Variability of regenerative potential among animals has long perplexed biologists. On the basis of their exceptional regenerative abilities, planarians have become important models for understanding the molecular basis of regeneration. However, planarian species with limited regenerative abilities are also found. Despite the importance of understanding the differences between closely related, regenerating and non-regenerating organisms, few studies have focused on the evolutionary loss of regeneration, and the molecular mechanisms leading to such regenerative loss remain obscure. Here we examine *Proctotyla fluviatilis*, a planarian with restricted ability to replace missing tissues, using next-generation sequencing to define the gene expression programs active in regeneration-permissive and regeneration-deficient tissues. We found that Wnt signalling is aberrantly activated in regeneration-deficient tissues. Notably, downregulation of canonical Wnt signalling in regeneration-deficient systems response shortly after amputation and a later burst near the wound site. In addition, regenerating tissues re-establish proper axial polarity along the anterior–posterior axis (Fig. 1a, b and Supplementary Fig. 1). The stage at which regenerative processes fail in this animal is unknown. Planarian regeneration encompasses several processes, including wound healing to cover exposed tissues and allow signalling between the wound epidermis and underlying mesenchymal cells, apoptosis, and stem cell (neoblast) proliferation. Following amputation, neoblast proliferation occurs in two waves: a systemic response shortly after amputation and a later burst near the wound site. In addition, regenerating tissues re-establish proper axial polarity, using a number of conserved signalling pathways. To identify the nature of regeneration failure in *P. fluviatilis*, we characterized several of these early regenerative processes following amputation in both regeneration-proficient (Reg+) and regeneration-deficient (Reg-) tissues. Histological staining and electron microscopy revealed that wound healing occurs properly following amputation in all tissues, regardless of regenerative potential (Fig. 1c, d). After amputation in Reg+ tissues, biphasic cell division occurs in both Reg+ and Reg- tissues (Fig. 1e, f and Supplementary Fig. 2). In addition, gut tissues seem to remodel in fragments that fail to regenerate (Supplementary Fig. 1). These data show that early phases of the regenerative response occur, although Reg- tissues fail to form a blastema.

To assess whether axial polarity is re-established properly following amputation in Reg+ tissues of *P. fluviatilis*, we characterized the spatiotemporal expression of homologues of *nou-darake* (ndk) and SFRP1, genes expressed specifically at the anterior of other planarian species. Both *Pfu-ndk* and *Pfu-sFRP1* are expressed at the anterior wound site shortly after amputation in Reg+ tissue (Fig. 1g, i). However, the expression of these genes was reduced in Reg- tissue following injury (Fig. 1h, j), indicating that the initial failure of regeneration occurs at or upstream of axial re-polarization.

Because these polarity markers are not expressed appropriately following amputation in Reg- fragments, we sought to identify gene expression differences between Reg+ and Reg- tissue after amputation. We generated a de novo *P. fluviatilis* transcriptome and used RNA sequencing (RNA-seq) to characterize transcripts from excised tissue fragments in Reg+ and Reg- body regions 24 h post amputation (Fig. 2a). We performed parallel analyses on tissues excised from intact animals at identical body regions to account for regional differences in transcripts, thereby identifying changes resulting from amputation (Fig. 2a). Analysis of amputated versus intact tissues revealed that...
10.7% of the assembled contigs (16,026/149,594) were significantly altered ≥ twofold ($P < 0.05$) after amputation in either Reg+ (red) or Reg– tissues. After collapsing contigs likely to be representing the same transcript based upon BLAST comparison similarity, we focused our analysis on 15,742 contigs that seem to be expressed differentially after amputation (based on the large number of contigs, many individual transcripts are likely to be represented by multiple contigs). Whereas a small number of contigs were simultaneously over- or under-represented in both conditions (74/15,742), many were over- or under-represented exclusively in either Reg+ or Reg– fragments (14,288/15,742). Other contigs were over-represented in Reg+ tissue and under-represented in Reg– tissue (537/15,742) or vice versa (842/15,742) (Supplementary Table 1). Upon close examination of transcripts over-represented in Reg+ tissues and under-represented in Reg– tissues (537/15,742) or vice versa (842/15,742) (Supplementary Table 1). Upon close examination of transcripts over-represented in Reg+ tissues and under-represented in Reg– tissues, we found that several represented genes were involved in Wnt signalling. Given the importance of Wnt signalling in defining anteroposterior polarity in other planarian species10,11,14, we focused on genes involved in this pathway.

RNA-seq revealed significant over-representation of many transcripts encoding Wnt ligands and receptors in Reg+ tissues after amputation (Fig. 2b), with some transcripts, such as Pfu-wnt11-1, upregulated as much as ~400-fold relative to intact controls. These same transcripts were downregulated in Reg– tissues relative to their position-adjusted intact controls (Fig. 2b). In addition, homologues of Wnt inhibitors, such as sFRP1 and sFRP2 (ref. 18), were downregulated in Reg+ tissues and upregulated in Reg– tissues (Fig. 2b). These patterns of gene expression were confirmed by quantitative PCR with reverse transcription (qRT–PCR) (Fig. 2c, d). Pfu-β-catenin1, the intracellular effector of Wnt signalling, was not expressed differentially following amputation in either tissue region (Fig. 2b), indicating that Pfu-β-catenin1 expression is not responsive to wounding in either Reg+ or Reg– tissues.

Because axial repolarization fails in Reg+ tissues after amputation, we propose that upregulation of posteriorly expressed genes, including Wnt ligands, may inhibit signals that lead to proper anterior–posterior patterning and, thus, block regeneration. To test this hypothesis, we disrupted Wnt signalling using RNA interference (RNAi) to target Pfu-β-catenin1, the intracellular Wnt signalling effector. Remarkably, Pfu-β-catenin1 RNAi resulted in blastema formation and regeneration of a complete head and brain in Reg+ fragments as assayed by regeneration of the photoreceptors ($n = 64/71$) (Fig. 3b, f), whereas control RNAi animals, injected with double-stranded RNA from a ccdB and camR-containing bacterial sequence, failed to form a blastema or neural structures (Fig. 3a, e). Pfu-β-catenin1 RNAi also affected posterior regeneration with the formation of heads at posterior facing wounds (Supplementary Fig. 3), which has been observed in other planarian species10. Knocking down Wnts individually or in combination did not rescue Reg+ tissue (Supplementary Table 2). Pfu-β-catenin1(RNAi) animals with rescued regeneration demonstrated anteriorly directed movements within 15 days after amputation (Supplementary Video 1), indicating complete and functional regeneration of the head. Rescue of regeneration in Pfu-β-catenin1(RNAi) animals reveals that Reg+ tissues are competent to express the head regeneration program, but either lack signals required for re-establishment of axial polarity or these signals are inhibited.

In contrast, knockdown of APC, an inhibitor of β-catenin and Wnt signalling, also resulted in blastema formation, but led to regeneration of tails at anterior-facing wounds ($n = 14/53$, Fig. 3c, g), as observed in Schmidtea mediterranea19. These data indicate that altering gene expression to create either an anterior or posterior polarity cue within Reg+ tissues can perpetuate downstream steps in regeneration, thereby allowing blastema formation and regeneration of heads or tails at anterior-facing wounds. Notum has recently been identified as a Wnt inhibitor expressed at anterior facing wounds in the planarian Schmidtea mediterranea19. However, Pfu-notum RNAi failed to initiate a similar posterior-regeneration program. To assay alterations in gene expression following regeneration rescue, we used qRT–PCR to characterize expression patterns of polarity genes following Pfu-β-catenin1 RNAi and Pfu-APC RNAi (Fig. 3h). After amputation in Pfu-β-catenin1(RNAi) animals, both Pfu-sFRP1 and Pfu-ndk were significantly upregulated, indicating that a latent anterior regeneration program is reactivated in Reg– tissues following Pfu-β-catenin1 RNAi.

Our data provide important clues about mechanisms regulating regeneration. In a related Dendrocoelid species with reduced regenerative capacity, anterior regeneration ability in posterior fragments was rescued through grafting irradiated anterior tissue onto Reg+ tissue20, suggesting that signals from differentiated anterior cells were sufficient to allow regeneration. Our results indicate that such signals...
are involved in re-establishing anterior–posterior polarity. Recent efforts have identified many requirements for the regenerative response to wounding, including proliferation, apoptosis and cell signalling, but the interrelationships between these processes are not yet well understood. Our data confirm that increased neoblast proliferation following amputation occurs independently of the re-establishment of anterior–posterior polarity (Fig. 1e, f and Supplementary Fig. 2). We have also shown that subsequent regenerative processes are inhibited until polarity is re-established. These observations suggest a checkpoint in the regeneration program that must be satisfied before downstream developmental processes can occur. Such a checkpoint would act as a vulnerable stage at which evolutionary modifications could alter regenerative potential.

Loss of regenerative ability would seem to carry a selective disadvantage, and possible causes for such losses have been the subject of much speculation. Identification of aberrant Wnt signalling following amputation in Reg+ tissues of P. fluviatilis suggests one mechanism limiting regeneration ability in planarians; however, we can only speculate about the driving force leading to regenerative loss. Unlike most planarians, P. fluviatilis is semelparous, reproducing only once, then dying within a single season. Life-history studies have shown that semelparous species invest more in reproduction, possibly at the expense of other developmental programs. Whereas highly regenerative, iteroparous planarian species resorb reproductive structures during periods of starvation and following amputation, semelparous species do not resorb testes after such events. We speculate that the establishment of anterior–posterior polarity (Fig. 1e, f and Supplementary Fig. 2) would act as a vulnerable stage at which evolutionary modifications could alter regenerative potential.

Next-generation sequencing technologies and functional analyses facilitate research on understudied, yet biologically informative, non-model organisms. Expanding use of these technologies will help elucidate causes for limited regeneration in other animals, potentially identifying inhibitory signals that must be overcome to elicit a regenerative response after wounding. Given that perturbation of a single gene’s function can rescue an entire regenerative program, identifying additional regeneration-inhibiting signals will increase our understanding of the evolution of regeneration loss and provide the intriguing prospect of restoring regenerative abilities in regeneration-deficient animals.

**METHODS SUMMARY**

**In situ hybridization.** In situ hybridizations were performed using formaldehyde fixation as described previously with minor modifications.

**Immunostaining.** Immunostaining was conducted as described previously using anti-synapsin mouse monoclonal, anti-phospho-histone H3 (Ser10) rabbit monoclonal, goat anti-mouse Alexa-488, and goat anti-rabbit Alexa-488 antibodies.

**Histology and scanning electron microscopy.** Samples were initially prepared as described previously. For histology, samples were sectioned and stained with toluidine blue. For scanning electron microscopy, samples were mounted on aluminium stubs and coated with Au/Pd.

**Transcriptome sequencing and RNA-seq.** Roche 454 pyrosequencing technology was used to sequence a reference transcriptome from the RNA of four intact P. fluviatilis. Reads were assembled de novo using iterations of SeqMan NGen and CLC Genomics. Solexa illumina sequencing was performed on RNA extracted from excised tissues. Reads were mapped to the reference transcriptome using CLC Genomics as described previously.

**Cloning.** Candidate genes were amplified via PCR from complementary DNA (Supplementary Table 3) and homology was assigned (Supplementary Fig. 4). PCR products were ligated with Eam1105I-digested pJC53.2 (ref. 30) and used for cloning.

**Quantitative RT–PCR.** cDNA was synthesized from RNA isolated as described and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions Both authors contributed to the design of the experimental strategy. J.M.S. conducted all experiments, analysed the data and drafted the manuscript, which was critically reviewed and revised by P.A.N. Both authors discussed the results and commented on the final version of the manuscript.

Author Information Sequence read archive (SRA) data reported in this paper were deposited at NCBI as a BioProject under accession number PRJNA205293. RNA-seq analyses have been deposited in the NCBI Gene Expression Omnibus under accession number GSE48497. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests.

Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.M.S. (jsikes@usfca.edu).
METHODS
Animal collection and culture. *Dugesia tigrina* was collected from streams at Blackhouse Point Conservation Park (Maryland, USA) or the LaRue Pine Hills/Otter Pond Research Natural Area (Illinois, USA). Animals were maintained in the laboratory at 18°C in Montjuich salts and fed bi-weekly. Planarians were starved for 1 week before use.

**In situ hybridization.** *In situ* hybridizations were performed using the formaldehyde-based fixation procedure as described previously with the following modifications. Planarians were killed in 8% *N*-acetyl cysteine for 5 min, fixed in 4% formaldehyde in PBSTX (PBS + 0.3% Triton X-100) for 25 min, and incubated in reduction solution for 5 min at room temperature. Samples were bleached in 6% H2O2 for 1 h. Samples were imaged with a Leica M205A stereoscope.

Immunostaining. Immunostaining was conducted as described previously using methacarn fixative. Primary antibody incubation was performed overnight at 4°C at the following concentrations: anti-synapsin mouse monoclonal antibody (1:75; Developmental Studies Hybridoma Bank, catalogue no. 3C11), anti-philosphistone H3 (Ser10) rabbit polyclonal antibody (1:100; Cell Signaling Technology, catalogue no. 3377). Animals were incubated in secondary antibody (goat anti-mouse Alexa-488, 1:400 or goat anti-rabbit, Alexa-488, 1:500; Molecular Probes, Invitrogen, catalogue no. A11029) overnight at 4°C. Animals were mounted in Vectashield. Images were captured with Zeiss SteREO LumarV12, Zeiss AxiosZoomV16, and Zeiss LSM710 confocal microscopes.

**Histology and scanning electron microscopy.** Animals were fixed and prepared as previously described. For histology, ethanol was gradually replaced with acetone, followed by infiltration with Araldite/Embed 812 (Electron Microscopy Sciences). Sections (1 μm) were collected on glass slides, dried over a heating plate, and stained with 1% toluidine blue for 10 s. Slides were mounted in Cytoseal 60 (Thermo Scientific) and imaged on a Nikon Eclipse TE200 inverted microscope. For scanning electron microscopy, once the samples reached 100% ethanol, they were critical-point dried using a Tousimis Samdri-PVT-3D, mounted on aluminium stubs, coated with Au/Pd using a Denton Desk II TSC turbo-pumped sputter coater and imaged on a Philips XL30 ESEM-FEG.

**Transcriptome sequencing and RNA-seq.** For sequencing the reference transcriptome, RNA from four random, intact *P. flava* adults was isolated using TRIzol (Invitrogen). DNase-treated, purified with an RNA Clean & Concentrator kit (Zymo Research), and submitted to the W. M. Keck Center for Comparative and Functional Genomics for Illumina sequencing. Reads were assembled *de novo* using iterations of SeqMan NGen (DNASTAR) and CLC Genomics (CLCbio). For RNA-seq experiments, RNA was isolated from tissue fragments of five worms, each excised ~2 mm posterior to the amputation sites. Control RNA was purified from corresponding control fragments excised from intact animals at equivalent body regions as described above. Samples were submitted to the W. M. Keck Center for Comparative and Functional Genomics for illumina sequencing. Reads were mapped to the reference transcriptome using CLC Genomics and compared as in ref. 29. Contigs with ≥2-fold change and P value < 0.05 (from two-sided unpaired t-tests) were used for detailed analysis. Selected contigs were screened to identify redundant contigs by using BLAST homology to the NCBI nr database to merge contigs with top hits to the same gene.

Cloning. To generate riboprobes, candidate genes were PCR amplified from cDNA generated from total RNA (iScript cDNA Synthesis Kit, Bio-Rad). For cDNA preparations, RNA was extracted using TRIzol Reagent (Invitrogen). For cloning, 2–3 μl of PCR product was ligated with 70 ng of Eam1105I-digested pJC53.2 (Rapid DNA Ligation kit, Roche) and used to transform DH5α Escherichia coli cells. *In vitro* transcriptions with the appropriate RNA polymerase were performed using standard approaches with the addition of digoxigenin-11-UTP (Roche). All primers used to amplify candidate genes are included in Supplementary Table 3.

**RNAi.** To generate dsRNA, templates cloned into pJC53.2 (ref. 30) were amplified with a modified T7 oligonucleotide (GGATCTCTAATACGACTCACTATAGGG), purified using a DNA Clean & Concentrator kit (Zymo Research), and eluted in 15 μl of water. 10.5 μl of each PCR product (Supplementary Fig. 5) was used as template for *in vitro* transcription in a reaction containing 5 μl 100 mM mix of ribonucleotide triphosphates (rNTPs) (Promega), 1 μl high-yield transcription buffer (0.4 M Tris pH 8.0, 0.1 M MgCl2, 20 mM spermidine, 0.1 M EDTA), 1 μl thermostable inorganic pyrophosphatase (New England Biolabs), 0.5 μl Optizyme recombinant RNase inhibitor (Fisher Scientific), and 2 μl T7 RNA polymerase. Samples were incubated at 37°C for 12 h and then treated with RNase-free DNase (Fisher Scientific, catalogue number FP2231) and cleaned/concentrated via ammonium acetate precipitation. Synthesized RNA was then annealed by heating at 95°C, 75°C and 50°C each for 3 min. dsRNA solution was mixed with dye and 65 nl (~1 μg μl−1) was microinjected into the gut of randomized adult planarians three times over the course of 1 week before amputation using a Nanoject II micromanipulator (Drummond Scientific). As a negative control, animals were injected with dsRNA synthesized from the cdlB- and camR-containing insert of pJC53.2 (ref. 30). Live (RNAi) animals were imaged and videos were captured with a Leica M205A.

**Quantitative RT–PCR.** To examine transcript levels following amputation in regeneration-proficient and regeneration-deficient tissues, RNA was extracted using TRIzol reagent from five random tissue fragments identical to those used for RNA sequencing. Following DNase treatment (DNA-free RNA Kit, Zymo Research), reverse transcription was performed (iScript cDNA Synthesis Kit) and quantitative PCR was conducted using Power SYBR Green PCR Master Mix (Applied Biosystems) and a 7900HT real-time PCR system (Applied Biosystems). Three biological replicates were performed and all samples were measured in triplicate to account for pipetting error. Absolute quantities of each transcript were determined for each gene and normalized to the level of *fdo-actin* in each sample. The mean value for each amputated treatment was then normalized to the intact tissue fragments extracted from an identical axial position to determine the relative changes in expression due to amputation. For qRT-PCR of genes following RNAi experiments, the mean values of control RNAi and experimental (RNAi) samples were graphed independently without normalization to intact fragments. All primers used for these studies are included in Supplementary Table 3.

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