Supporting Information

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SI Materials and Methods

Isolation, Cloning, and Sequencing of S. mediterranea Genes. Planarian homologs of the Wnt pathway were identified from the S. mediterranea genomic database (Washington University, St. Louis, MO) through a BLAST search. The corresponding full-length transcripts were amplified by rapid amplification of cDNA ends using a GeneRacer Kit (Invitrogen).

Whole-Mount in Situ Hybridization. Gene expression analysis was carried out using an Intavis InSituPro hybridization robot. Digoxigenin-labeled riboprobes for Smed-dvl-1, Smed-dvl-2, Smed-vang-1, Smed-vang-2, Smed-div, Smed-OtxB, SmedOtp (kindly provided by Mette Handberg-Thorsager, European Molecular Biology Laboratory, Heidelberg, Germany), Smed-Gpas, Smed-TCEN49 (1), Smed-HoxD (1), Smed-AblBA (1), and Smed-Cintillo (2) were synthesized using an RNA in vitro transcription kit (Roche). Primer details are available on request.

RNAi Silencing. dsRNA was synthesized by in vitro transcription (Roche) and injected into planarians as described previously (3). Primer details are available on request. All the dsRNAs were injected at the same concentration (450–500 ng/μL) except for Smed-vang-1/2 and Smed-div dsRNAs, which were injected at a concentration of 800 ng/μL for the Smed-dvl-1/2 dose-dependent experiment, and Smed–β-catenin1/2 (bcat1) dsRNA, which was injected at a concentration of 300 ng/μL in the low-dose RNAi experiment. For regeneration experiments, animals were amputated pre- and postpharyngeally after 3 consecutive days of injections (one round of injection). In the case of Smed-wnt5, Smed-evi/ Wntless, Smed-vang-1/2, and Smed, we injected a second round of dsRNA 1 wk after the first round. For low-dose RNAi experiments, we injected dsRNA just 1 d before amputation. For homeostasis experiments, we followed the same protocol of injection but without amputation. All specimens were analyzed between 3 and 6 wk after amputation. Control animals were injected with water or GFP (in the case of combinatorial experiments).

Motility Analysis. Live animals were placed in Petri dishes and left until control planarians adapted to the surface and began to display normal motility. Animals were placed in the center of the dish with a Pasteur pipette, and a light stimulus was then introduced. Movement away from the light was recorded with a Zeiss Stemi SV6 stereomicroscope.

Immunostaining and Confocal Imaging. Immunostaining was carried out as described elsewhere (4). The following primary antibodies were used: antiarrestin (1:15,000; kindly provided by Hidelumi Orii, Himeji Institute of Technology, Hyogo, Japan), anti-synapsin (anti-SYNORF1, 1:50; Developmental Studies Hybridoma Bank), anti-α-tubulin (1:20; Developmental Studies Hybridoma Bank), antiactin JLA20 (undiluted; Developmental Studies Hybridoma Bank), anti-Smed–β-catenin2 (1:2,000) (5) and anti-Neuropeptide F (NPF; 1:1,000) (6). Note that differences in the expression pattern of NPF compared with previous reports (7) are explained by the fixation protocol used. When analyzing cilia, we used cavity slides to avoid squashing the cilia. Confocal laser scanning microscopy was performed with a Leica TCS 4D (Leica Lasertechnik) adapted for an inverted microscope (DMIRB; Leitz). A region located ~1 mm from the anterior end of the planarian was chosen for imaging ventral cilia and the actin network. To build 3D reconstructions, confocal sections were deconvolved using Huygens Deconvolution Software (Scientific Volume Imaging) and processed using Imaris Software (Bitplane).

Electron Microscopy. For SEM, animals were encapsulated to avoid manipulation problems and pretreated carefully with 2% L-cysteine, pH 7.2, to clear the mucus present on the surface. Animals were fixed in phosphate-buffered 2.5% (vol/vol) glutaraldehyde. For TEM, animals were fixed in phosphate-buffered 2.5% (vol/vol) glutaraldehyde and 4% (weight/vol) paraformaldehyde.

Fig. S2. Conservation of different functional domains in the brain of dvl-1/2 RNAi animals. The expression pattern of the homeobox genes otp and otxB reveals the conservation of different functional domains despite the abnormalities in brain morphogenesis after silencing of Smed-dvl-1/2. (Upper) Anterior. dR, days after cutting. (Scale bar: 400 μm.)

Fig. S1. Phylogenetic analysis and mRNA expression pattern of Smed-dvl paralogs. (A) Phylogenetic tree of Dvl proteins inferred from the accurate multiple amino acid sequence of Dvl proteins obtained using MAFFT, version 6. The phylogenetic tree was prepared using MEGA, version 3.1. Vertebrates are labeled in yellow, ecdysozoans in blue, and lophotrocozoans in pink. Accession numbers of each protein sequence are indicated in the tree. Accession numbers of Smed-Dvl-1 and Smed-Dvl-2 corresponding to the complete mRNA coding sequence are EU130787.1 and EU130788.1, respectively. Bf, Branchiostoma floridae; Bm, Brugia malayi; Ce, Caenorhabditis elegans; Ci, Ciona intestinalis; Dg, Dugesia japonica; Dm, Drosophila melanogaster; Dr, Danio rerio; Hs, Homo sapiens; Lv, Lytechinus variegatus; Mm, Mus musculus; Nv, Nematostella vectensis; Nvii, Nasonia vitripennis; Pn, Pediculus humanus corporis; Sg, Saccoglossus kowalevskii; Sm, Schistosoma mansoni; Smed, Schmidtea mediterranea; Tc, Tribolium castaneum. (B) In intact animals, Smed-dvl-1 and Smed-dvl-2 are expressed in the CG, VNCs, pharynx, and parenchyma. During regeneration, expression of both genes is detected from 2dR; they are expressed in the anterior and posterior blastemas and in the regenerating CNS. It is important to note that Smed-dvl-1 is expressed at much higher levels, particularly in the anterior region of the animal and in the pharynx. (Left) Anterior. dR, days after cutting. (Scale bar: 400 μm.)
Fig. S3. Eye differentiation is not completely abolished during homeostasis or in regenerating animals after low doses of dvl-1/2 RNAi. Stereomicroscopic views of live regenerating and intact animals and of anti-VC1 immunostaining show the development of aberrant ectopic eyes (white arrowheads). In regenerating animals, the eyes are asymmetrically distributed and visual axons are absent or abnormally oriented. Eye and visual axon differentiation is affected in a dose-dependent manner. Anti-Syn staining reveals that during regeneration, the progression of the anteriorization event also occurs in a dose-dependent manner. Anti-Syn and anti-VC1 images correspond to confocal z-projections. (Left) Anterior. dvl-1/2, Smed-dvl-1/2; Syn, synapsin; VC1, arrestin. (Scale bar: 400 μm.)

Fig. S4. Time course of CNS regeneration after wnt5 silencing. Anterior regeneration (A) and posterior regeneration (B). Anti-αTub staining reveals that silencing of wnt5 leads to lateral displacement of the anterior CNS already after 3dR. As regeneration proceeds, lateral expansion and disaggregation of the CNS become much more apparent. Red arrows indicate the growth direction of preexisting VNCs, white arrowheads indicate regenerating CNS in a control situation, and orange arrowheads indicate aberrant displacements of the regenerating CNS in the case of wnt5 RNAi planarians. Anterior (C) and posterior (D) regions of a 20-d-old regenerated trunk. Anti-αTub staining allows us to visualize the lateral nerves, which project from the VNCs toward the edge of control animals. In contrast, after silencing wnt5, these lateral nerves fail to branch properly toward the edge of the animal and appear disorganized. All images correspond to regenerating trunk pieces. Anti-αTub images correspond to confocal z-projections. (Left) Anterior. dR, days after cutting; αTub, α-tubulin; wnt5, Smed-wnt5. (Scale bar: 150 μm.)
Fig. S5. Dvl-2 paralog is required for the stabilization of intracellular β-catenin. (A) Summary of the phenotypes generated after dvl-2 silencing. (B) Expression analysis of the posterior marker AbdBA and the central identity marker TCEN revealed a reduction of posterior identity in dvl-2 RNAi animals. Note the asymmetrical position of the AbdBA signal. Images correspond to 30-d-old regenerated trunk fragments. (C) Stereomicroscopic view of small live head fragments showing the appearance of ectopic posterior eyes (yellow arrowheads). Expression of Gpas, which specifically labels the cephalic branches and the pharynx, confirms the differentiation of a posterior ectopic brain. Images correspond to 18-d-old regenerated head fragments. (D) Very slight reduction of bcat-1 activity could largely explain the overall phenotype observed after dvl-2 silencing. (E) Immunostaining with anti-Syn in control and dvl-2 intact animals. The anti-Syn staining images correspond to confocal z-projections. (Left) Anterior. bcat-1, Smed-β-catenin1; dvl2, Smed-dvl-2; Gpas, Smed-Gpas; Syn, synapsin. (Scale bar: A, B, D, and E, 250 μm; C, 500 μm.)

Fig. S6. Eye differentiation is abolished only after dvl-1/2 silencing. Stereomicroscopic view of live animals after RNAi for bcat1, wnt5 (alone or in combination), and evi reveals the differentiation of normal and ectopic eyes (white arrowheads). In contrast, dvl-1/2 RNAi animals do not have eyes. (Left) Anterior. bcat1, Smed-β-catenin1; dvl1/2, Smed-dvl-1/2; evi, Smed-evi; wnt5, Smed-wnt5. (Scale bar: 400 μm.)
Fig. S7. Sequence analysis and mRNA expression pattern of Smed-vang paralogs and Smed-div. (A) Alignment of Smed-Vang-1 and Smed-Vang-2 with Vang homologs from phylogenetically distant animals showing protein sequence conservation. (B) Alignment of the ankyrin-repeat domain of Smed-Div with Dgo/Div/ARD6 (ankyrin-repeat domain-containing protein) homologs from phylogenetically distant animals. Accession numbers of Div homologs are as follows: Dm-Dgo NP_724973.1, Dr-Div AAL39075, and Hs-Div BAA76801.2. (C) Phylogenetic tree of Vang proteins inferred from the accurate multiple amino acid sequence of Vang proteins obtained using MAFFT, version 6. The phylogenetic tree was prepared using MEGA version 3.1. Vertebrates are labeled in yellow, ecdysozoans in blue, and lophotrochozoans in pink. Accession numbers of Vang proteins are indicated in the tree. Ce, Caenorhabditis elegans; Ci, Ciona intestinalis; Dm, Drosophila melanogaster; Dr, Danio rerio; Hmag, Hydra magnipapillata; Hs, Homo sapiens; Hv, Hydra vulgaris; Nv, Nematostella vectensis; Lv, Legend continued on following page.
Lytechinus variegatus; Mm, Mus musculus; Nv, Nematostella vectensis; Pd, Platynereis dumerilii; Ph, Pediculus humanus corporis; Sk, Schistosoma mansoni; Sm, Schmidtea mediterranea; TC, Tribolium castaneum; Xl, Xenopus laevis. (D) In intact animals, Smed-vang-1, Smed-vang-2, and Smed-div are expressed in the CG, VNCs, pharynx, and parenchyma. During regeneration, expression of the three genes is detected from 2dR: Smed-vang-1 and Smed-vang-2 are expressed within the anterior and posterior blastemas and in the regenerating CNS. Smed-div is expressed in the regenerating brain and in the pharynx and parenchyma. (Left) Anterior. dR, days after cutting. (Scale bar: 400 μm.)

Fig. S8. (A) Vang-1/2 and div RNAi animals exhibit disorganization of structures within the epithelial sheet. Anti-αTub immunostaining reveals a reduction in the density of cilia, which also appear incorrectly orientated. Anti-Actin immunostaining reveals abnormal establishment of the actin network. The x-y confocal projections are shown. Yellow arrowheads indicate anterior. (Scale bar: 10 μm.) (B) Bcat1 and evi RNAi do not lead to abnormal organization of the cilia within the epithelial sheet. SEM shows the multiciliated epithelium. As in controls, a dense and well-orientated conglomerate of cilia is apparent in bcat and evi RNAi animals. Yellow arrowheads indicate anterior. (Scale bar: 10 μm.) (C) Vang-1/2 and div RNAi animals do not show any defects in brain morphogenesis or axis establishment. Stereomicroscopic view of live animals and anti-Syn shows apparently normal regeneration of vang-1/2 and div RNAi animals. Anti-Syn images correspond to confocal z-projections. (Left) Anterior. (Scale bar: 400 μm.) bcat1, Smed-β-catenin1; div, Smed-div; evi, Smed-evi; αTub, α-tubulin; Syn, synapsin; vang-1/2, Smed-vang-1/2.
Movie S1. Ventral-to-dorsal views of planarians stained with anti-α-tubulin antibody: head of a control animal. Note that CG structures appear dorsal to VNCs.

Movie S1

Movie S2. Ventral-to-dorsal views of planarians stained with anti-α-tubulin antibody: posterior ectopic head of a Smed-dvl1/2 RNAi animal. Note that CG structures appear dorsal to VNCs.

Movie S2
Movie S3. Ventral-to-dorsal views of planarians stained with anti-α-tubulin antibody: posterior ectopic head of a Smed-evi RNAi animal. Note that CG structures appear dorsal to VNCs.

Movie S4. Ventral-to-dorsal views of planarians stained with anti-α-tubulin antibody: head of a Smed-wnt5 RNAi animal. Note that CG structures appear dorsal to VNCs.
Movie S5. Ventral-to-dorsal views of planarians stained with anti-α-tubulin antibody: posterior ectopic head of a Smed-β-catenin1 RNAi animal. Note that CG structures appear dorsal to VNCs.

**Movie S7.** Ventral-to-dorsal views of planarians stained with anti-α-tubulin antibody: tail of a Smed-wnt5 RNAi animal.

**Movie S8.** Motility analysis: control animal. Planarian movement is governed by ventral motile cilia and by a muscle network.

**Movie S9.** Motility analysis: Smed-dvl-2 RNAi planarian. After silencing Smed-dvl-2, the movement of planarians is governed by muscular contractions instead of ventral motile cilia.
Movie S10. Motility analysis: Smed-vang-1/2 planarian. After silencing *Smed-vang-1* and *Smed-vang-2*, the movement of planarians is governed by muscular contractions instead of ventral motile cilia.

Movie S10

Movie S11. Motility analysis: Smed-div RNAi animal. After silencing *Smed-div*, the movement of planarians is governed by muscular contractions instead of ventral motile cilia.

Movie S11