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

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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/ActR-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.

nervous system regeneration | *Schmidtea mediterranea*

Well-known signaling cascades continuously coordinate axes of polarity during homeostasis and regeneration in planarians (1). Wnt signaling through β-catenin induces posterior identity (2–5), guiding the animal's body plan during homeostasis and, after injury, supporting regeneration programs and directing reestablishment of axial polarity (6, 7). Hedgehog signaling also promotes posteriorization, partly through regulation of *Wnt* gene expression (8, 9), whereas the α/β-hydrolase Notum, an inhibitor of Wnt signaling, plays a critical role in anterior polarity reestablishment (10). The differentiated cells that secrete polarity cues must be respecified after injury, a process that can occur in the absence of planarian stem cells (5, 6, 11), pluripotent cells called neoblasts (12). Although the mechanisms underlying the restoration of polarity after amputation are unknown, they likely involve interpretation of existing gradients of polarity, as evidenced by the observation that very thin fragments with insufficient gradient depth fail to reestablish polarity (13). Nonetheless, robust regeneration of polarity gradients must involve redundant signaling; for example, concomitant knockdown of *wnt1* with *notum* negates its headless phenotype, leading to faithful resetting of anterior polarity through some other, unknown mechanism (10).

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. 1 *A* and *B*). Consistent with a previously published study (7), *follistatin* expression is upregulated 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. 1*A* and Fig. S1*A*). Double fluorescence in situ hybridization (FISH) experiments showed that the cluster of *follistatin*$^+$ cells in the head of the planarian also expresses *notum* (Fig. 1*B* and Fig. S1*B*), 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. S1*E*). 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. 1*C* and Fig. S1 *E* and *F*). However, *follistatin*$^+$/*ChAT*$^+$ cells were infrequently observed in regenerating organisms (Fig. S1*G*), 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. 1*D*). In contrast, expression of *follistatin* outside the anterior focus was unaffected (Fig. 1*D*). 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. S1*B*). 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. 1*D*) that were diminished even without amputation (Fig. S1*A*). Neither *foxD(RNAi)* nor *notum(RNAi)* treatment eliminated early *follistatin* up-regulation after a superficial cut (Fig. S1*A*), 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 underly 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.

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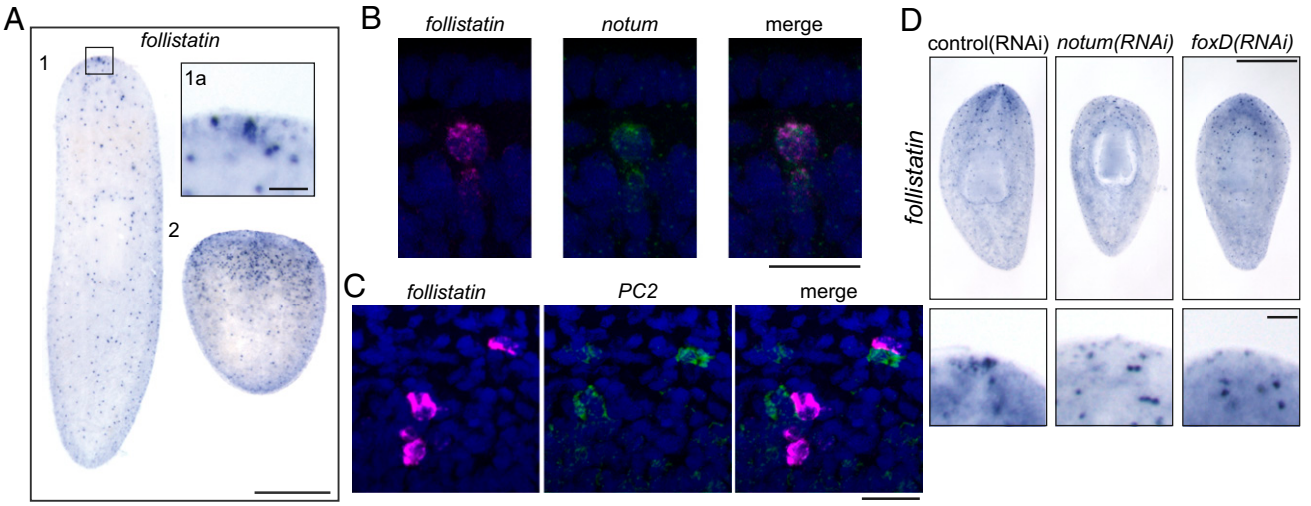

Fig. 1. Planarian *follistatin* is expressed in the head. (*A*) Whole-mount in situ hybridization with a *follistatin* probe (1) reveals strong expression in a few anterior cells (boxed area, 1a) and weaker expression in numerous cells throughout the planarian body. Puncta of *follistatin* expression are markedly increased in a tail piece regenerating a new head 2 d after amputation (2). (*B*) *follistatin* and *notum* transcripts, viewed by FISH, are present in the same few cells in the anterior of the planarian. The *follistatin* transcripts are visualized in magenta, whereas *notum* transcripts are in green. (*C*) *follistatin*-expressing cells (magenta) and *PC2*-expressing cells (green) are often near each other, but coexpression of *follistatin* and *PC2* was not observed by FISH. (*D*) *follistatin* expression in the tip of the head is absent or disorganized in 17 of 20 *notum(RNAi)* and 6 of 8 *foxD(RNAi)* animals after 5 d of regeneration. Expression of *follistatin* in the remainder of the animal is not affected. [Scale bars, 500 μm (*A* and *D*), 20 μm (*B* and *C*), and 50 μm (*A*, *Inset*).] The anterior of each animal is oriented toward the top.

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. 2*C* 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 respecification 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 *nou darake* (*ndk*) mRNAs showed reduced expression in *follistatin(RNAi)* planarians (Fig. 2*D*). We also observed that *notum* expression was disrupted in *follistatin(RNAi)* animals (Fig. 2*D*), 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 dramatic 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. 2*E* and Fig. S2*F*). 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. 2*F* and Fig. S2*G*). 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* along 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. 3*A*). 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. 3*B* and Fig. S3*A*). 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. 3*C*). These ventral puncta become the dominant expression pattern during regeneration (Fig. 3*C*). Although the patterns of *activin* and *follistatin* expression in regenerating worms appear superficially similar (Figs. 1*A* and 3*C*), the two genes are not expressed in the same cells (Fig. S3*C*).

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. 3*D*), and 87.5% of these animals regenerated two eyespots (Fig. 3*E* and Fig. S3*E*). RNAi against planarian *smad2/3* also partially rescued the *follistatin(RNAi)*

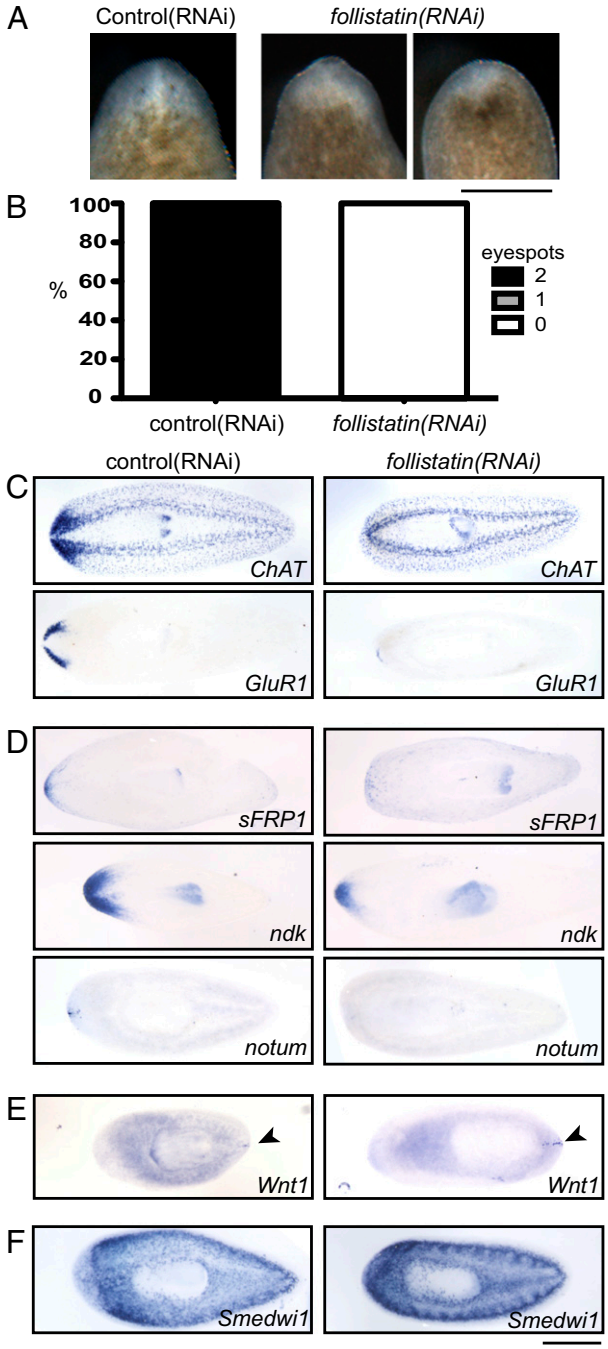

Fig. 2. *follistatin* plays a critical role in anterior regeneration. (*A*) After 5 d of head regeneration, *follistatin(RNAi)* animals display small blastemas compared with control animals and are missing eyespots. (*B*) Nearly all control animals regenerated eyespots within 5 d of amputation, but eyespots were missing from all *follistatin(RNAi)* animals ($n \geq 50$ each). (*C*) Cephalic ganglia are absent or dramatically reduced in size in *follistatin(RNAi)* animals 5 d after amputation of the head. Both control(RNAi) and *follistatin(RNAi)* animals were subjected to in situ hybridization with *ChAT* and *GluR1* probes to mark the entire central nervous system and the brain branches, respectively. (*D*) Anterior marker expression was reduced after 5 d of head regeneration in *follistatin(RNAi)* animals. *sFRP-1*, *ndk*, and *notum* probes each mark different anterior cell populations. (*E*) A posterior marker, *wnt1*, was expressed in an expanded posterior region (arrowheads) but was not expressed inappropriately in the anterior of *follistatin(RNAi)* animals. (*F*) In situ hybridization with a probe for a neoblast marker, *Smedwi-1*, indicates that neoblasts were present after *follistatin(RNAi)*. (Scale bars, 500 μm.) Anterior is up (*A*) or to the left (*C–F*).

phenotype (Fig. 3 *D* and *E* and Fig. S3*E*), although with less efficiency. Knockdown of *ActR-1* alone led to a behavioral phenotype, with worms adopting a flattened posture (Fig. S3*F*) and movement defects, consistent with a previous report (28). Additionally, *ActR-1(RNAi)* animals amputated in front of and behind the pharynx occasionally regenerated with supernumerary pharynges (Fig. S3*D*). Knockdown of *smad2/3* alone resulted in a mild head regeneration phenotype, with cyclopia or absence of eyes in some regenerating animals (Fig. 3*E* and Fig. S3*G*). Consistent with these phenotypes, *ActR-1* and *smad2/3* expression was broad, with an enrichment of expression in the eyes and cephalic ganglia (Fig. 3*F*). Whereas double-RNAi experiments indicate that ActR-1 and Smad2/3 function downstream of Activin, detection of secondary phenotypes in *ActR-1 (RNAi)* and *smad2/3(RNAi)* animals that were not present in *activin(RNAi)* animals suggests 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. S3*H*). 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

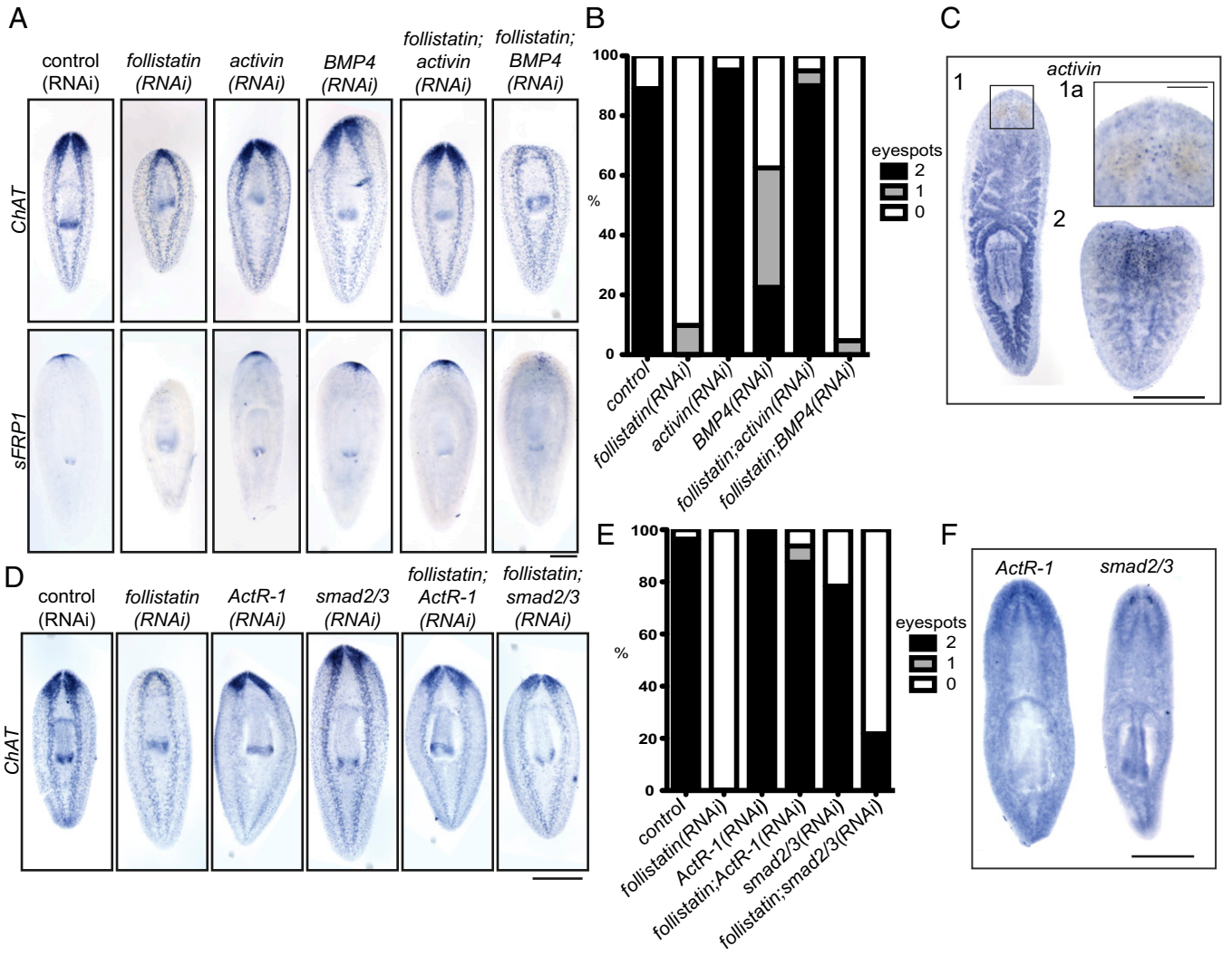

Fig. 3. Planarian *follistatin* inhibits *activin*. (*A*) *activin(RNAi)* combined with *follistatin(RNAi)* rescues nervous system regeneration and anterior polarity, whereas a combination of *BMP4(RNAi)* and *follistatin(RNAi)* does not lead to rescue of these phenotypes. In situ hybridization with *ChAT* and *sFRP-1* probes illustrates nervous system and anterior polarity, respectively, in 5-d regenerates. (*B*) Concomitant *activin(RNAi)* and *follistatin(RNAi)* rescues the *follistatin (RNAi)* phenotype, as evidenced by eyespot regeneration ($n \geq 40$ each). (*C*) Whole-mount in situ hybridization with an *activin* probe indicates expression in the gut and pharynx (1) and in ventral spots along the animal and in the head (boxed area, 1a). The punctate expression pattern is dominant in 2-d regenerating tail pieces (2). (*D*) Simultaneous knockdown of *follistatin* with *ActR-1* or *smad2/3* by RNAi rescues the *follistatin(RNAi)* phenotype. dsRNA-fed animals 5 d after amputation were subjected to in situ hybridization with the *ChAT* probe to illustrate regeneration of the nervous system. (*E*) Eyespot quantitation after RNAi and 5 d of head regeneration. Concomitant treatment with *ActR-1(RNAi)* rescues the *follistatin(RNAi)* phenotype, and treatment with *smad2/3(RNAi)* partially rescues the *follistatin(RNAi)* phenotype ($n \geq 30$ each). (*F*) *ActR-1* and *smad2/3*, detected by whole-mount in situ hybridization, are broadly expressed but neurally enriched. [Scale bars, 500 μm (*A*, *C*, *D*, and *F*) and 100 μm (*C*, *Inset*).] Anterior is up.

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. 4 *A* 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. 4*D*). 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

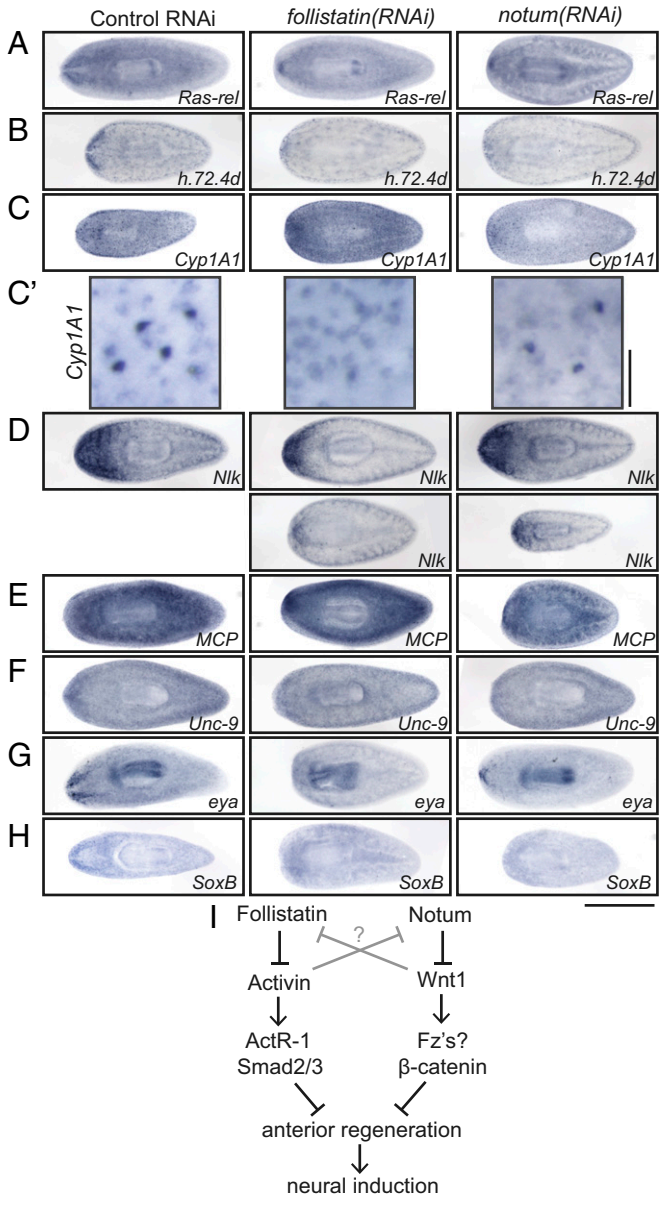

Fig. 4. Follistatin influences fate decisions in planarians. In situ hybridization of control(RNAi), *follistatin(RNAi)*, or *notum(RNAi)* animals 5 d after head amputation, using *Ras-related* (*A*), *h.72.4d* (*B*), *Cyp1A1* (*C*; higher magnification in *C′*), *nemo-like kinase* (*nlk*; *D*), *MCP* (*E*), *Unc-9* (*F*), *eya* (*G*), or *SoxB* (*H*) probes. (*I*) Model illustrating the role of Follistatin and Notum signals in providing a positive feedback loop to stabilize signaling of anterior polarity in planarians. [Scale bars, 500 μm (*A*–*H*) and 50 μm (*C′*).] Anterior is to the left.

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. 4*G*). 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. 4*H*). 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. 4*I*). 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. 4*I*). 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 asexual planarians (ClW4) 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 pJC53.2 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 InsituPro 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) H_2O_2 in PBS overnight. Animals were blocked in 6 mg/mL BSA (Jackson ImmunoResearch) and 0.45% 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 M205A 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.

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