Supporting Information

Roberts-Galbraith and Newmark 10.1073/pnas.1214053110

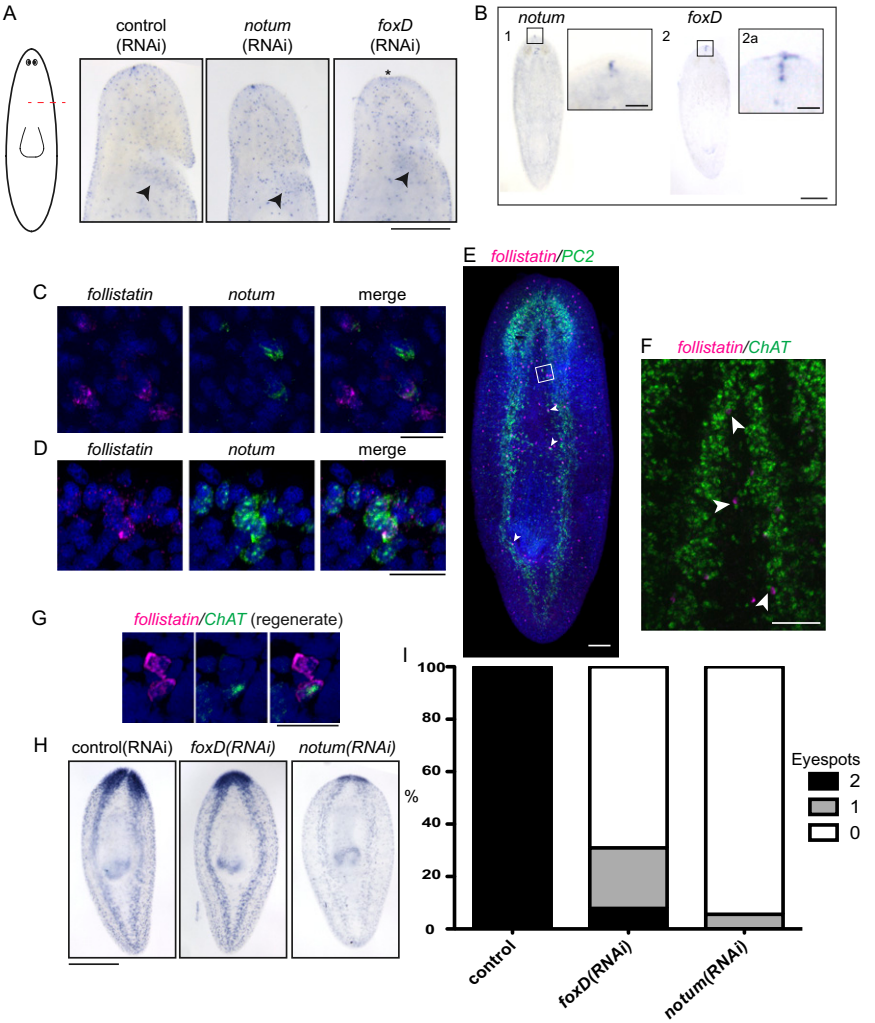

Fig. S1. Planarian *follistatin* expression in the head. (*A*) After control(RNAi), *notum(RNAi)*, or *foxD(RNAi)*, animals were cut as indicated in the diagram and were killed and fixed 6 h later. Animals were probed for *follistatin* expression by in situ hybridization (ISH), which revealed some up-regulation 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. 1*C*. 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.

1. Wagner DE, Wang IE, Reddien PW (2011) Clonogenic neoblasts are pluripotent adult stem cells that underlie planarian regeneration. *Science* 332(6031):811–816.

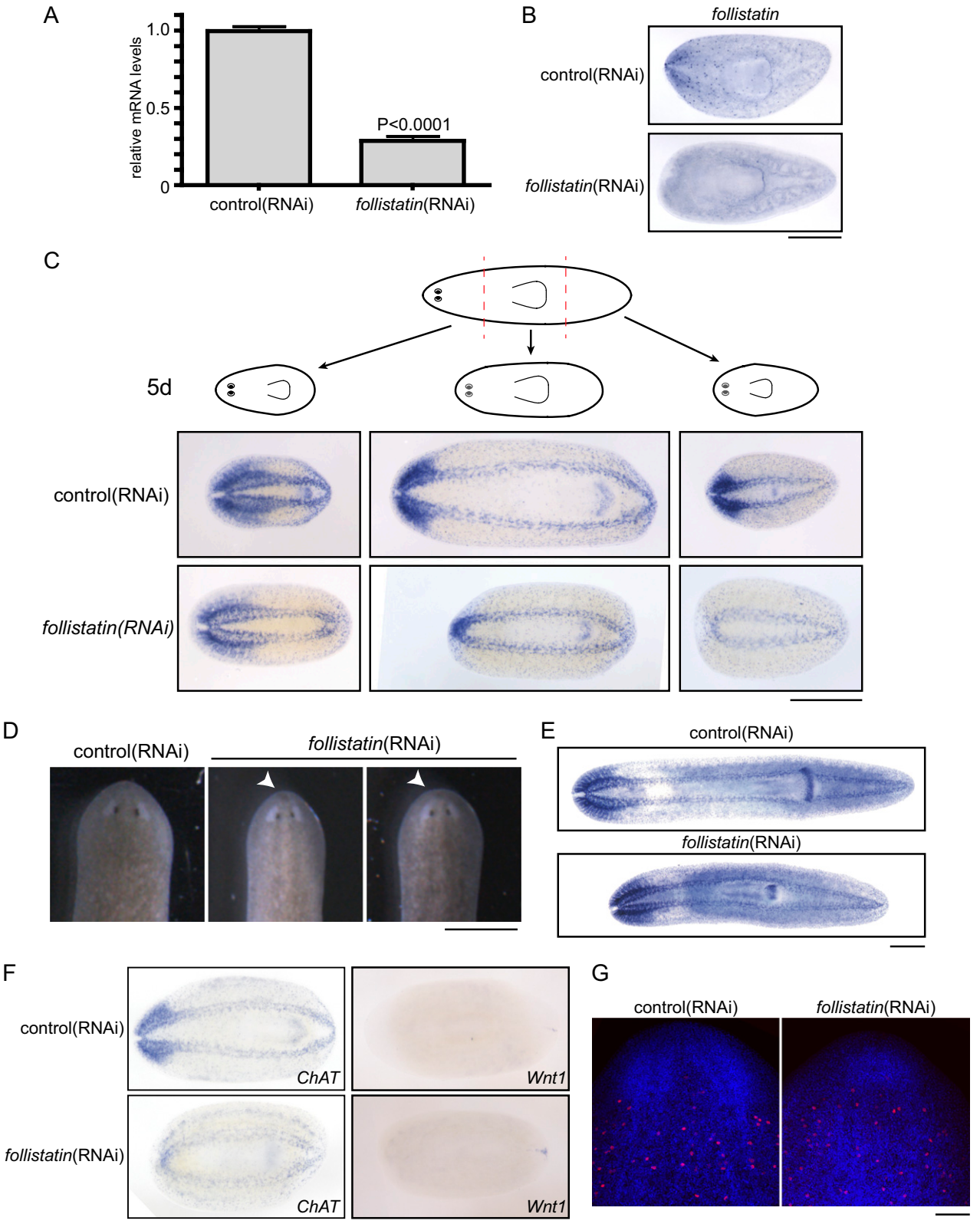

Fig. S2. *follistatin* plays a critical role in anterior regeneration. (*A*) RT-quantitative PCR of *follistatin* transcript levels in control(RNAi) and *follistatin(RNAi)* animals in a representative experiment. Significance was determined using a Student's *t* test. (*B*) ISH showing loss of *follistatin* 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

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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*).

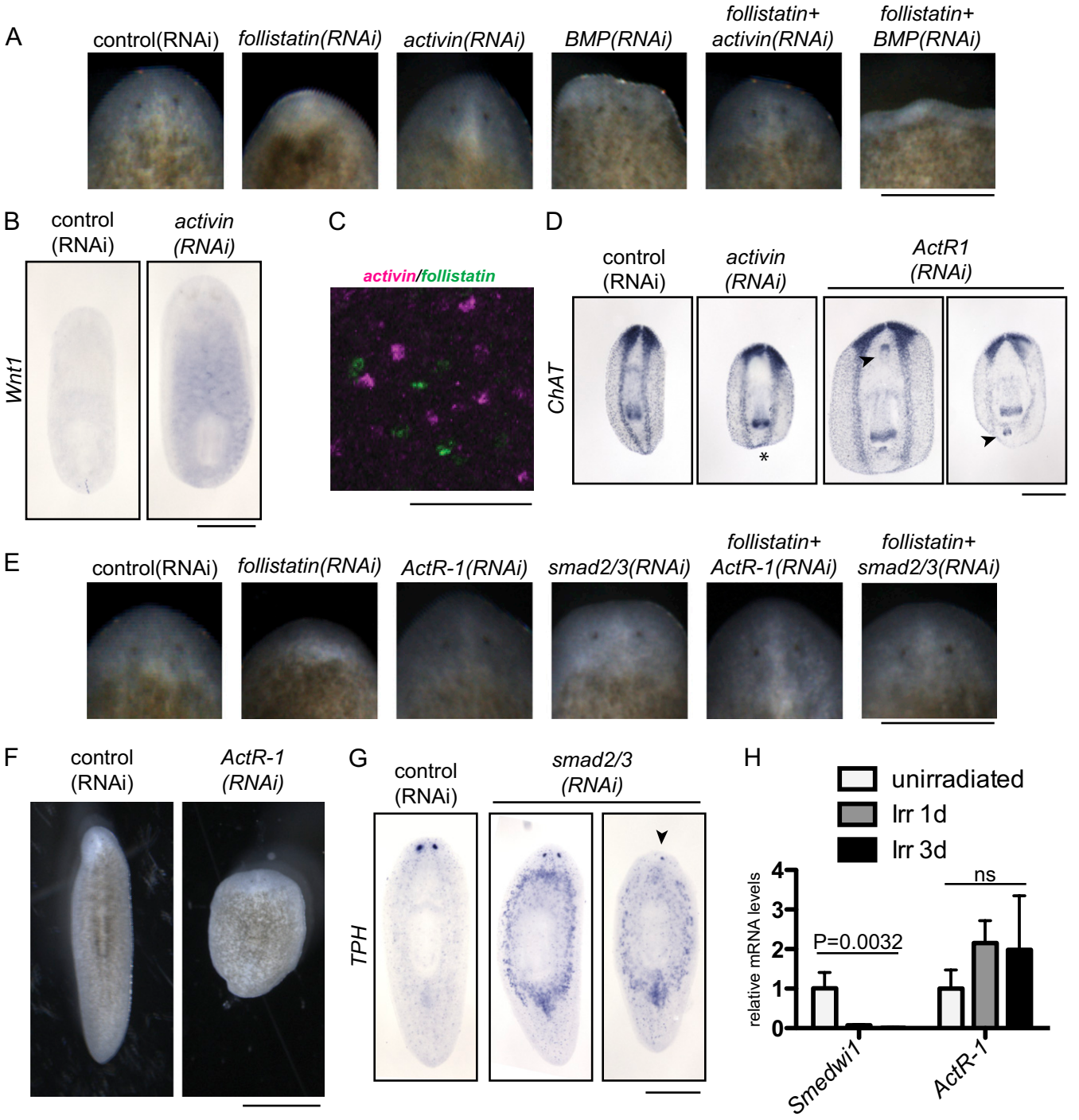

Fig. S3. Planarian *follistatin* inhibits *activin* upstream of an Activin receptor (*ActR-1*) and *smad2/3*. (*A*) Live animals with eyespots visible in an *activin* rescue experiment, quantitated in Fig. 3*B*. (*B*) After tail amputation, *activin(RNAi)* animals reestablish a smaller or absent *Wnt1*-expressing 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)* trunks 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. 3*G*. (*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.

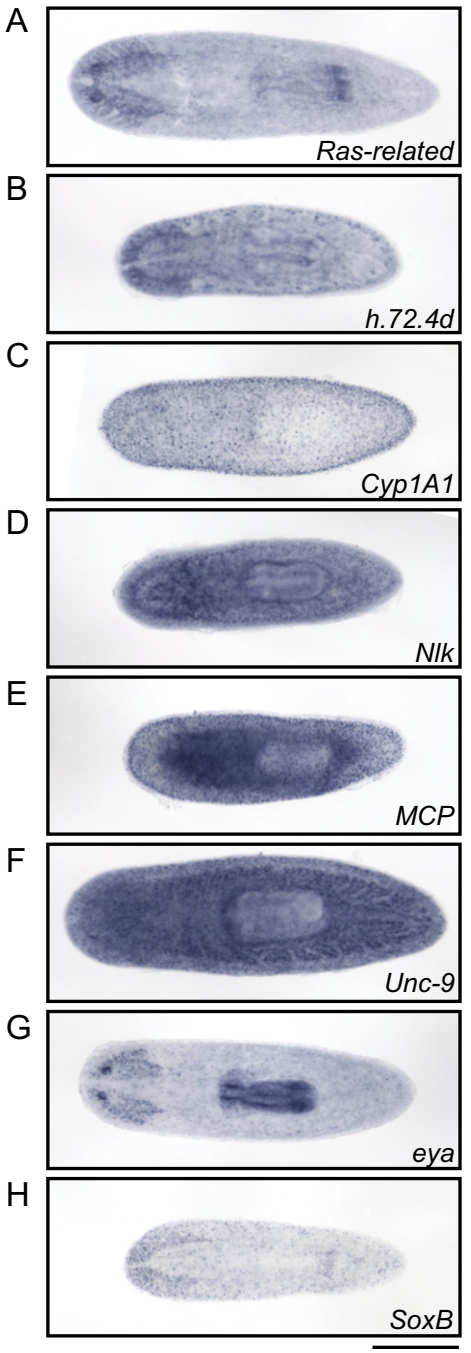

Fig. S4. Category 3 genes and neuronal transcription factors in planarians. Whole-mount in situ hybridization with *Ras-related* (*A*), *h.72.4d* (*B*), *Cyp1A1* (C), *nemo-like kinase* (*nlk*; *D*), *mitochondrial carrier protein* (*MCP*) (*E*), *Unc-9* (*F*), *eyes absent* (*eya*; *G*), or *SoxB* (*H*) probes. (Scale bar, 500 μm.) Anterior is to the left.