**Follistatin antagonizes Activin signaling and acts with Notum to direct planarian head regeneration**

Rachel H. Roberts-Galbraith and Phillip A. Newmark

Howard Hughes Medical Institute and Department of Cell and Developmental Biology, University of Illinois, Urbana, IL 61801

Edited by Joseph G. Gall, Carnegie Institution of Washington, Baltimore, MD, and approved December 5, 2012 (received for review August 13, 2012)

Animals establish their body plans in embryogenesis, but only a few animals can recapitulate this signaling milieu for regeneration after injury. In planarians, a pluripotent stem cell population and perpetual signaling of polarity axes collaborate to direct a steady replacement of cells during homeostasis and to power robust regeneration after even severe injuries. Several studies have documented the roles of conserved signaling pathways in maintaining and resetting axial polarity in planarians, but it is unclear how planarians reestablish polarity signaling centers after injury and whether these centers serve to influence identity decisions of stem cell progeny during their differentiation. Here we find that a planarian Follistatin homolog directs regeneration of anterior identity by opposing an Activin/Acr-1/Smad2/3 signaling pathway. Follistatin and Notum, a Wnt inhibitor, are mutually required to reestablish an anterior signaling center that expresses both cues. Furthermore, we show that the direction of cells down particular differentiation paths requires regeneration of this anterior signaling center. Just as its amphibian counterpart in the organizer signals body plan and cell fate during embryogenesis, planarian Follistatin promotes reestablishment of anterior polarity during regeneration and influences specification of cell types in the head and beyond.

**Results and Discussion**

**Characterization of Planarian Follistatin.** To better understand reestablishment of anterior polarity, we have characterized a Follistatin homolog in the planarian species *Schmidtea mediterranea*. Smed-follistatin mRNA is evident in a ventrally enriched punctate distribution throughout the body of the animal, with a strong focus of expression in a small cluster of several cells at the anterior midline tip, beneath the epithelium (Fig. 1A and B). Consistent with a previously published study (7), follistatin expression is up-regulated after injury, with more puncta evident as early as 6 h after a cut in the side of the animal and continuing to at least 2 d after amputation (Fig. 1A and Fig. S1A). Double fluorescence in situ hybridization (FISH) experiments showed that the cluster of follistatin+ cells in the head of the planarian also expresses notum (Fig. 1B and Fig. S1B), whereas follistatin+ cells outside this region are notum+. After amputation, follistatin+/notum+ cells appeared near the wound within 1 d and an anterior focus of double-positive cells is clear within 2 d (Fig. S1 C and D), follistatin+ cells outside the anterior center often resided in or near the central nervous system (Fig. S1E). Double FISH indicated that these cells often abutted neurons [prohormone convertase-2 (PC2)+ or choline acetyltransferase (ChAT)+ cells], but that they were not themselves neural in nature (Fig. 1C and Fig. S1 E and F). However, follistatin+/ChAT+ cells were infrequently observed in regenerating organisms (Fig. S1G), suggesting a possible transient overlap in the two differentiation trajectories.

We next investigated whether follistatin expression in the head depends on Notum. After notum(RNAi), follistatin expression in the tip of the head was absent or disorganized (Fig. 1D). In contrast, expression of follistatin outside the anterior focus was unaffected (Fig. 1D). The group of follistatin+/notum+ cells in the planarian head also evoked the expression pattern of foxD, a forkhead transcription factor of unknown function that was identified in the planarian *Dugesia japonica* (14) (Fig. S1B). The forkhead transcription factor FoxL2 had previously been shown to regulate follistatin expression in the context of the murine ovary and pituitary (15, 16), so we evaluated whether the anterior group of follistatin+ cells depended on Smed-foxD. Indeed, we found that foxD(RNAi) animals had absent or disorganized anterior follistatin foci (Fig. 1D) that were diminished even without amputation (Fig. S1L). Neither foxD(RNAi) nor notum(RNAi) treatment eliminated early follistatin up-regulation after a superficial cut (Fig. S1L), suggesting that foxD and notum function more specifically in reestablishment and/or maintenance of follistatin expression in the anterior-most focus and that other regulatory mechanisms under additional follistatin expression, foxD(RNAi), like notum(RNAi) (10), led to aberrant eyespot number and altered nervous system morphology after amputation (Fig. S1 H and I), indicating that both gene products play roles in proper anterior regeneration.

**Follistatin Is Essential for Planarian Head Regeneration.** We next tested whether Follistatin itself is required for proper head regeneration. follistatin(RNAi) animals were amputated and, after 5 d of regeneration, these animals were significantly impaired in their ability to regenerate heads compared with controls.
Outwardly, follistatin(RNAi) animals had small blastemas that were devoid of regenerated eyespots (Fig. 2 A and B and Fig. S2 A and B). In situ hybridization for ChAT and glutamate receptor (GluR1), which are expressed in the entire nervous system and branches of the cephalic ganglia (brain), respectively (17, 18), indicated impairment of cephalic ganglia regeneration after follistatin(RNAi) (Fig. 2C and Fig. S2 C and F). These defects appeared to be contingent upon amputation, as long-term treatment with follistatin(RNAi) caused animals to display slightly regressed tips of the head without an overall deterioration of the central nervous system (Fig. S2 D and E).

A defect in neural regeneration could be due to failure of either neurogenesis or specification of anterior polarity. To determine whether anterior polarity was reestablished after amputation, follistatin(RNAi) animals were subjected to in situ hybridization to detect transcripts with roles in anterior polarity (2, 3, 10, 19), secreted frizzled-related protein 1 (sFRP-1) and nov darake (ndk) mRNAs showed reduced expression in follistatin(RNAi) planarians (Fig. 2D). We also observed that notum expression was disrupted in follistatin(RNAi) animals (Fig. 2D), indicating a reciprocal requirement between Notum and Follistatin. The interdependence of follistatin and notum signals at the tip of the head could assist in focusing the anterior signaling center, ensuring accurate and robust organization of the body after traumatic injury.

One explanation for the follistatin(RNAi) phenotypes was that RNAi resulted in posteriorization of the planarians, but this seemed not to be the case, as the zone of wnt1 expression was broader but still confined to the true posterior end of treated animals (Fig. 2E and Fig. S2F). Furthermore, we ruled out the possibility that defects in regeneration were due to neoblast depletion or lack of mitotic cells in follistatin(RNAi) planarians (Fig. 2F and Fig. S2G). Finally, ventral nerve cord regeneration after tail amputation occurred (Fig. S2 C and F), indicating that some nerve tissue regeneration could occur after follistatin(RNAi). Taken together, our data indicate that Follistatin plays a positive role in establishing anterior polarity during planarian regeneration.

**Follistatin Antagonizes Activin Signaling.** Follistatin homologs in vertebrates and *Drosophila* bind to and inhibit members of the TGF-β family, in particular Activin and bone morphogenetic protein (BMP) family members (20–24). No planarian Activin homolog has been described, but BMP signaling drives dorsal polarity and is important for maintenance of the midline (11, 25, 26). To determine whether planarian Follistatin functions by inhibiting one of these pathways, we performed RNAi of activin and BMP4 alone with follistatin(RNAi). activin(RNAi) animals regenerated anterior structures and concomitant RNAi targeting both activin and follistatin rescued the phenotype of follistatin(RNAi), both in terms of cephalic ganglia regeneration (assayed by strong ChAT expression) and anterior polarity specification (reflected by sFRP-1 expression) (Fig. 3A). Quantitatively, 90% of activin(RNAi); follistatin(RNAi) animals regenerated two eyespots within 5 d of amputation, compared with zero planarians treated with follistatin(RNAi) alone (Fig. 3B and Fig. S3A). In contrast, BMP4(RNAi) did not rescue follistatin(RNAi) in terms of nervous system regeneration, anterior polarity specification, or eyespot formation (Fig. 3 A and B).

Knockdown of planarian activin alone did not result in a dramatic phenotype, but activin(RNAi) animals had a diminished capacity to regenerate posterior tissues, sometimes regenerating with notched tails or regenerating with a smaller wnt1-expressing domain (Fig. S3 B and D). activin is expressed in the planarian gut and pharynx during homeostasis, as well as in cells distributed along the ventral side of the animal (Fig. 3C). These ventral puncta become the dominant expression pattern during regeneration (Fig. 3C). Although the patterns of activin and follistatin expression in regenerating worms appear superficially similar (Figs. 1 A and 3C), the two genes are not expressed in the same cells (Fig. S3C).

Activin signals are transduced by receptor serine/threonine kinases, which phosphorylate and activate Smad2/3 transcription factors (for a review, see ref. 27). We cloned eight planarian TGF-β receptors and one Smad2/3 homolog. We found that knockdown of one type I Activin/TGF-β receptor (ActR-1) also rescued the follistatin(RNAi) phenotype. ActR-1(RNAi); follistatin(RNAi) animals showed near-control regeneration of the cephalic ganglia (Fig. 3D), and 87.5% of these animals regenerated two eyespots (Fig. 3E and Fig. S3E). RNAi against planarian smad2/3 also partially rescued the follistatin(RNAi)
are absent or dramatically reduced in size in different anterior cell populations. (follistatin(RNAi) in animals were subjected to in situ hybridization with follistatin(RNAi) after amputation of the head. Both control(RNAi) and missing from all control animals and are missing eyespots. (follistatin(RNAi) animals display small blastemas compared with control animals and are missing eyespots. (follistatin(RNAi) animals suggest that the receptor and downstream transcription factor also play roles in mediating the effects of additional TGF-β family members. Knockdown of notum or follistatin results in the absence of head regeneration after amputation, but simultaneous knockdown of wnt1 or activin, respectively, rescues anterior polarity (10). Taken together, these data suggest that either pair of signals is dispensable as long as other redundant polarity cues remain. However, the interdependence of follistatin and notum expression in the tip of the head and the influence of Follistatin and Activin on wnt1 expression in the tail imply cross-talk between the two pathways. The benefit of parallel signals of anterior polarity could be to reinforce and refine polarity reestablishment after injury, thus supporting the robustness of planarian regeneration through verification of cellular position. Both Follistatin and Notum function as inhibitors, blocking Activin and Wnt signaling, respectively. It remains unclear whether cells of the planarian body (neoblast progeny in particular) receive and integrate signals from one or both pathways, although the ActR-1 transcript is not enriched in cycling neoblasts themselves (Fig. S3H). Our determination that ActR-1 is expressed broadly suggests that a wide variety of cell types could be responding to the Follistatin–Activin balance, and the large number of Frizzled-related receptors of Wnt signals also suggests a wide audience of cells able to respond to the level of Wnt signaling (2). The impact of Hedgehog on Wnt signaling as well (8, 9) further illustrates the complex mechanisms underlying assignment of place in the anteroposterior axis.

**Follistatin Directs Fate Decisions in Planarians.** Although it is known that axial gradients organize the body plan during regeneration and homeostasis in the planarian, it is not clear how these gradients are received and interpreted in other cells. Of critical importance, it is not clear whether neoblast progeny respond to Wnt, TGF-β, or other polarity signals (directly or indirectly) to determine migration direction or cell identity. By contrast, a wealth of literature documents the effects of similar ligands (and their inhibitors) during development or for differentiation and maintenance of pluripotent stem cells (for a review, see ref. 29). For example, inhibition of Activin and/or BMP signaling (by Noggins, Follistatin, Chordin, or chemical inhibitors) drives neuronal fate of pluripotent stem cells in vitro and also during development of a range of vertebrate and invertebrate organisms (22, 30–39). The expression of follistatin in cells near the nervous system and the absence of cephalic ganglia in regenerating follistatin(RNAi) animals led us to investigate whether Follistatin could be inducing specific fates (neural or otherwise) in planarians as well.

Because molecular markers along the path to neural differentiation have not yet been described in planarians, we first used markers that are available to label subtypes of differentiating cells. A previous report documented a number of genes (called category 3 genes) that are down-regulated 7 d after ablation of neoblasts by irradiation (40). Although gene expression in differentiated cells remains unchanged at this time point, neoblast
progeny would be depleted. Thus, down-regulated genes of this category were interpreted to label cells during the process of differentiation. Furthermore, because expression of pairs of category 3 genes often do not overlap (40), each category 3 gene might represent neoblast progeny within a certain fate trajectory or during a certain window of differentiation (Fig. S4).

We used genes in this category to evaluate whether specific populations of differentiating cells were affected after knockdown of follistatin or notum. We found that Ras-related and h.72.4d, category 3 genes with expression near the nervous system, were indeed expressed in fewer cells in follistatin(RNAi) and notum(RNAi) animals (Fig. 4A and B). We next examined the expression of Cyp1A1, a category 3 gene that marks two populations of cells throughout the planarian body. Surprisingly, one of the two populations of cells (the darker-staining population present ventrally) disappeared in follistatin(RNAi) animals but not in notum(RNAi) animals (Fig. 4 C and C′). This population was absent not only anteriorly but in posterior regions as well, indicating that the effect of Follistatin on this population of cells is Notum-independent and is likely not acting through Follistatin’s role in anterior polarity alone. Nemo-like kinase, a gene expressed in neoblasts and the brain (41), also showed a mildly to moderately reduced domain of expression in both follistatin(RNAi) and notum(RNAi) animals (Fig. 4D). Some subtypes of differentiating cells were insensitive to follistatin(RNAi) treatment, including those expressing mitochondrial carrier protein (MCP) and unc-9 (Fig. 4 E and F), indicating that Follistatin is not responsible for instruction of cell differentiation in general.

Although the influence of Follistatin on category 3 gene-expressing cells suggests a role for Follistatin in differentiation, we were specifically interested in whether Follistatin might drive cells toward neuronal fates. Toward this end, we examined
the expression of eyes absent (eya), a transcription factor and phosphatase expressed in differentiating photoreceptors (among other cell types) (42, 43), and found that eya expression was diminished in follistatin(RNAi) and notum(RNAi) animals (Fig. 4G). Sox transcription factors often function in neural induction in other organisms (44), with Xenopus SoxD and Drosophila SoxN being inhibited by BMP/Dpp signaling and Xenopus Sox-2 being induced by Chordin (45–47). We identified a planarian Sox transcription factor expressed in the nervous system, smed-SoxB (48), that is also dramatically down-regulated in follistatin(RNAi) and notum(RNAi) animals (Fig. 4H). Together, these results demonstrate that planarian Follistatin does influence differentiation, possibly directing some neoblast progeny toward anterior fates.

**Conclusion**

Organization of the body plan and determination of cell fates during embryonic development often rely on signaling centers such as the amphibian organizer, which secretes BMP/Activin inhibitors—including Follistatin—and Wnt inhibitors (49). In this study, we suggest that regenerating organisms use and reuse similar mechanisms, with follistatin and notum expression within a population of cells with organizer-like activity that promotes anterior identity and morphogenesis of the cephalic ganglia and eyespots (Fig. 4f). Here we have characterized the function of planarian Follistatin, a signaling molecule with a critical role in head regeneration. We have further investigated the mechanism of Follistatin activity, using double-RNAi rescue experiments to reveal that Follistatin inhibits an Activin/ActR-1/Smad2/3 signaling pathway that itself represses anterior regeneration (Fig. 4f). Together, our results indicate that redundancy in signals that promote anterior polarity sustains the body plan in planarians, with signals reinforcing one another to ensure accurate organization of the body, especially after severe injury. Furthermore, the intimate association between follistatin*+* cells and neurons raises the intriguing possibility that Follistatin could be influencing neuronal fate and function directly. In the future, it will be important to dissect direct and indirect functions of Follistatin and to separate, if possible, the roles of follistatin*+* cells in the anterior versus the remainder of the planarian body.

**Methods**

**Planarian Experiments.** A clonal line of asean planarians (CIW4) was maintained as previously described (50), except that animals were kept in Instant Ocean salts (Spectrum Brands) at 0.5 g/L in ultrapure water and 50 μg/mL gentamicin (Gemini Bio-Products). Each riboprobe or dsRNA was synthesized as previously described from 400- to 500-bp fragments of genes cloned into the pUC53.2 vector (51, 52). For single-RNAi experiments, animals were fed 3 μg dsRNA in 35 μL of a liver:salts puree (3:1). For double-RNAi rescue experiments, animals were fed a total of 4 μg dsRNA (either 4 μg control, 2 μg control + 2 μg experimental, or 2 μg each experimental). Volumes were also kept constant in these experiments. For all experiments, unless otherwise noted, animals were fed dsRNA on days 0, 6, and 12, before being cut prepharyngeally on day 17. On day 22 (day 5 postamputation), regenerating animals were evaluated for phenotypes (counting eyespots or imaging) or were killed and fixed (for in situ hybridizations).

In situ hybridizations were performed as per ref. 53 with some modifications, either by hand or using an In situ Pro VS (Intavis). For immunofluorescence experiments, planarians were killed in 2% (vol/vol) HCl, fixed for 1 h at 4 °C in 4% (vol/vol) formaldehyde in PBS, and then bleached in 6% (vol/vol) H2O2 in PBS overnight. Animals were blocked in 6 mg/mL BSA (Jackson ImmunoResearch) and 0.45% MCP (Jackson ImmunoResearch) and 0.04% fish gelatin (Sigma-Aldrich) in PBS + 0.3% Triton X-100. Animals were incubated with rabbit anti-phospho-histone H3 (S10; Cell Signaling) primary antibodies at a 1:5,000 dilution overnight at 4 °C. The secondary antibody (Molecular Probes) was used at a 1:2,500 dilution.

RT-quantitative PCR and irradiation experiments were performed as previously described (51, 54), except for an increase to 100-Gy dosage for irradiation.

**Image Acquisition and Processing.** Live animals and chemically developed in situ hybridization experiments were imaged with a Leica MZ255A stereomicroscope running LAS software 3.6.0 (Leica). Immunofluorescence and fluorescence in situ hybridization experiments were imaged using a Zeiss LSM 710 confocal microscope with either a 20× (Plan-Apochromat 206/0.8) or a 63× objective (Plan-Apochromat 636/1.4) using Zen software (Carl Zeiss).

The complete mRNA sequence for *S. mediterranea* follistatin, as well as incomplete mRNA sequences for activin, ActR-1, and smad2/3, have been deposited in GenBank under accession numbers KC161222–5.

**ACKNOWLEDGMENTS.** We are grateful to members of the P.A.N. laboratory for advice and technical help during the course of this project. We also thank Bret Pearson for suggesting the use of Instant Ocean salts and Ryan King for suggested improvements to the in situ protocol. R.H.R.-G. is a fellow of the Jane Coffin Childs Memorial Fund for Medical Research. This work was supported by National Institutes of Health Grant R01 HD043403 (to P.A.N.). P.A.N. is an investigator of the Howard Hughes Medical Institute.


Supporting Information

Roberts-Galbraith and Newmark 10.1073/pnas.1214053110

Fig. S1. Planarian follistatin expression in the head. (A) After control(RNAi), notum(RNAi), or foxD(RNAi), animals were cut as indicated in the diagram and were killed and fixed 6 h later. Animals were probed for follistatin expression by in situ hybridization (ISH), which revealed some up-regulation after injury in each treatment (arrowheads). Additionally, loss of follistatin expression at the anterior-most tip of the animal is visible after foxD(RNAi) treatment (asterisk). (B) Whole-mount in situ hybridization using notum and foxD probes. Both transcripts are expressed within a small number of cells in the tip of the head, visible in magnifications of boxed areas. (C) Double fluorescence in situ hybridization (FISH) with follistatin (magenta) and notum (green) probes. A double-positive cell is apparent in an animal 1 d after head amputation. Single-positive cells are also evident. (D) Double FISH of the anterior tip of an animal 2 d into head regeneration, showing cells expressing follistatin (magenta) and notum (green). (E) Whole-mount confocal microscopy image of FISH using follistatin and prohormone convertase-2 (PC2) probes. The square indicates the region presented at higher magnification in Fig. 1C. Other adjacent PC2+ and follistatin+ cells are indicated with arrowheads. (F) Follistatin- and choline acetyltransferase (ChAT)-expressing cells are also often adjacent (arrowheads), but follistatin and ChAT are not coexpressed. (G) Rarely observed colocalization of ChAT (green) and follistatin (magenta) transcripts in the same cell in a 3-d regenerating anterior end. ChAT transcript localization in the nucleus has previously been show to occur in differentiating neurons (1). (H) foxD(RNAi) and notum(RNAi) animals display impaired cephalic ganglia regeneration 5 d after head amputation. The central nervous system is detected by in situ hybridization with a ChAT probe. (I) foxD(RNAi) and notum(RNAi) animals do not regenerate anterior structures properly. After 5 d of regeneration, negative-control animals have regenerated eyespots, but foxD(RNAi) and notum(RNAi) animals do not (n = 25). [Scale bars, 500 μm (A and H), 200 μm (B), 20 μm (C, D, and G), 100 μm (E), and 50 μm (B, Insets and F); Anterior is up.]

Fig. S2. follistatin plays a critical role in anterior regeneration. (A) RT-quantitative PCR of follistatin transcript levels in control(RNAi) and follistatin(RNAi) animals in a representative experiment. Significance was determined using a Student’s t test. (B) ISH showing loss of follistatin transcript after follistatin(RNAi). (C) Regeneration of head, trunk, and tail fragments after 5 d. Control(RNAi) and follistatin(RNAi) animals with nervous systems stained via ISH with a ChAT probe. Nervous system regeneration is perturbed in follistatin(RNAi) animals. Lack of pharyngeal ChAT signal in anterior and posterior pieces indicates that pharynx regeneration or innervation occurs aberrantly in follistatin(RNAi) animals as well. (D) Live images of planarian heads after 100 d of control(RNAi) or follistatin(RNAi) treatment every 6 d. A slight discoloration and regression of the head tissue is apparent in follistatin(RNAi) animals (arrowheads). (E) ISH of

Roberts-Galbraith and Newmark www.pnas.org/cgi/content/short/1214053110

Legend continued on following page
uncut animals after long-term RNAi treatment using a ChAT probe. (F) Animals were fed RNAi for 70 d (every 6 d) and amputated pre- and post-pharyngeally. ISH of ChAT or Wnt1 was performed on trunk pieces killed and fixed 6 d into head and tail regeneration. The cephalic ganglia did not regenerate in follistatin(RNAi) animals, but some ventral nerve cord regeneration is evident in the tail. Wnt transcript was not visible in the anterior blastema of follistatin(RNAi) animals by DAPI staining, but an expanded Wnt1-positive area was visible in the tail blastema after follistatin(RNAi). (G) Control(RNAi) and follistatin(RNAi) planarians after 5 d of regeneration, stained with anti-phospho-histone H3 (red) and DAPI (blue). Failure of cephalic ganglia regeneration is evident in the follistatin(RNAi) animal by DAPI staining, but mitotic neoblasts are visible. [Scale bars, 500 μm (B, C, E, and F), 2 mm (D), and 100 μm (G).] Anterior is to the left (B, C, E, and F) or up (D and G).
Planarian follistatin inhibits activin upstream of an Activin receptor (ActR-1) and smad2/3. (A) Live animals with eyespots visible in an activin rescue experiment, quantitated in Fig. 3B. (B) After tail amputation, activin(RNAi) animals reestablish a smaller or absent Wnt1-expressing posterior region. (C) Double FISH illustrates that the punctate activin and follistatin expression patterns are not overlapping in a 2-d regenerate. (D) After amputation in front of and behind the pharynx, activin(RNAi) trunk fragments sometimes have a minor defect in tail regeneration, such as a notched tail (asterisk). Following head and tail amputation, ActR-1(RNAi) trunk fragments sometimes regenerate an extra pharynx (arrowheads). The central nervous system and pharyngeal plexus are both visible using ChAT in situ hybridization. (E) Live animals from ActR-1 and smad2/3 rescue experiments, with eyespots quantitated in Fig. 3G. (F) ActR-1(RNAi) animals, imaged live, displayed a flattened behavior both before (not pictured) and after head amputation and regeneration. (G) smad2/3(RNAi) animals, subjected to in situ hybridization using a TPH (tryptophan hydroxylase) probe, regenerated eyespots and anterior regions with slightly reduced efficiency. Cyclopia was occasionally observed (arrowhead). (H) ActR-1 transcript levels were evaluated by RT-quantitative PCR either in unirradiated animals or in animals 1 or 3 d after treatment with 100 Gy of gamma radiation. Significance was calculated using one-way ANOVA; ns, not significant. [Scale bars, 500 μm (A, B, D, E, and G), 50 μm (C), and 1 mm (F).] Anterior is up.
Fig. S4. Category 3 genes and neuronal transcription factors in planarians. Whole-mount in situ hybridization with Ras-related (A), h.72.4d (B), Cyp1A1 (C), nemo-like kinase (nlk; D), mitochondrial carrier protein (MCP) (E), Unc-9 (F), eyes absent (eya; G), or SoxB (H) probes. (Scale bar, 500 μm.) Anterior is to the left.