the failure of *fst(RNAi)* animals to regenerate, we asked whether similar defects occur in irradiated, amputated animals. It has previously been observed that irradiated, amputated animals can display either higher or lower levels of wound-induced expression, depending on the gene examined [8]. Indeed, some wound-induced genes were similarly affected between irradiated and *fst(RNAi)* animals, while others were oppositely affected (Fig 3 – supplement 1). As was the case for the failed apoptotic response of *fst(RNAi)* animals, the missing-tissue gene expression defects of *fst(RNAi)* animals cannot therefore be explained as a simple side-effect of regenerative failure.

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In addition to producing a regeneration blastema and new tissues, amputated animals must also reorganize and rescale what remains of their body in a process termed morphallaxis [4, 5]. This process includes producing a pharynx internally if one is absent, shrinking organs that are too large for the reduced animal size, and redistributing gene expression gradients to accommodate new animal dimensions. Some of these processes do not require neoblasts and occur in lethally irradiated animals. For example, the gene *wntP-2* (also known as *wnt11-5* [15]) is normally expressed in the planarian tail region [15, 16]. In tail fragments, following amputation, the *wntP-2* expression domain rapidly rescales along the anteroposterior (AP) axis within 48h of amputation (becoming more restricted) whether regeneration proceeds or not [15], suggesting that this process is in fact an intrinsic response to missing tissue. *fst(RNAi)* animals did not rescale the *wntP-2* expression domain 48h following amputation, further supporting a model in which *fst* is required for responding to missing tissue (Fig 3E, Fig

3 – supplement 1). In addition, these animals were defective in several other measures of morphallaxis. Following head amputation, the head fragment must not only produce missing tissues (i.e., posterior-specific cell types), but also reduce the numbers of existing tissues (i.e., anterior-specific cell types). *fst(RNAi)* animals were defective in reducing the numbers of over-abundant cell types following amputation (Fig 3F). Finally, *fst(RNAi)* animals were unable to produce pharynges *de novo* (which normally occurs in the pre-existing tissue of head and tail fragments) (Fig 3 – supplement 1). We tested whether this defect occurs commonly in RNAi conditions that result in regeneration failure. *smad1(RNAi)* tail fragments, a different RNAi condition blocking blastema formation, produced a pharynx normally, indicating this defect is not a simple consequence of blastema formation failure (Fig 3 – supplement 1). We conclude that *fst* is required broadly for missing-tissue-specific wound responses, and that these defects likely underlie the inability of *fst(RNAi)* animals to regenerate.

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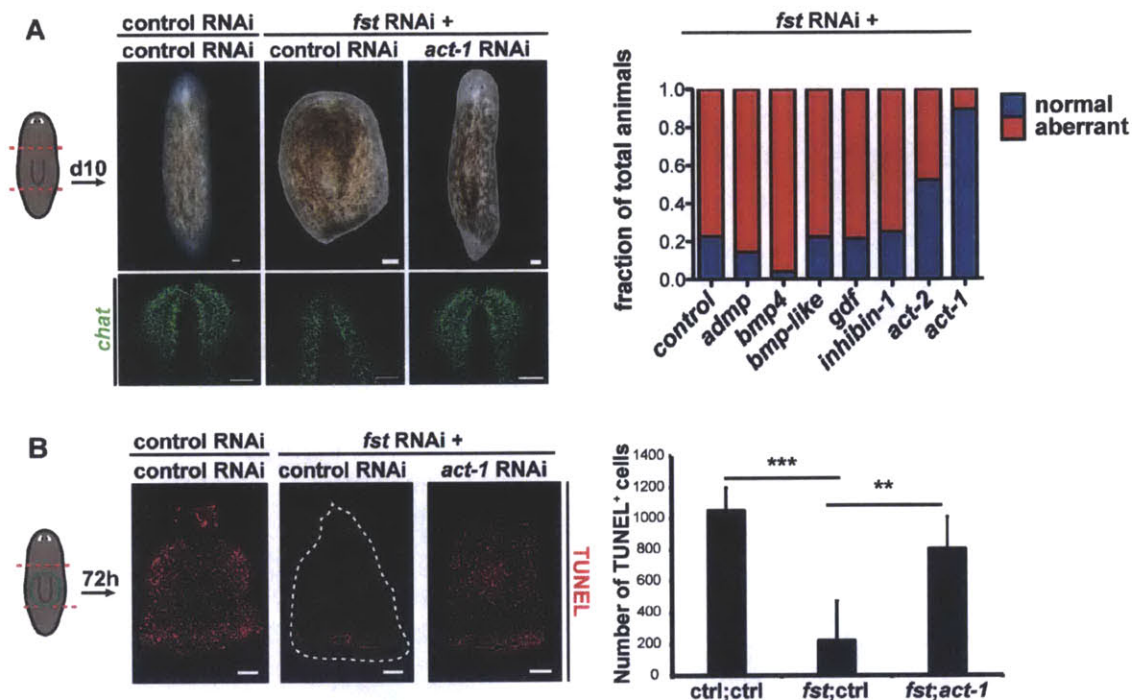


Figure 4. *act-1* is required for the *fst* RNAi phenotype (A) *fst*(RNAi) animals subsequently treated with control dsRNA did not produce blastemas or form a brain after amputation (n=17/22), while *fst*(RNAi) animals treated with *act-1* dsRNA produced normal blastemas and brain (left; n=25/28, p<.0001, Fisher's exact test). Treatment with dsRNA of other candidate genes to *fst*(RNAi) animals did not significantly suppress the *fst* RNAi phenotype (for *act-2*: n=12/23, p=.065, Fisher's exact test; n>9 for all others). Aberrant animals were scored as having greatly decreased or absent brain. (B) *fst*(RNAi) animals treated with control dsRNA failed to display an apoptotic response 3d after amputation as assayed by quantification of pharyngeal TUNEL⁺ cells, while *fst*(RNAi) animals treated with *act-1* dsRNA displayed a normal apoptotic response (p<.001 between control RNAi and *fst*;ctrl RNAi; p<.01 between *fst*;ctrl RNAi and *fst*;act-1 RNAi, two-tailed t test for both). Dotted white line = pharynx outline. Scale bars = 100μm. Anterior up.

Smed-activin-1 is required for the *follistatin* phenotype

Because Follistatin proteins are well-characterized extracellular inhibitors of TGF-β ligands [17, 18], we sought to identify putative TGF-β ligands that *Smed-Follistatin* might regulate to promote regeneration.

Through sequence homology searching of the *S. mediterranea* genome we identified seven putative TGF-β superfamily members. The Bmp family members *Smed-bmp* and *Smed-admp* have previously been described in detail [19-23], and expression of a putative *inhibin* homolog has also been described [8]. In addition to these genes, we identified

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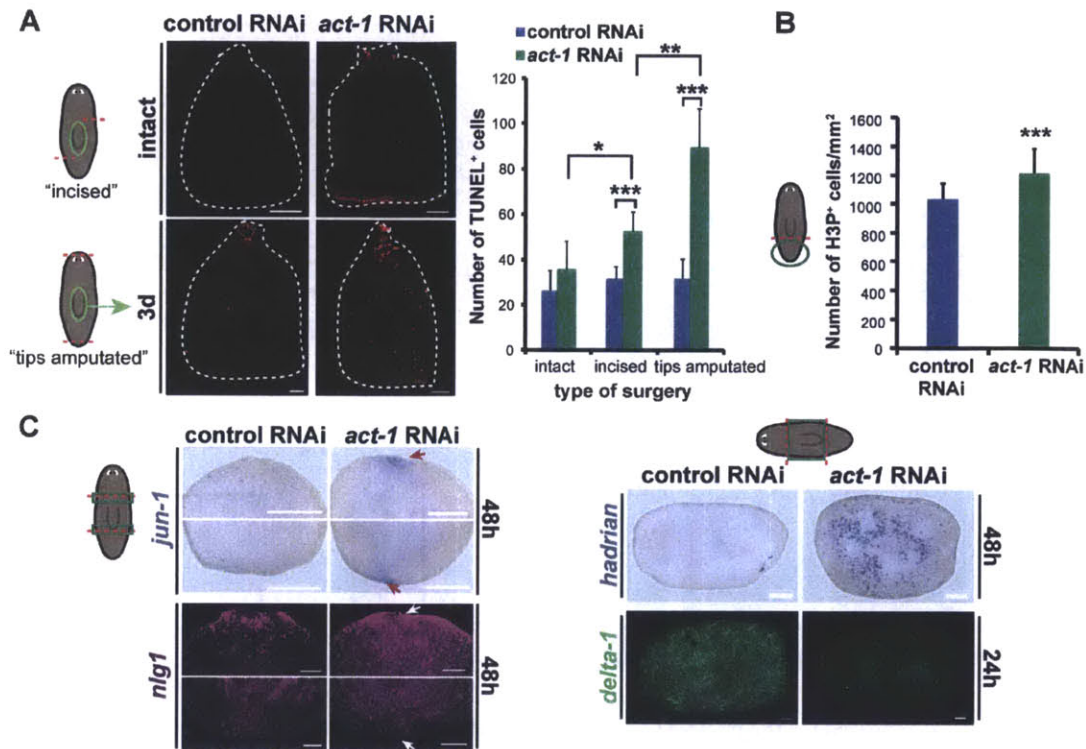


Figure 5. *act-1* suppresses missing tissue wound responses (A) *act-1(RNAi)* animals displayed higher apoptotic numbers than controls 3d after either small incisions or head and tail tip amputation ($p < .001$ for both, two-tailed t test). Apoptotic numbers after incision and tip amputation in *act-1(RNAi)* animals were higher than baseline levels ($p < .05$ and $p < .01$, respectively, two-tailed t test), and higher than control animals ($p < .001$ for both, two-tailed t test). Dotted white line = pharynx outline. (B) *act-1(RNAi)* animals displayed increased numbers of mitoses 72h after amputation as compared to controls ($p < .001$, two-tailed t test). (C) *act-1(RNAi)* animals displayed increased wound induced gene expression 48h after amputation (arrows, *jun-1*: 12/13 correctly scored blindly, $p < .01$, Fisher's exact test; *nlg1*: 8/8; *hadrian*: right, 24/28), with the exception of *delta-1*, which was lower (right, $n = 14/14$). Scale bars = 100 μ m. Anterior up in (B) and left of (C). Anterior left in right of (C).

two *activin*-like genes, *Smed-activin-1* (Fig 4 – supplement 1) and *Smed-activin-2*, a putative *gdf* homolog, *Smed-gdf*, and finally a gene that we named *Smed-bmp-like*. We reasoned that if a protein encoded by one of these genes is regulated by Fst in regeneration, then inhibition of that gene with RNAi

might suppress the *fst* RNAi phenotype. We therefore tested whether any of these genes was required for the *fst* RNAi phenotype by inhibiting both *fst* and the candidate TGF- β gene using RNAi (see materials and methods for details). The efficacy of RNAi in these animals was confirmed by *in situ*

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hybridization (Fig 4 – supplement 1). RNAi of one gene, *Smed-activin-1* (*act-1* in short), robustly suppressed regeneration failure in *fst(RNAi)* animals as well as the failure of *fst(RNAi)* animals to regenerate a brain (Fig 4A). *act-1* RNAi also suppressed the failure of *fst(RNAi)* animals to initiate a missing-tissue apoptotic response 72h post-amputation (Fig 4B). These data demonstrate that *act-1* expression is required for the *fst* RNAi phenotype and, given that Follistatin proteins have been shown to directly regulate Activin proteins in other organisms [17, 18], suggest that Follistatin promotes planarian regeneration by inhibiting the function of Activin-1 protein.

***activin-1* inhibits regeneration-specific wound responses**

Because *act-1* inhibition suppressed the regeneration failure of *fst(RNAi)* animals, we considered the possibility that *act-1* functions to inhibit regeneration-specific wound responses. A prediction of this hypothesis is

that inhibition of *act-1* should produce more potent or longer-lasting responses to injuries. To test this prediction, we first investigated whether *act-1* RNAi caused an elevated apoptotic response to injury. Following small amputations at the tips of animal heads and tails, *act-1(RNAi)* animals indeed displayed a greatly increased 72h apoptotic response as compared to controls (Fig 5A). Strikingly, *act-1(RNAi)* animals that were subjected to only a small incision, an injury that does not stimulate missing-tissue wound responses in control animals [10], displayed an ectopic 72h apoptotic response (Fig 5A). Because Activin proteins signal through the downstream effector Smad4 in other organisms, we tested whether *smad4* inhibition also caused this defect. Indeed, *smad4(RNAi)* animals displayed greatly increased apoptotic levels 72h after incisions (Fig 5 – supplement 1). Together, these data indicate that Activin-1, through Smad signaling, suppresses the apoptotic missing-tissue response.

We next assessed whether *act-1* RNAi caused higher than normal levels of

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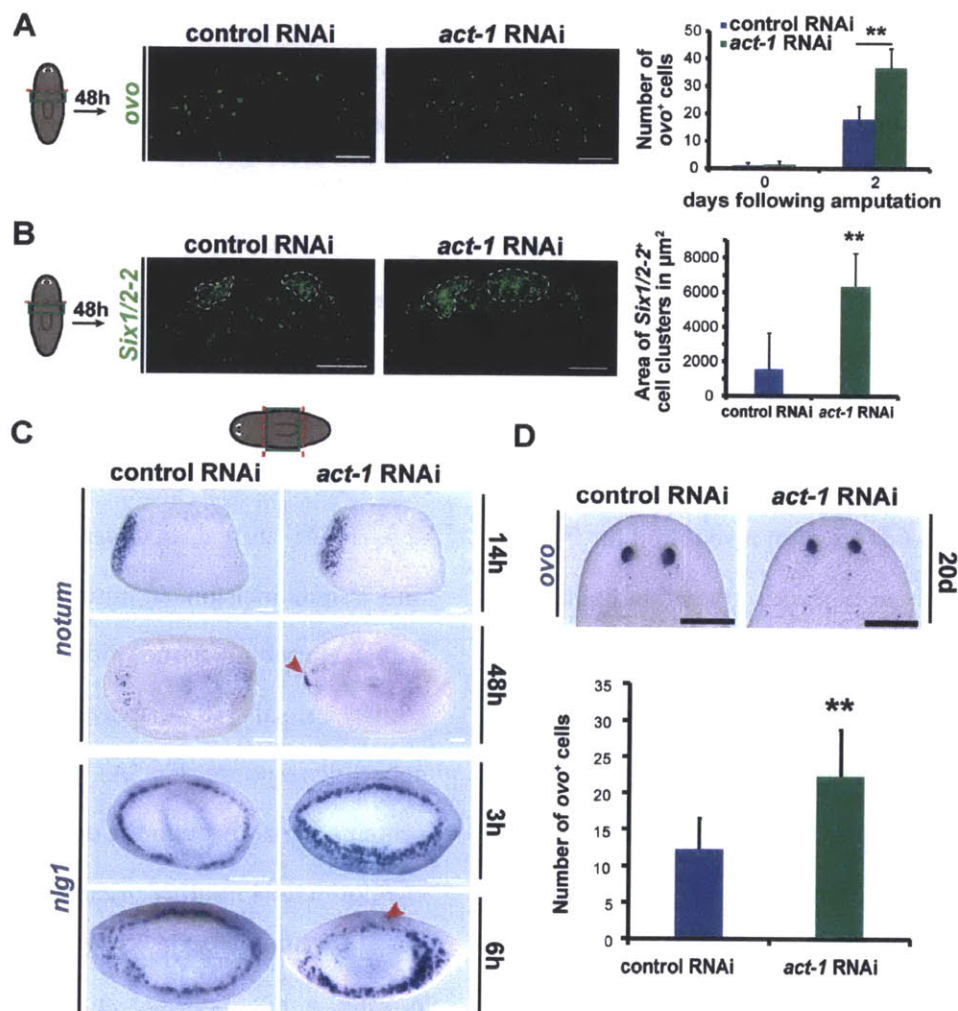


Figure 6. *act-1* RNAi accelerates regeneration but causes a failure to reduce progenitor formation (A) *act-1*(RNAi) animals displayed a greater induction of *ovo*⁺ photoreceptor progenitors 2d after head amputation ($p < .01$, two-tailed t test). (B) *act-1*(RNAi) animals displayed larger aggregations of *Six1/2-2*⁺ cells at wound sites (dotted white outline) 48h after head amputation ($p < .01$, two-tailed t test). (C) *act-1*(RNAi) animals displayed premature anterior coalescence of *notum* expression at 48h post amputation (top, arrowhead, $n = 15/15$) and had reduced dorsal expression of *nlgl1* at 6h post amputation (bottom, arrowhead, $n = 5/6$). (D) *act-1*(RNAi) animals displayed higher numbers of *ovo*⁺ photoreceptor progenitors 20d after amputation as compared to controls ($p < .01$, two-tailed t test). Anterior up in (A)(B)(D). Anterior left in top of (C). Anterior out of page in bottom of (C). Scale bars = 100 μm .

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neoblast proliferation following amputation. *act-1(RNAi)* animals displayed normal numbers of neoblasts prior to amputation (Fig 5 – supplement 1) and normal 6h and 48h peaks of mitoses following amputation (Fig 5 – supplement 1); however, they displayed increased mitotic numbers compared to controls by 72h post amputation (Fig 5B). This was also observed 72h after a minor amputation of animal head and tail tips (Fig 5 – supplement 1). These results suggest that *act-1* is required for reducing mitotic activity as regeneration progresses.

Finally, we investigated whether wound-induced genes displayed higher than normal expression following amputation in *act-1(RNAi)* animals. Indeed, *act-1* RNAi resulted in greater levels and longer lasting wound-induced gene expression following amputation than did control animals (Fig 5C). By contrast, expression levels of *Smed-delta-1*, which scale inversely with the severity of injury and were higher than normal in *fst(RNAi)* animals, were lower than normal in *act-1(RNAi)* animals (Fig 5C). Higher than

normal wound-induced gene expression was not observed following an incision, however, suggesting that animals are still able to distinguish major (missing-tissue) from simple (non-missing-tissue) injuries in some respects (Fig 5 – supplement 1). Taking these data together, we conclude that *act-1* inhibits regenerative responses to missing tissue and that the failure of *fst(RNAi)* animals to regenerate likely involves increased Act-1 signaling.

Regeneration occurs faster than normal in *activin-1(RNAi)* animals

Given that *act-1* inhibits several missing-tissue responses, we investigated the consequences of *act-1* RNAi on regeneration. *act-1(RNAi)* animals were capable of regenerating (Fig 6 – supplement 1), but displayed several abnormal features. The gene *ovo* is expressed exclusively in mature eyes and trails of photoreceptor and optic cup progenitors as they migrate to form mature eyes [14]. Therefore, the number of *ovo*⁺ trail

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cells can provide a quantitative measurement of the number of photoreceptor progenitors present and the rate of photoreceptor regeneration. Whereas *act-1(RNAi)* animals displayed normal numbers of *ovo*⁺ cells prior to amputation, greatly increased numbers as compared to controls were present following amputation (Fig 6A). In addition to the eyes, regeneration of the planarian excretory system (comprised of protonephridia) can also be measured. Regeneration of planarian protonephridia is characterized by the aggregation of progenitors into tight clusters within the regeneration blastema that express the marker *Six1/2-2* [24]. Therefore, the size of *Six1/2-2*⁺ clusters can be quantified at time points early in regeneration to measure the extent of protonephridial regeneration. *act-1(RNAi)* animals displayed increased aggregation of *Six1/2-2*⁺ cells compared to controls 48h after amputation (Fig 6B). These results raised the possibility that *act-1(RNAi)* animals regenerate faster than do control animals.

In addition to forming new tissues, animals must also rescale and reposition gene expression domains during regeneration. For example, the generic wound-induced expression of several genes becomes polarized either along the anteroposterior (AP) or dorsoventral (DV) axes as regeneration proceeds [8, 16, 25]. We examined the rate of regeneration further by observing how quickly disrupted gene expression domains return to their normal distributions following amputation. Wound-induced *Smed-notum* (*notum*) expression begins as a diffuse domain at anterior wound sites but coalesces to an anterior point of expression representing the regenerating anterior pole 3d after amputation [25]. Strikingly, we observed coalesced *notum* expression at a presumptive anterior pole as early as 48h after amputation in *act-1(RNAi)* animals, faster than ever observed in control animals (Fig 6C). Importantly, *notum* expression in *act-1(RNAi)* animals was indistinguishable from controls 14h after amputation, indicating that the coalesced expression observed at 48h was the result of faster coalescence as opposed to an aberrant

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pattern of induction (Fig 6C). Another wound-induced gene, *Smed-nlg1*, is initially expressed both dorsally and ventrally at all wound sites but normally becomes polarized to the ventral side of wounds 24h after amputation [8]. In *act-1(RNAi)* animals, *nlg1* expression was restricted to the ventral side of animals as early as 6h following amputation, whereas control animals displayed no polarization at this time point (Fig 6C). As was the case with *notum*, *nlg1* expression in *act-1(RNAi)* animals was indistinguishable from controls at an earlier time point following amputation. Taken together, these results indicate that *act-1* inhibition causes faster than normal regeneration and supports a model in which *act-1* normally acts to suppress several aspects of regeneration. *act-1* is therefore the first planarian gene described to inhibit regenerative processes, with RNAi of the gene accelerating regeneration.

If inhibition of *act-1* accelerates regeneration, what is the practical function of *act-1* expression in regenerating animals? One possibility is that *act-1* may serve as a

brake on regeneration that ultimately allows for the restoration of homeostatic levels of tissue turnover, with slower neoblast proliferation and progenitor production, after regeneration is complete. We therefore tested whether *act-1* RNAi caused perduring progenitor production following the time at which regeneration is normally completed. In normal animals, production of *ovo*⁺ progenitors becomes greatly reduced after the photoreceptors have been completely regenerated [14]. By contrast, *act-1(RNAi)* animals displayed elevated numbers of *ovo*⁺ progenitor cells as compared to controls 20d after amputation (Fig 6D). Importantly, elevated *ovo*⁺ progenitor numbers were not observed in unamputated *act-1(RNAi)* animals that were maintained under RNAi conditions for several months, indicating that amputation and regeneration were required to produce this state (Fig 6 – supplement 1). These data suggest that *act-1* is required for terminating regenerative processes.

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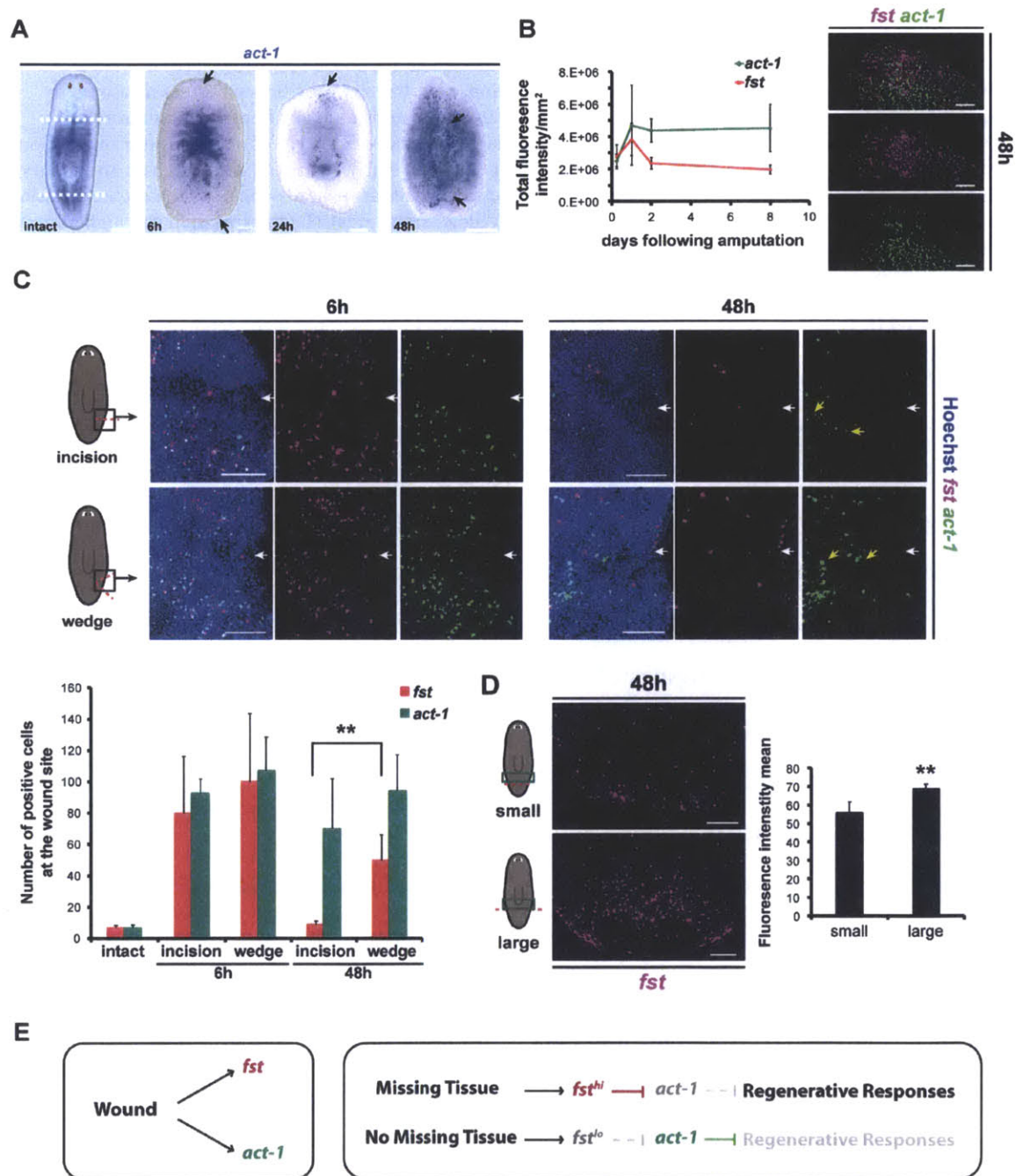
The amount of missing tissue regulates the relative levels of follistatin and activin-1 expression following injury

We next asked whether *act-1* expression was, like *follistatin*, wound induced. *act-1* displayed intestinal and pharyngeal expression in unamputated animals. However, expression was robustly induced following amputation and persisted at high levels throughout regeneration, with no significant decrease in expression level observed as late as 8d after amputation (Fig 7A, Fig 7 – supplement 1). This result was unusual, as all wound-induced genes previously examined become reduced in expression level and restricted to their pre-amputation domain of expression by this time [8, 15, 16, 25]. We therefore compared *act-1* expression to *fst* expression at several time points following amputation. Whereas *fst* was more highly expressed than *act-1* immediately following amputation, *act-1* expression persisted at much higher levels than *fst* starting 24h after amputation and for several days thereafter, as assayed by both *in situ* hybridization and quantitative PCR

(Fig 7B, Fig 7 – supplement 1). Interestingly, however, *act-1* expression was largely excluded from wound sites (Fig 7B). These results are consistent with the requirement of *fst* for regenerative wound responses and support the possibility that *act-1* is required for terminating these responses.

We next tested how the relative expression of *fst* to *act-1* varies across different injuries. If *act-1* inhibits regenerative processes following simple injury, but is inhibited by Fst following major injury, *fst* expression relative to *act-1* might be high following amputation, but low following an incision or puncture. To test this prediction, we assessed *fst* and *act-1* expression at wound sites following either an incision or the excision of a wedge of tissue. The level of *fst* and *act-1* expression at wounds was low prior to injury (Fig 7 – supplement 1), and similar 6h after either incision or wedge excision (Fig 7C). By 48h after injury however, *fst* expression was only detected at wedge excision wound sites (Fig 7C). By contrast, *act-1* was expressed highly at both

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Figure 7. *fst* induction is regulated by the amount of missing tissue following injury (A) *act-1* expression was detected in the intestine and pharynx in intact animals and was induced following amputation, beginning at wound sites (6h and 24h, arrows) and then spreading throughout the body (48h, arrows). (B) left: *act-1* was expressed more highly than *fst* throughout regeneration, except at very early time points, as measured by quantifying fluorescent *in situ* hybridization signal intensity (see materials and methods); right: *fst* expression was enriched at wound sites, while *act-1* expression was largely excluded from wound sites 48h after amputation (n>10) (C) Incised animals displayed wound-induced expression of both *fst* and *act-1* expression 6h after injury, but by 48h after injury, only *act-1* expression was detected (n>5, white arrowheads = injury site). *act-1* expression at 48h was more distant from the wound site than at 6h (yellow arrowheads). (D) *fst* expression was higher in level after an amputation resulting in a large amount of missing tissue than after an amputation resulting in little missing tissue, as measured by quantifying fluorescent *in situ* hybridization signal intensity (p<.01, two-tailed t test) (E) A proposed genetic model for *fst* and *act-1* function in regeneration. Wounds induce expression of both *fst* and *act-1* (left). If there is missing tissue following injury, then *fst* induction is high, Act-1 signaling is inhibited, and regeneration-specific responses are initiated. If there is no missing tissue following injury, then *fst* expression is low, Act-1 signaling is not inhibited, and regeneration-specific responses are repressed. Anterior up. Scale bars = 100µm.

types of wounds at this time, with expression having receded from the wound site (Fig 7C). These results indicate that *fst* expression

persists longer at wounds that result in tissue absence. To further investigate how *fst*

expression is regulated by tissue absence, we assessed expression of *fst* at wounds following amputations that resulted in different amounts

of missing tissue. Indeed, the level of *fst* expression at the wound site was greater 48h following an amputation that resulted in more missing tissue (Fig 7D). Together, these data are consistent with a model in which both *fst* and *act-1* are induced by injury, with the level of *fst* expression regulated by the amount of missing tissue. In this model, wound-induced *act-1* inhibits the regenerative response, and

wound-induced *fst* relieves this inhibition specifically following major injury, allowing regeneration to occur (Fig 7E).

Discussion

Regeneration initiation and termination

All long-lived animals face the prospect of injury and must possess regenerative mechanisms. Planarians are an exceptional example of the regenerative potential of animals as they are capable of robust whole-body regeneration. Importantly, distinct cellular and molecular programs for responding to simple injury versus amputation have been described in

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planarians. In the case of amputation, animals mount unique mitotic and apoptotic responses and produce an extended program of wound-induced gene expression [7, 8, 10]. These events represent the earliest described divergent behaviors following major injuries requiring regeneration versus simple injuries that require only wound healing without new tissue formation. A central question has therefore become how these distinct responses are mediated.

We uncovered a homolog of the TGF- β inhibitor *follistatin* that is wound induced and that is required for regeneration and for regeneration-specific cellular and molecular responses to injury. Conversely, we identified a wound-induced *activin* gene that suppresses regeneration-specific responses to injury. Our data suggest that inhibition of Act-1 signaling by Fst is therefore required for initiating a regenerative response at wounds following major injuries (that necessitate significant new tissue formation), and raise the possibility that Act-1 is subsequently required for restoring homeostatic levels of tissue turnover

after regeneration is complete. Finally, the observation that the level of *fst* expression relative to *act-1* expression is higher following major injury than following simple injury suggests a model in which the level of wound-induced *fst* expression allows for regenerative responses to be initiated specifically as a consequence of tissue absence.

The nature of the planarian missing-tissue signal

The ability of *act-1(RNAi)* animals to activate a regeneration-specific apoptotic response following simple injury (incision) suggests that some aspects of the missing-tissue-specific regeneration program can be triggered by the combination of generic injury signals and reduced *act-1* levels.

It is important to recognize, however, that inhibition of *act-1* in the absence of amputation is insufficient to induce all aspects of a regeneration-like state. Specifically, incised *act-1(RNAi)* animals do not display wound-induced gene expression after 24h,

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as would occur in an amputated animal.

Therefore, some aspects of the missing-tissue response to injury require an as yet unknown “missing-tissue” signal or signals produced by amputation, whereas others can be induced by simple injury coupled with inhibition of *act-1* expression.

Similarly, not all missing-tissue responses are abolished following *fst* inhibition. Specifically, we still observed migration of neoblasts to amputation sites in *fst(RNAi)* animals, despite their failure to activate a normal proliferative response. This suggests that despite the suppression of regeneration by Activin signaling, there exist processes that are triggered independently of this system. The identification of this “missing-tissue” signal will be crucial to building a complete description of the decision process that governs whether a regenerative response is activated following injury.

TGF- β signaling across regenerative contexts

Our findings describe a system in which Activin signaling negatively regulates regeneration through mitigation of proliferation, apoptotic responses, and wound-induced gene expression. In this system, suppression of Activin signaling is required for regeneration to proceed, with negative regulation of regeneration by Activin possibly involved in restoring homeostasis after regeneration is complete. The possibility therefore exists that Activin signaling may serve similar functions in other organisms. Indeed, TGF- β signaling has been implicated as a negative regulator of regeneration in a variety of contexts. For example, TGF- β is wound-induced and inhibits proliferation following partial hepatectomy in mammals [26-28]. Similarly, the addition of Activin to the embryonic chick retina is sufficient to block its regeneration [29], Follistatin may promote renal regeneration following ischemia/reperfusion injuries [30], and Follistatin in mouse greatly facilitates regeneration of skeletal muscle through its

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interaction with the TGF- β superfamily ligand Myostatin [31]. Given the relevance of these systems to human medicine, it will be important to investigate to what extent these regenerative regimes recapitulate the mechanisms observed in planarians.

Interestingly, a number of systems use TGF- β signaling to promote rather than suppress regeneration. Investigations of mammalian wound repair have indicated that *activin* expression is induced by wounding and that exogenous TGF- β is able to speed the rate of healing [32-34]. Moreover, putative gain-of-function TGF- β signaling mice more reliably regenerate following hole-punching of the ear [35]. Likewise, recent work in zebrafish tail-fin regeneration indicated that wound-induced *activin* is required for cell proliferation and migration following fin amputation [36]. Similar findings have been reported with TGF- β signaling in axolotl limb regeneration, and in *Xenopus* tail regeneration [37, 38]. Therefore, although TGF- β signaling plays a major role in nearly all forms of regeneration

studied, its specific function appears to vary. Nonetheless, the consistent presence of TGF- β signaling and suppression of signaling as major regulators of regeneration across a variety of contexts suggests that parallels might exist. For example, wounding could produce either increased TGF- β signaling or TGF- β suppression depending on the specific context to activate similar responses. The observation that in regeneration *activin* can block proliferation in some cases and be required for it in others supports this possibility. Therefore, uncovering “missing-tissue” signals in planarians, describing how these signals interact with Activin signaling, and identifying the key factors regulated by these signals, will undoubtedly inform a broader understanding of core regenerative mechanisms.

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Acknowledgements

We thank Reddien Lab members for comments and discussion, Irving Wang and Mirjam Mayer for comments on the manuscript. P.W.R. is an early career scientist of the Howard Hughes Medical Institute and an associate member of the Broad Institute of Harvard and MIT. We acknowledge support from the NIH (R01GM080639) and the Keck Foundation.

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Supplemental Figures

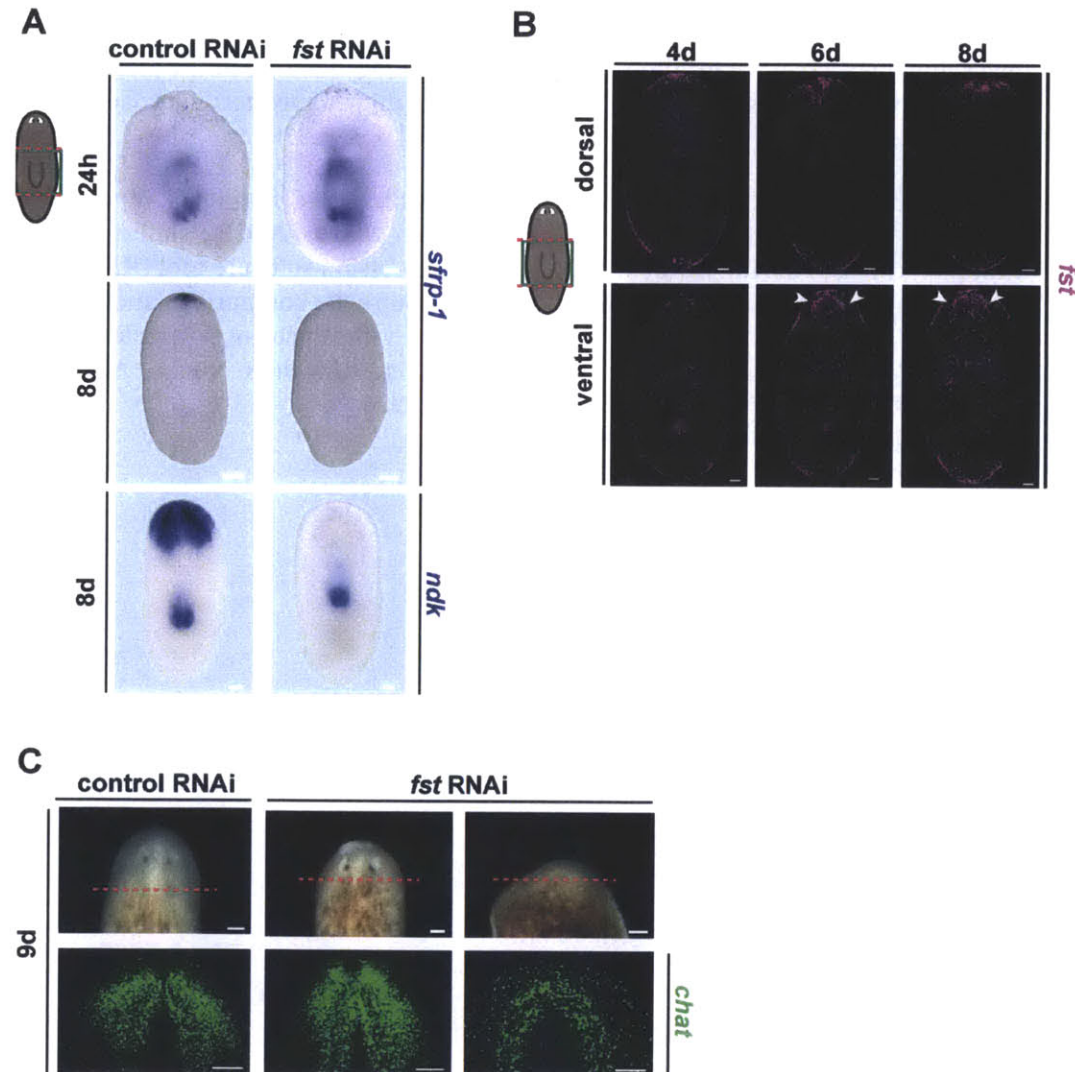
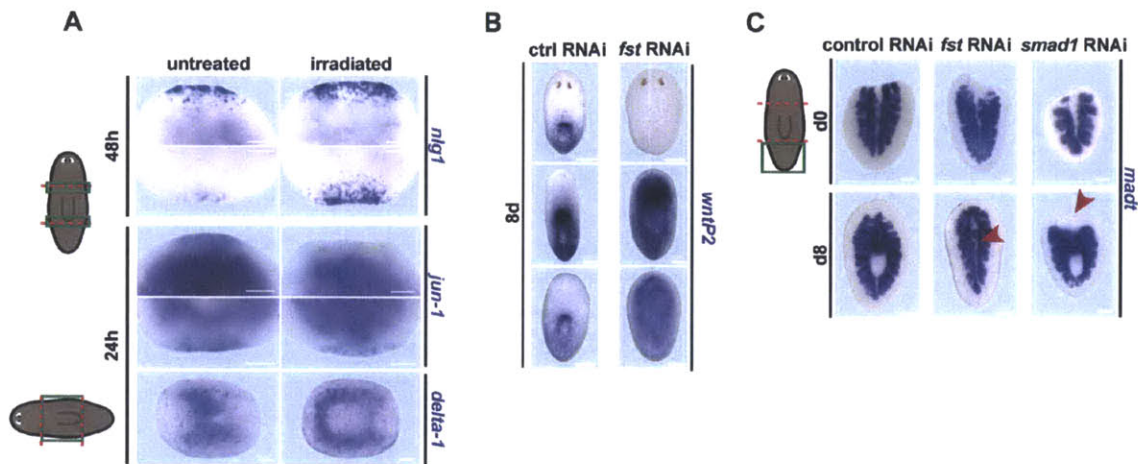


Figure 1 – Figure Supplement 1. Wound induced *fst* expression and post-amputation RNAi phenotypes (A) *fst*(RNAi) animals displayed normal anterior *sfrp-1* expression 24h after amputation (n=11/11, top), but by 8d displayed none (n=11/12, middle). *fst*(RNAi) animals also failed to regenerate anterior *ndk* expression (bottom, n=7/8) (B) *fst* is expressed at wound sites throughout regeneration, with additional expression in the brain at later timepoints (arrows, 6d and 8d). (C) Animals amputated and then injected twice with *fst* dsRNA within 24h of amputation developed aberrant brains as labeled by *chat* expression, and in some cases produced no blastemas (n=7/10 aberrant, 1/10 no blastema), while animals injected twice with control dsRNA regenerated normally (10/10 normal). Scale bars = 100µm. Anterior up in all.

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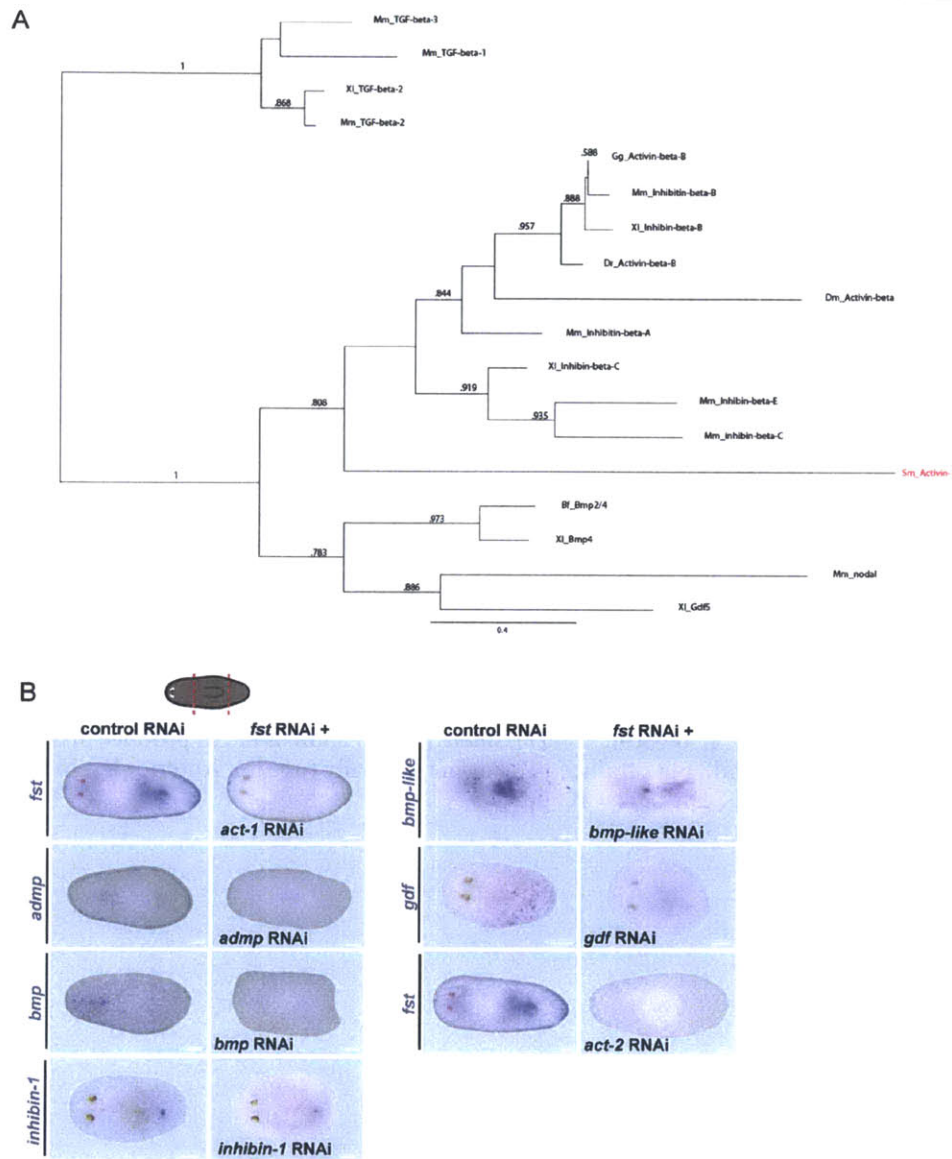


Figure 4 – Figure Supplement 1. Smed-Act-1 phylogeny and suppression RNAi controls (A) Phylogeny of selected TGF-beta proteins. The maximum likelihood tree is shown with support values above 0.5 for each branch. Smed-Act-1 is shown in red. The phylogenetic position of Smed-Act-1 supports orthology with Activin proteins. Xl = *Xenopus laevis*, Mm = *Mus musculus*, Gg = *Gallus gallus*, Bf = *Branchiostoma floridae*, Dm = *Drosophila melanogaster*, Dr = *Danio rerio*, Sm = *Schmidtea mediterranea*. (B) Animals treated with both *fst* dsRNA and *act-1* dsRNA display no *fst* RNAi phenotype even though expression of *fst* is greatly reduced (top left). Animals treated with *fst* dsRNA and another candidate dsRNA display *fst* RNAi phenotypes even though expression of candidate genes are greatly reduced ($n > 6$ for all). Animals treated with both *fst* dsRNA and *act-2* dsRNA displayed some reduction in the *fst* RNAi phenotype even though expression of *fst* is greatly reduced (bottom right). Anterior left, scale bars = 100µm.

Activin signaling regulates the planarian response to injury

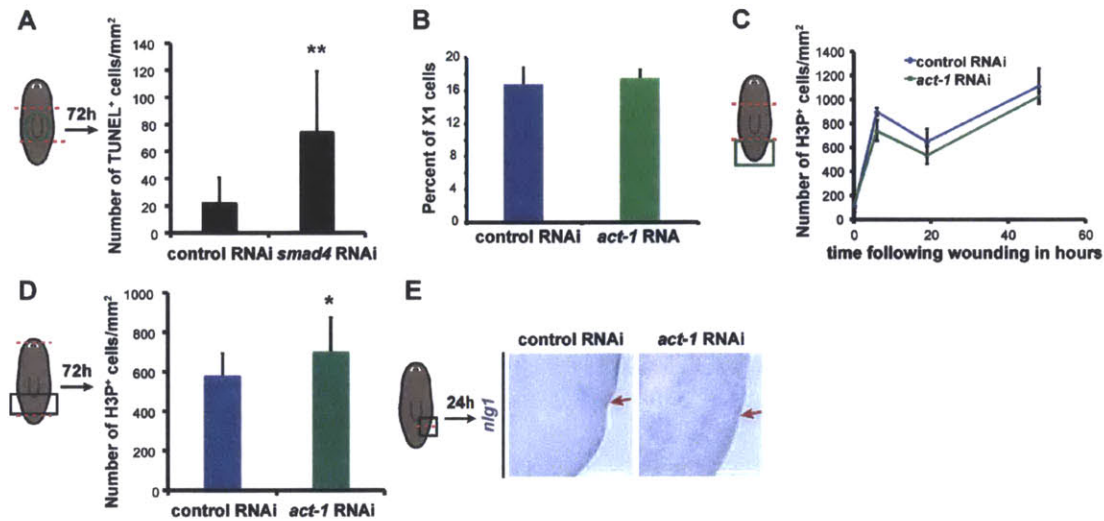


Figure 5 – Figure Supplement 1. Additional aspects of the *act-1* RNAi phenotype (A) *smad4* RNAi caused increased apoptotic numbers compared to control RNAi 3d after incision ($p < .01$, two-tailed t test), a phenotype similar to that observed in *act-1*(RNAi) animals. (B) *act-1*(RNAi) animals displayed normal numbers of X1 cells as counted by flow cytometry. (C) *act-1*(RNAi) animals displayed normal mitotic numbers at 0h, 6h, 18h, and 48h (D) *act-1*(RNAi) animals displayed higher mitotic numbers 72h after head and tail tip amputation than control animals ($p < .05$, two-tailed t test) (E) *nlgl1* expression was absent from both control and *act-1*(RNAi) animals by 24h after incision ($n=5/5$).

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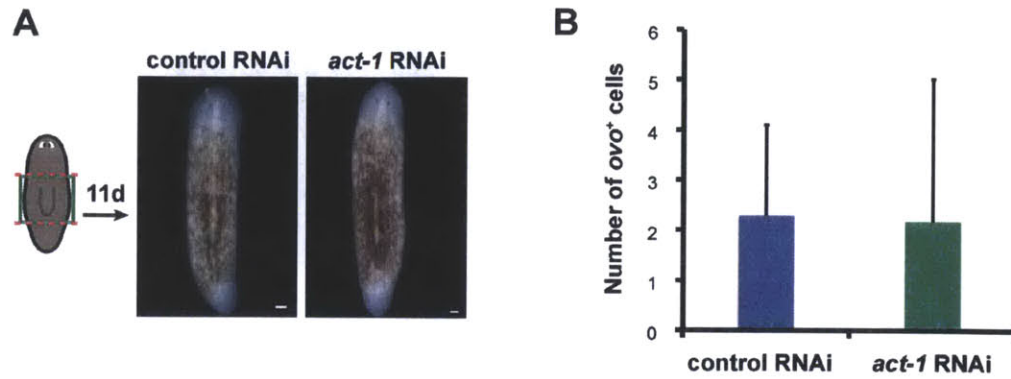


Figure 6 – Figure Supplement 1. Additional controls for *act-1* RNAi phenotypes (A) *act-1*(RNAi) animals formed blastemas and regenerated following head and tail amputation (n>200). (B) Animals treated with *act-1* dsRNA for over 100 days in the absence of amputation did not display an increase in the production of *ovo*⁺ photoreceptor progenitors (n>7). Anterior up, scale bars = 100μm.

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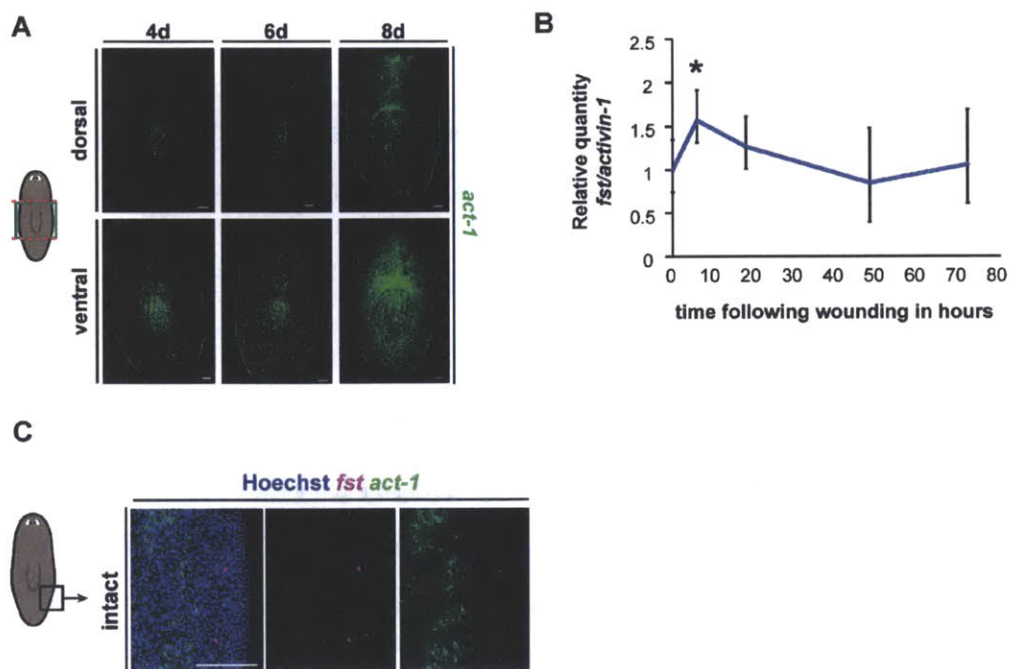


Figure 7 – Figure Supplement 1. *fst* and *act-1* expression during regeneration (A) *act-1* expression persisted at high levels throughout the animal during regeneration until at least 8d after amputation. (B) *fst* expression relative to *act-1* expression was higher than in intact animals 6h following amputation as quantified by qPCR ($P(H_1) < .05$). (C) Lateral, post-pharyngeal expression of *fst* and *act-1* is minimal prior to injury (*act-1* signal present is intestinal). Anterior up, scale bars = 100 μ m.

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Materials and Methods

Gene cloning

For RNA probes, genes were cloned into pGEM and amplified using nested PCR with T7-promoter-containing primers or existing cDNA clones. For RNAi, genes were cloned into pPR244 as described [12].

Identification of TGF- β superfamily homologs

A BLAST search was performed on an assembly of the *S. mediterranea* genome (<http://genome.wustl.edu>) using *Xenopus bmp4* to identify TGF- β homologs. Each gene containing a putative TGF- β domain was isolated by PCR from asexual *S. mediterranea* cDNA. Genes other than *Smed-act-1* were named based on the consensus of top blast hits.

Phylogenetic analysis

The homology of *Smed-act-1* was determined using the maximum likelihood method. The *Smed-act-1* sequence was aligned with other TGF- β sequences using CLUSTALW [39, 40]. The alignments were trimmed using GBlocks [41] allowing for smaller final blocks, gap positions within the final blocks, and less strict flanking positions. Maximum likelihoods were calculated using PhyML [42] with default parameters and 100 bootstrap replicates.

RNAi experiments

The control dsRNA for all RNAi experiments was *unc-22* from *C. elegans*. RNAi experiments were performed by feeding the animals a mixture of liver and bacteria expressing dsRNA [11]. Twenty milliliters of bacterial culture was pelleted and resuspended in 60 μ l of liver. For *fst* and *act-2* RNAi regeneration experiments, animals were fed on day 0, day 4, day 8, and day 12 and amputated on day 16/17, and then either soaked for 6h in dsRNA (a final concentration of 1 μ g/ μ l - TUNEL experiments), soaked for 2h in dsRNA (wound-induced gene expression

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experiments) or not soaked in dsRNA except as noted below. For suppression experiments, totals given represent pooled results from two separate experiments: 1) animals were fed *fst* dsRNA on day 0, day 4, day 8, and day 12, and fed candidate gene dsRNA on days 16, 20, and 23 and then amputated on day 24. 2) Animals were amputated and injected 4 times with a 30nL equimolar mixture of *fst* dsRNA and candidate gene dsRNA on day 0, injected in the same manner without amputation on day 1, amputated and injected on day 4, and injected only on day 5. Animals were scored and fixed 8d after amputation for both experiments. To test the requirement of *fst* specifically during regeneration (Fig 1 - supplement), animals were amputated and then injected 4 times with 30nL *fst* dsRNA immediately following amputation. This injection protocol was repeated a second time 6h after amputation.

in situ hybridizations

Whole-mount *in situ* hybridizations and fluorescence *in situ* hybridizations (FISH) were performed as described [43]. For double/triple labeling, HRP-inactivation was performed between labelings in 4% formaldehyde, 30min.

qPCR

Total RNA was isolated from asexual *S. mediterranea* animals. cDNA was prepared using SuperScript III (Invitrogen) with oligo-dT primers and qPCR was performed using SYBR Green (Applied Biosystems). Data were normalized to *clathrin* expression as previously described [44]. *act-1* (left: GCGAGCTACCTTTCAATGCT, right: AAAAACTGTTGCACTCCCGT) and *fst* (left: CCAGGCGAAAGAAATCCAG, right: TGTATCAAATGCCCCACCTC) primers were used to evaluate gene expression. Ratios of *fst* expression to *act-1* expression were used for relative changes and normalized by time “zero” control samples. Samples without reverse transcriptase were used as negative control template. REST was used for determination of significance in expression differences (P(H1)) [45].

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Immunostaining

Immunostainings were performed as previously described [12] using tyramide signal enhancement.

TUNEL assays

TUNEL was performed as previously described [10].

Exposure to γ -irradiation

For lethal irradiation (elimination of all neoblasts), planarians were exposed to 6000 rad (6K, ~72 min) using a cesium source (~83 rad/min).

Flow cytometry

Animals were amputated in cold CMFB, and cells were prepared as described [24]. For quantification of X1 cells, five animals were used per RNAi condition, and triplicate experiments were performed. Analyses and sorting were performed using a Moflo3 FACS sorter and FlowJo.

Imaging and analyses

Quantification of cell numbers positive for any given marker or an area of positive cells, equal numbers of optical stacks were taken of each specimen, collapsed, and quantified using Automeasure in the AxioVision software (Zeiss) and/or manually. For quantification of fluorescence intensity, 7 optical stacks were acquired from the entire ventral surface of the animals, collapsed, and values were determined using the Automeasure module (Densitometric sum) in the AxioVision software (Zeiss). Images were acquired using an AxioImager with an Apotome (Zeiss) or an LSM 700 (Zeiss).

Conclusions**Chapter 4****Conclusions**

Conclusions

I. Embryonic DV patterning and conservation of the *admp/bmp* circuit

Previously to the studies described in this document, an *admp/bmp* regulatory circuit for DV polarity establishment had been described exclusively in *Xenopus* embryogenesis. *admp* homologs however had also been characterized in the zebrafish *Danio rerio* and the chicken *Gallus gallus* [1, 2]. In both of these systems, *admp* is also negatively regulated by Bmp signaling and functions as a Bmp ligand. Therefore, although the buffering function of this circuit in these systems has not been directly tested, it is likely that the function of oppositely regulated *admp* and *bmp* described in *Xenopus* is conserved in these systems.

As mentioned in chapter two, putative *admp* homologs also exist in several Lophotrochozoans, namely *Helobdella*, *Lottia*, and *Capitella*. Recent work has found that *bmp2/4* and *admp* homologs are expressed broadly in overlapping domains in *Helobdella*

embryos, instead of in spatially opposing domains [3]. In this system, expression of a *bmp5/8* homolog is instead dorsally polarized and required for establishment of DV polarity [3]. Although a detailed mechanism of how DV patterning works in this system has not been described, these results suggest that DV patterning in *Helobdella* proceeds by a previously unobserved mechanism. These observations lead to several questions. Firstly, what is the function of *bmp2/4* and *admp* in *Helobdella* if they are not central components of the DV axis? Interestingly, a homolog of the Bmp inhibitor *gremlin* was also identified in *Helobdella* and was observed to inhibit *bmp2/4* rather than *bmp5/8*, suggesting that *bmp2/4* may yet play an important role in the establishment of DV polarity in this system [3]. A second question that arises from these results is to what extent developmental systems rely on variant mechanisms of DV polarity establishment. It is important to note that DV patterning in *Drosophila* does not rely on an *admp/bmp* circuit and that *Drosophila* has no *admp* homolog. However, *Drosophila* embryos do express another Bmp

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ligand in addition to the *bmp* homolog *dpp*. This factor, *screw*, is expressed broadly in the embryo [4]. Screw is transported dorsally as a heterodimer with Dpp and through this action helps to canalize early *Drosophila* DV pattern [5]. One reason why this occurs is that the Dpp/Screw heterodimer is less sensitive to gene dosage effects than either homodimer [5]. Additional ways in which the use of this heterodimer canalizes DV patterning have also been proposed through mathematical modeling of the system [6]. Therefore, the usage of a Dpp/Screw heterodimer as a main source of signal buffers the system to perturbations of expression. However, early *Drosophila* morphogology and organization is significantly different than in vertebrates and displays many derived as opposed to ancestral developmental mechanisms [7-9]. Therefore, in *Helobdella* and *Drosophila*, developmental contexts with unique needs may have facilitated the innovation of variant Bmp-based DV patterning systems.

This general conclusion is applicable in vertebrate systems as well. Both mice and

humans do not have an identified *admp* homolog. Indeed, mammalian axis formation has unique features not found in *Xenopus* or zebrafish, and embryogenesis is significantly different [10]. In mammals then, does a core *bmp/admp*-like system function in DV polarity establishment? This could be the case even in the absence of a direct *admp* homolog if a functional equivalent exists. Given the identification of “expander-repressor”-like regulatory topologies in at least the *Drosophila* wing disc and *Xenopus* DV axis, it would not be surprising to discover a similar system at work in mice or human embryogenesis. It will be interesting to observe as studies of mammalian embryogenesis progress whether such a factor exists, or whether DV polarity in mammals, like in *Helobdella* and *Drosophila*, uses a variant regulatory system in its establishment of a Bmp gradient.

Finally, some organisms like *C. elegans* do not rely on Bmp signaling at all for establishing DV polarity [11], indicating that other mechanisms for this process also exist. Unlike the other systems discussed here,

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however, *C. elegans* develops by an invariant set of cell divisions [12]. Therefore, it is possible that this particular mode of highly stereotyped development made Bmp-based polarity establishment ultimately dispensable.

The identification of an *admp/bmp* regulatory circuit in planaria, a Lophotrochozoan, is highly significant as it allows us to propose that such a regulatory circuit was an ancestral feature of the Bilateria. We can similarly conclude due to their conservation that Bmp inhibitors such as *noggin* and *chordin* are also ancestral features of DV patterning systems. With these findings and others, a basic depiction of this ancestral system is now beginning to emerge (See Chapter 1 Fig 1). As more features and mechanisms of this system become apparent, it will become increasingly feasible to infer key evolutionary changes that have occurred in specific systems, and possibly to associate these changes with particular morphologies or other unique features. Therefore, molecular studies of DV polarity in novel developmental contexts have the potential to identify: 1) core

properties of Bmp signaling that allow for its widely conserved use in DV patterning; as well as 2) alternate mechanisms by which organisms generate DV polarity, and the conditions that permit these mechanisms to evolve.

Furthermore, significant evidence is presented here that *admp* is required for maintenance and regeneration of medio-lateral (ML) polarity in planaria (see chapter 2), a function not previously described. For example, *admp(RNAi)* animals do not regenerate following lateral amputation, an injury requiring both DV and ML regeneration, and they lose proportion along their ML axis even in the absence of amputation. This phenotype is accompanied by corresponding disruptions in ML polarized gene expression. Therefore, it seems likely that opposing *bmp* and *admp* expression (in this case, medial *bmp* and lateral *admp*) can carry out its central function (self-regulation and scaling) in a non-DV setting (ML). Examining whether this function is conserved in other systems, and identifying which other aspects

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of the core circuit are present, will likely enhance our understanding of the *admp/bmp* regulatory topology. Furthermore, this finding suggests that, as has proven to be true with many developmental pathways, the *admp/bmp* circuit may have derived additional functions in specific developmental contexts.

II. Planarian regeneration and the use of *activin* and *follistatin*

Recent work has identified the decision to mount a regenerative response following injury as a key step of planarian regeneration. Animals respond differently to simple injuries, such as a puncture or incision, than to injuries that remove significant tissue, such as an amputation. In the former case, animals mount a transient proliferative response 6h after injury [13], display an increase in apoptotic numbers at the wound site [14], and transiently express hundreds of wound-induced genes [15]. In the latter case, all of these responses are mounted but also second, later, responses are observed. These

responses include increases in proliferation [13] and apoptotic numbers [14] 2-3d after amputation, and persistent wound-induced gene expression for a period of days [15]. This second set of responses can be referred to as regenerative responses. A central question in planarian regeneration has become how the decision to mount a regenerative response as opposed to a simple injury response, is made.

The identification of *fst* and *act-1* as key regulators of this process represents the first molecular description of the mechanisms that animals use to distinguish between injuries of varying severity and drive regenerative responses. *fst* and *act-1* are both wound induced but function oppositely: *act-1* suppresses regenerative responses whereas *Fst* inhibits *Act-1* signaling and thereby promotes regenerative responses. Following injuries that require significant regeneration, *fst* is induced potently relative to *act-1* and regeneration occurs. Following minor injuries, *fst* is induced weakly relative to *act-1* and regenerative responses are repressed. Finally, *act-1* expression persists at high

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levels for over a week after amputation, and is required for repressing regenerative responses after the bulk of regeneration is complete. This regulatory system is required for producing a regenerative response of the proper magnitude in response to an injury, as well as terminating regenerative responses after regeneration is complete.

Conservation of broader developmental functions of activin and follistatin

Whereas planarian regeneration uses the conserved vertebrate functions of Bmp and Admp for establishing DV polarity, the vertebrate functions of planarian *activin* and *follistatin* do not seem to be similarly conserved.

In vertebrate embryogenesis, Activin-like molecules are important for the establishment of organizer type regions, Spemann's organizer in *Xenopus* and Hensen's node in chick, and consequently the main body axes [16-19]. A number of factors expressed in vertebrate organizers are wound-induced and present at wound sites in planaria

[15, 20]. From these observations, one could speculate that a transient "organizer" may exist at wound sites shortly following injury. However, existing evidence argues against this. Firstly, *act-1* expression is largely excluded from wound-sites and therefore seems unlikely to function as an inducer of this structure. Secondly, *act-1* inhibition does not result in a failure to express "organizer" genes (wound induced factors), but in fact has the opposite effect in that wound-induced gene expression is potentiated. There are a number of alternate candidate TGF- β genes in planaria (see chapter 3). However, none of these genes produced any noticeable phenotype following RNAi ([15], and M.G. unpublished data). This is inconsistent with a role in organizer induction, as any factor important for wound induced gene expression should be required for a number of regenerative processes. In addition, none of these genes, except *act-1* and an *inhibin*-like gene, display wound-induced expression.

Alternatively, it has been suggested that perhaps the ventral midline of intact

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planarians is analogous to the vertebrate organizer [21]. However, other than displaying expression of *admp* and a *noggin* homolog (see chapter 2), this region does not display expression of other organizer genes. Moreover, given the ability of any part of the animal to regenerate following injury, including lateral domains that lack a midline, it does not mechanistically make sense to ascribe an organizing function to this domain of gene expression. Finally, there is no evidence to suggest that any Activin-like factor uniquely regulates this domain of gene expression. Taking these observations together, it seems unlikely that a structure analogous to the embryonic organizer exists during planarian regeneration, and therefore unlikely that the canonical function of embryonic Activin in establishing the vertebrate organizer is conserved in planaria.

As is the case with Activin-like molecules, the role of *follistatin* seems distinct between vertebrate embryogenesis and planarian regeneration. The chief function of *follistatin* in early amphibian development

is to antagonize Bmp signaling, and it is consequently expressed in the organizer [22][23]. In addition to this role in DV pattern specification, *follistatin* inhibition also causes defects in the formation of anterior structures in *Xenopus* embryos [23]. Notably, this phenotype is also observed following inhibition of a number of other Bmp inhibitors [24, 25]. This ventro-posteriorization can be interpreted in two fundamental ways: 1) In addition to their roles in embryonic DV patterning, Bmp proteins and Follistatin have a separable second role in embryonic AP patterning; or 2) The central function of Bmp signaling and Bmp inhibition by Follistatin is to pattern the DV axis whereas AP defects are a secondary consequence of this function that arise due to the embryonic morphology of *Xenopus*. Comparative studies of other developmental systems support the second conclusion. Namely, Bmp signaling has been nearly universally observed in systems studied to establish polarity along the DV axis [26]. In most systems in which this is the case, Bmp signaling has no role in AP development,

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suggesting that this aspect of Follistatin function is a vertebrate derived function. Consistent with this, *follistatin* inhibition does not seem to affect Bmp signaling in planaria and, furthermore, Bmp pathway inhibition in planaria does not disrupt the AP polarity of animals [27, 28]. Rather, the Wnt pathway controls AP polarity establishment in planaria [29-32], and Follistatin has not previously been observed to interact with Wnt proteins. Rather, evidence here (see chapter 3) indicates that Follistatin instead interacts with Activin-like molecules in planaria. Therefore, it seems likely that the anterior requirement for *follistatin* is either due to secondary effects of the regeneration phenotype or due to an as of yet undescribed mechanism. One hypothetical model explaining how the broader regeneration phenotype could cause head patterning defects could be that morphallaxis is required for brain formation and that brain formation is integral to acquiring anterior identity. For all of these reasons, it is likely that the anterior patterning phenotype of *follistatin* inhibition in planaria is unrelated to similar phenotypes observed in

vertebrates, and that the AP patterning roles ascribed to *follistatin* in these two contexts likely represent distinct derived functions.

III. Uncovering conserved developmental programs and mechanisms of canalization in regeneration

As has been demonstrated here, developmental pathways used in embryogenesis can be conserved in planarian regeneration. Though I have focused on the conservation of DV patterning mechanisms in regeneration, other developmental pathways are seemingly also conserved, among them the use of Wnt signaling for establishing AP polarity and the use of *slit* and *netrin* for midline patterning [31, 32]. Comparisons of regeneration with embryogenesis can yield insights broadly into how common developmental pathways are co-opted for novel functions. Furthermore, studying how these pathways are regulated to be kept active in adult animals may yield insights of medical relevance.

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The canalization of regeneration

Much like embryogenesis, regeneration is a developmental phenomenon that must occur with unerring accuracy. Regeneration is unique, however, in that there is no fixed starting tissue from which regenerative programs begin; they must produce a common output with an input that can be essentially random. Inherent in this process therefore must be mechanisms that account for the variety of injuries encountered in order to canalize the process. Regenerative models therefore have a potentially unique level of canalization in all of their developmental programs. For these reasons, it seems likely that future investigations into the mechanisms of regeneration will allow not only for descriptions of how specific developmental modules are made robust, but also for the identification of key regulatory topologies of regulating canalized processes. The expander-repressor model discussed earlier is one such example. What other developmental processes utilize this regulatory motif? To what extent is this motif modulated to adapt to regenerative

systems, if at all? Finally, how diverse are the regulatory motifs that are used broadly among animals? All of these questions will require a much broader sampling of developmental and regenerative systems. As planarians have proven to be a genetically tractable system in which unparalleled feats of regeneration are possible, they present an attractive model for addressing this need. Future investigations into how planarian signaling systems are reset and rescaled following injury should therefore facilitate a greater understanding of how common signaling pathways are canalized as well as how patterning mechanisms in general can be structured to withstand perturbation.

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