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# Smed-Evi/Wntless is required for β-catenin-dependent and -independent processes during planarian regeneration

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Planarians can regenerate a whole animal from only a small piece of their body, and have become an important model for stem cell biology. To identify regenerative processes dependent on Wnt growth factors in the planarian *Schmidtea mediterranea* (*Smed*), we analyzed RNAi phenotypes of Evi, a transmembrane protein specifically required for the secretion of Wnt ligands. We show that, during regeneration, *Smed-evi* loss-of-function prevents posterior identity, leading to two-headed planarians that resemble *Smed-β-catenin1* RNAi animals. In addition, we observe regeneration defects of the nervous system that are not found after *Smed-β-catenin1* RNAi. By systematic knockdown of all putative *Smed* Wnts in regenerating planarians, we identify Smed-WntP-1 and Smed-Wnt11-2 as the putative posterior organizers, and demonstrate that Smed-Wnt5 is a regulator of neuronal organization and growth. Thus, our study provides evidence that planarian Wnts are major regulators of regeneration, and that they signal through β-catenin-dependent and -independent pathways.

**KEY WORDS: Anteroposterior axis, Brain patterning, Evi/Wls, Planarians, Regeneration, Wnt signaling**

## INTRODUCTION

Planarians have a nearly unlimited capability of renewing lost body structures (reviewed by Saló, 2006). Candidates for providing positional information during planarian regeneration are gradient-forming growth factors, such as Wnts. Wnts are secreted glycoproteins that behave as major organizers during embryonic development in various metazoan organisms (reviewed by Logan and Nusse, 2004). Canonical Wnt signaling is transduced through β-catenin, and mediates developmental processes, including axis specification (reviewed by Grigoryan et al., 2008). Some Wnts, such as mammalian Wnt5 (Slusarski et al., 1997), can signal through β-catenin-independent mechanisms, and control processes, such as cell polarity and directional movement (Witze et al., 2008).

Recently, β-catenin has been demonstrated to be essential in the establishment of anteroposterior (AP) identity during regeneration of the planarian species *Schmidtea mediterranea* (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). *Smed-β-catenin1* silencing leads to a loss of posterior identity and complete anteriorization ('radial-like hypercephalyzed' planarians) (Iglesias et al., 2008). However, although these studies suggest that Wnt proteins might be essential organizers of regeneration in planarians, direct evidence is missing.

Wnt secretion requires the transmembrane protein Evenness interrupted [Evi; also known as Wntless (Wls) and Sprinter] (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). In *Drosophila* and *Caenorhabditis elegans*, Wnts are not secreted in the absence of Evi, leading to a loss of Wnt signaling in the surrounding tissue. Owing to the lack of non-canonical phenotypes in *evi* mutant flies (Bartscherer et al., 2006), it is currently not known whether Evi also controls β-catenin-independent Wnt signaling.

Here, we report the effect of RNAi-mediated silencing of *Smed-evi* and all putative *S. mediterranea* Wnt genes during planarian regeneration. We show that, like *Smed-β-catenin1*, *Smed-evi*, as well as *Smed-wnt11-2* and *Smed-wntP-1*, are required for posterior identity in regenerating animals. In addition, we demonstrate that *Smed-evi* RNAi causes the same β-catenin-independent defects as does *Smed-wnt5* loss-of-function, suggesting that Smed-Wnt5 is a non-canonical Wnt that requires Smed-Evi for its secretion, and which acts to control neuronal growth during regeneration of the planarian nervous system.

## MATERIALS AND METHODS

### Animals

The planarians used belong to an asexual race of *S. mediterranea*, and were maintained as described elsewhere (Molina et al., 2007).

### Identification and cloning of *S. mediterranea* genes

*Smed-evi* and the nine Smed-Wnt genes were identified from the *S. mediterranea* genomic database (http://genome.wustl.edu/) through a BLAST search (http://ncbi.nlm.nih.gov). The full-length transcripts of the incomplete genes were amplified by rapid amplification of cDNA ends (RACE) using the Invitrogen GeneRacer Kit (Invitrogen).

### Accession numbers

*Smed-evi*, FJ463748; *Smed-wnt5*, FJ463749; *Smed-wntA*, FJ463750; *Smed-wnt11-2*, FJ463751; *Smed-wntP-4*, FJ463752.

### RNAi silencing

dsRNA microinjection was performed as described elsewhere (Sánchez-Alvarado and Newmark, 1999). dsRNAs were synthesized as described (Boutros et al., 2004). Primer details are available upon request. Control animals were injected with water. Injected planarians were amputated pre- and post-pharyngeally, and the head-, trunk-, and tail-pieces were allowed to regenerate for the times indicated.

### Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out as described previously (Nogi and Levin, 2005; Umesono et al., 1999). Digoxigenin-labelled riboprobes were synthesized using an in vitro transcription kit (Roche) (Iglesias et al., 2008). Primer details are available upon request.

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### Whole-mount immunostaining

Immunostaining was carried out as described previously (Cebrià and Newmark, 2005). Antibodies used were: anti-Arrestin (Sakai et al., 2000) at a 1:15,000 dilution, and anti-Synapsin (anti-SYNORF1, Developmental Studies Hybridoma Bank) at 1:25.

## RESULTS AND DISCUSSION

### An Evi homolog is expressed in *S. mediterranea*

We found a single *evi* gene in the genome of *S. mediterranea* (Fig. 1A; see also Fig. S1 in the supplementary material). *Smed-evi* mRNA mainly localized to the nervous system, the brain/cephalic ganglia (CG) and the ventral nerve cords (VNCs), as well as to the pharynx, and to the mouth. In addition, it was expressed in discrete cells of the posterior parenchyma (Fig. 1B).

To analyze the expression of *Smed-evi* in regenerating animals, we dissected heads and tails from the trunk parts of the animals and followed their regeneration. *Smed-evi* was expressed in the regenerating nervous system and upregulated in the pharynx primordia, as well as in the tips of posterior wounds (blastemas; Fig. 1C), suggesting that *Smed-evi* might be especially important for posterior regeneration. As Evi is required for the secretion of Wnts, the *Smed-evi* expression pattern might indicate sites of Wnt production and release in planarians.

### *Smed-evi* is required for posterior identity

To reveal Wnt-dependent processes in planarian regeneration, we depleted *Smed-evi* by RNAi, and dissected heads and tails from the trunk parts of the animals. Within 25 days of regeneration, all head fragments developed an ectopic posterior head with eyes and a brain (Fig. 2A,B). In addition, the expression of the central-posterior Hox gene *Smed-hoxD* was lost (Fig. 2C). These results indicate that, in the absence of *Smed-evi*, posterior fate is suppressed to the gain of anterior structures.

We obtained similar results for *evi* RNAi trunk fragments, as most of them developed an ectopic posterior head (Fig. 2D,E). In all animals, the anterior head showed several ectopic eyes. Both, anterior and posterior eyes differentiated laterally along the anteroposterior axis and were connected by their visual axons. In most cases, they did not cross the commissure and the optic quiasm was not formed (Fig. 2D). In ~50% of the *Smed-evi* RNAi trunks, the posterior head dissociated spontaneously during regeneration, a process we refer to as ‘scission’. Tail pieces regenerated a head with ectopic eyes (for *Smed-evi* RNAi phenotypes, see Fig. S2 in the supplementary material).

The role of planarian Evi in AP polarity, but not in dorsoventral (DV) polarity (see Fig. S3 in the supplementary material), is consistent with the recently described *Smed-β-catenin1* RNAi phenotype. *Smed-β-catenin1* RNAi causes a loss of AP polarity and complete anteriorization of regenerating planarians (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008) (Fig. 2F). However, we never observed full anteriorization after *Smed-evi* knockdown, which might be due to inefficient knockdown of *Smed-evi*, or to a stronger blockage of signaling after the loss of *Smed-β-catenin1*.

Together our results suggest that Smed-Evi is required for the release of Wnts that act through a β-catenin-dependent pathway to organize AP axis polarity during planarian regeneration.

### *Smed-evi* is required for neuronal growth regulation during regeneration

To analyze the brains of *Smed-evi* RNAi animals in more detail, we tested head fragments for Synapsin expression (Fig. 2G-J). In wild-type animals the CG were located dorsally above the VNCs and

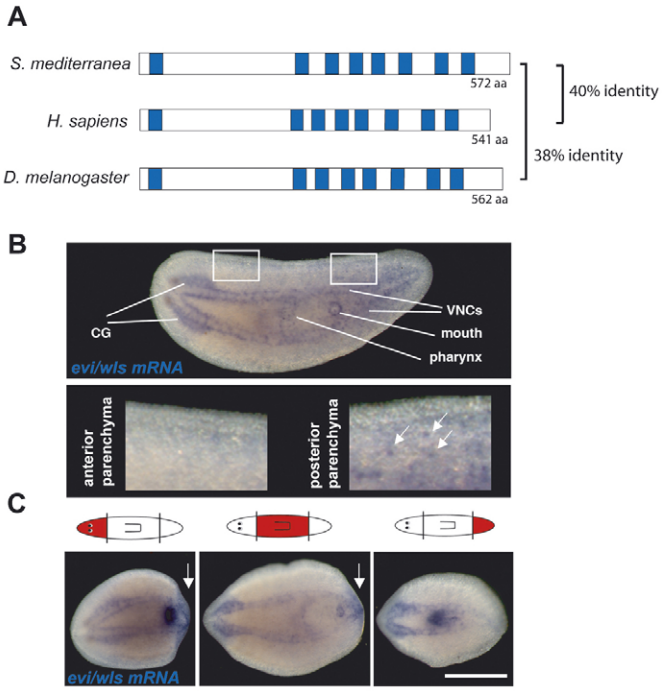



**Fig. 1. An Evi homolog is expressed in planarians.** (**A**) Human, fly and planarian Evi proteins. Protein sizes and sequence identities are indicated. Putative transmembrane domains are blue. (**B**,**C**) Whole-mount in situ expression analysis of *evi* mRNA in *Schmidtea mediterranea*. In intact animals, *Smed-evi* mRNA is expressed in the cephalic ganglia (CG), the ventral nerve cords (VNCs), the mouth, the pharynx, and cells of the posterior parenchyma (B). During regeneration (C), *Smed-evi* is expressed in the regenerating nervous system, and is upregulated in the pharynx and in posterior blastemas (arrows). Shown are head, trunk and tail fragments six days after dissection. Anterior is left, posterior is right. Scale bar: 500 μm.

extended as a pair into the regenerating tail (Fig. 2G; see Movie 1 in the supplementary material). However, in the posterior head of *Smed-evi* RNAi animals, both CG grew laterally along the VNCs. Confocal sections revealed that the posterior part of the CG projected into the ventral region (Fig. 2G′; see Movie 2 in the supplementary material). We refer to this phenotype as ‘deflected-brain phenotype’. Even though *Smed-β-catenin1* knockdown also resulted in the formation of a posterior brain, the ectopic CG never appeared deflected from the VNCs (Fig. 2H).

In bipolar regenerating trunk fragments, Synapsin expression showed the same deflected-brain phenotype. In addition, longitudinal neuronal tissue projected from posterior CG into the ventral half of the animal (Fig. 2I′; see Movie 4 in the supplementary material; a control trunk fragment is shown in Movie 3). We can only speculate whether these projections were new VNCs growing from posterior to anterior. As they grew laterally to the old VNCs, it is possible that old ones might repel new neuronal tissue, causing the lateral protrusions we observed in regenerating trunks (Fig. 2D,I′). Consistent with the absence of any optical chiasm, Synapsin staining revealed that the commissure was lost in anterior and posterior brains. We also observed a disconnection between the old and the new VNCs. *Smed-β-catenin1* RNAi animals showed neither a deflection nor any disconnections of the nervous tissue (Fig. 2J). Thus, we propose

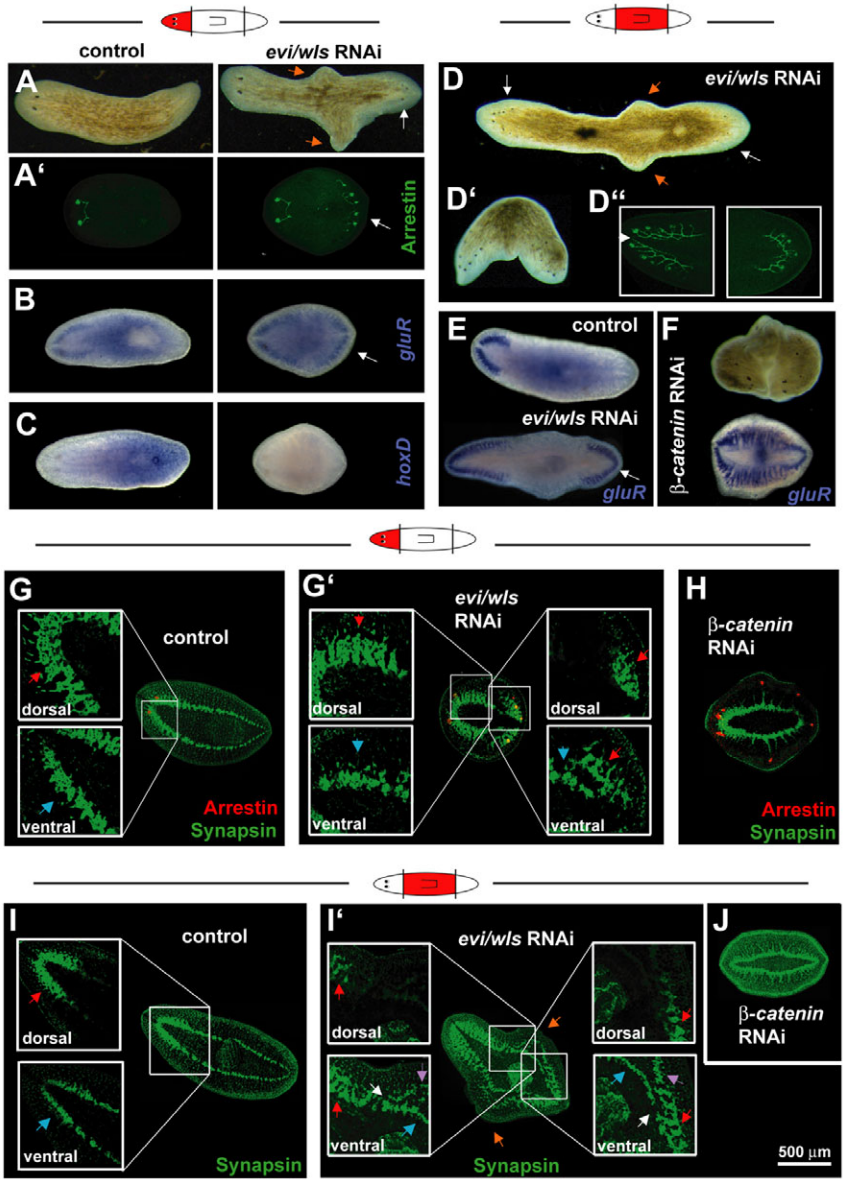



**Fig. 2. *Smed-evi* RNAi phenotypes.** (**A-E**) *Smed-evi* RNAi results in anteriorized planarians. Images of live animals (A,D,D′), and animals stained for visual Arrestin (A′,D″). Note the formation of a posterior head, indicated by white arrows pointing towards ectopic eyes. Orange arrows indicate ectopic lateral protrusions (see also I′). Loss of the optical chiasm is indicated by the white arrowhead in D″. (B,E) Whole-mount in situ hybridization against *Smed-glutamate receptor* (*gluR*) mRNA shows the formation of an ectopic brain instead of a tail in *Smed-evi* RNAi animals (arrow). (C) Whole-mount in situ hybridization against *Smed-hoxD* mRNA indicates the loss of posterior and medial identities after *Smed-evi* RNAi. (**F**) RNAi against *Smed-β-catenin1* leads to fully anteriorized planarians. (**G**,**G′**,**I**,**I′**) *Smed-evi* RNAi results in growth and patterning defects of the regenerating nervous system. The nervous system was labeled with an anti-Synapsin antibody (green). The position of Arrestin-positive eyes is indicated in red (pseudocolor). In control head fragments (G) CG (red arrows) are located dorsally above the VNCs (blue arrows). After *Smed-evi* RNAi (G′), the posterior CG are detected in dorsal and ventral sections, laterally to the VNCs (deflected-brain phenotype). (I,I′) Regenerating trunk fragments. After *Smed-evi* RNAi, both anterior and posterior nervous tissue show the deflected-brain phenotype. CG ,red arrows; VNCs, blue arrows, putative VNCs projected from posterior CG, purple arrows; disconnections between the old and the new nervous tissue, white arrows. (**H**,**J**) Synapsin expression in regenerating head and trunk fragments after *Smed-β-catenin1* RNAi. Regenerating head and trunk animals correspond to 20 and 25-30 days of regeneration. Images are *z*-projections of several confocal sections. Dorsal and ventral images are single sections. Anterior is left, posterior is right.

that Smed-Evi is required for the release of a Wnt protein that does not signal through β-catenin, and which restricts and defines the position of neuronal tissue during planarian regeneration.

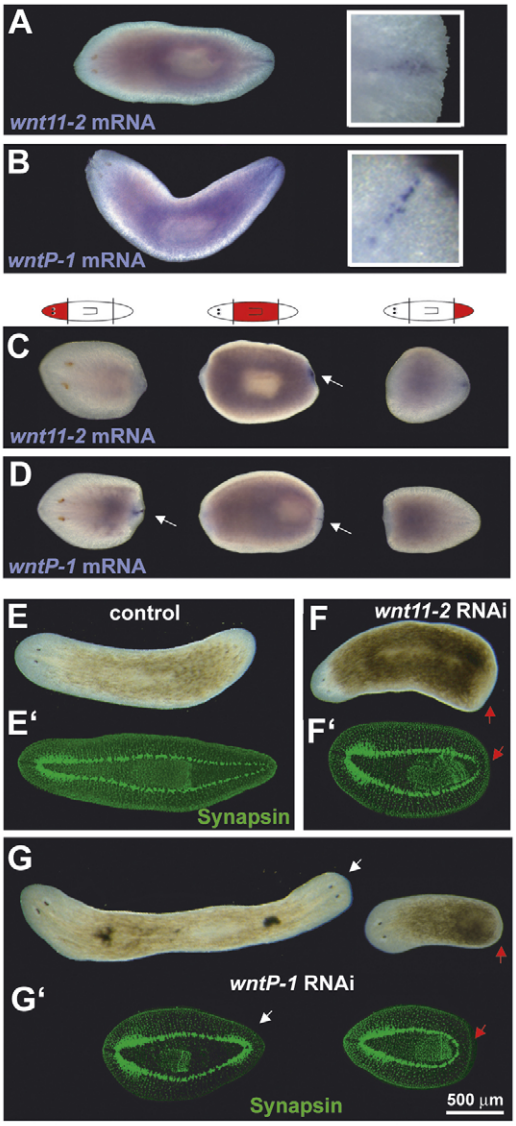
## Wnt genes in the *S. mediterranea* genome

To identify all putative Wnt genes in *S. mediterranea*, we searched the genome for sequences homologous to Wnts from several species. We identified nine Wnt genes: *Smed-wntP-1*, *Smed-wntP-2*, *Smed-wntP-3*, *Smed-wnt11-1* and *Smed-wnt2-1*, have been recently reported (Petersen and Reddien, 2008); *Smed-wntA* and *Smed-wnt5* encode proteins homologous to Wnts from other planarian species (Kobayashi et al., 2007; Marsal et al., 2003); and *Smed-wnt11-2* and *Smed-wntP-4* have not been described before. Phylogenetic analysis demonstrates that Smed-Wnt5, Smed-Wnt11-1/2 and Smed-Wnt2-1 can be assigned to established Wnt subfamilies. However, the other planarian Wnts appear as internal duplications in this phylum, and cannot be classified with high certainty (see Fig. S4 in the supplementary material).

## Posterior identity requires *Smed-wntP-1* and *Smed-wnt11-2*

Next, we set out to identify the Wnts responsible for the specification of posterior structures during regeneration. In situ hybridization experiments showed that *Smed-wnt11-2*, as well as *Smed-wntP-1* (Petersen and Reddien, 2008), was expressed in discrete cells along the most posterior midline of the *S. mediterranea* body (Fig. 3A,B). In regenerating animals, *Smed-wnt11-2* and *Smed-wntP-1* mRNA levels were upregulated in discrete cells of the posterior blastemas, indicating an important role in the control of posterior identity (Fig. 3C,D).

We tested whether posterior regeneration was affected by *Smed-wnt11-2* and *Smed-wntP-1* RNAi. Silencing of either mRNA led to a ‘tailless’ morphology, which was characterized by a shorter and more rounded posterior end, in which VNCs terminated shortly behind the pharynx (Fig. 3F,G). Furthermore, ~10% of regenerating trunks, and more than half of all regenerating head fragments of *Smed-wntP-1* RNAi animals resulted in two-headed planarians (Fig. 3G), a phenotype that was




G''

| *Smed-wntP-1* RNAi | | 20 d |
|---|---|---|
| Heads | Two-headed | 13/21 (62%) |
| | Tailless | 8/21 (38%) |
| Trunks | Two-headed | 2/21 (10%) |
| | Tailless | 19/21 (90%) |

**Fig. 3. *Smed-wnt11-2* and *Smed-wntP-1* are required for posterior identity during planarian regeneration.** (**A-D**) Whole-mount in situ hybridization analysis of *Smed-wnt11-2* and *Smed-wntP-1* mRNAs (arrows) in intact (A,B) and regenerating animals at day 4 of regeneration (C,D). (**F**,**G**) RNAi against *Smed-wnt11-2* and *Smed-wntP-1* leads to a 'tailless' phenotype (red arrows, compare with control in E). In some animals (see quantification in G″), *Smed-wntP-1* RNAi causes the generation of ectopic posterior heads (white arrows in G). Shown are images of live regenerating trunk fragments (E-G), and confocal *z*-projections of trunk fragments stained with anti-Synapsin (E′-G′), at day 20 of regeneration.

not enhanced by the simultaneous knockdown of *wnt11-2* (not shown). As *Smed-β-catenin1* RNAi also causes anteriorization of planarians (Fig. 2), Smed-Wnt11-2 and Smed-WntP-1 are likely to signal through β-catenin to permit posterior fate during regeneration.

## Smed-Wnt5 controls neuronal growth and patterning during regeneration

To identify Wnt proteins responsible for the deflected-brain phenotype of *Smed-evi* RNAi animals, we silenced all remaining *S. mediterranea* Wnt transcripts. *Smed-wnt2-1*, *Smed-wntP-3* and *Smed-wntP-4* had no obvious phenotype, possibly owing to inefficient knockdown. Consistent with the phenotype after DjwntA silencing in the planarian species *Dugesia japonica* (Kobayashi et al., 2007), we detected an abnormal elongation of the new brain and visual axons in *Smed-wntA* RNAi animals (see Fig. S5 in the supplementary material).

In *Smed-wnt5* RNAi animals, we discovered a deflection and ventral expansion of the regenerating CG (Fig. 4D; see also Movie 5 in the supplementary material). In the regenerating tail, new Synapsin-positive tissue appeared, which was thicker than, and appeared disconnected from, the old nervous tissue (Fig. 4D). Expression analysis of *Smed-glutamate receptor* (*gluR*) mRNA showed that the new posterior neuronal tissue was not of brain identity (Fig. 4E), suggesting that it was new VNCs that grew deflected from the old ones. These phenotypes were similar to those observed after *Smed-evi* silencing (Fig. 2I; see also Movie 5 in the supplementary material).

Consistent with this, we found that *Smed-wnt5* mRNA localized mainly to distinct cells along the CG and VNCs, and was upregulated in the regenerating nervous system and the blastemas (Fig. 4B). Together, our data suggest that Smed-Evi restricts and coordinates the growth of regenerating neuronal tissue by regulating the secretion of Smed-Wnt5.

## Concluding remarks

Our study reveals several regenerative processes that are regulated by Wnts in planarians (for a summary, see Table S1 in the supplementary material). Using *Smed-evi* RNAi to block Wnt secretion, we identify AP axis polarity and neuronal growth as being Wnt-regulated processes. Specifically, we identify *Smed-wntP-1* and *Smed-wnt11-2* as being the secreted molecules responsible for AP axis polarity. Consistent with the role of Wnts as morphogens (reviewed by Bartscherer and Boutros, 2008), Smed-WntP-1 and Smed-Wnt11-2 might activate posterior fate, from their site of production in the tail (Fig. 3), along the AP body axis. As posterior identity also depends on *Smed-β-catenin1*, we propose that Smed-WntP-1 and Smed-Wnt11-2 signal through β-catenin to permit posterior fate during regeneration.

Furthermore, we show that knockdown of *Smed-wnt5* results in a deflected-brain phenotype. Our data are consistent with the reported role of Wnt5 in axonal growth in several organisms (Yoshikawa et al., 2003; Fradkin et al., 2004; Zhang et al., 2007). Smed-Wnt5 belongs to the Wnt5 family (see Fig. S4 in the supplementary material), which has been linked to non-canonical Wnt signal transduction (Wong et al., 1994; Olson and Papkoff, 1994; Shimizu et al., 1997) and seems to regulate cell motility rather than specification (Moon et al., 1993; Wallingford et al., 2001; Witze et al., 2008). As we did not observe any deflected-brain phenotype after *Smed-β-catenin1* RNAi, we suggest that Smed-Wnt5 signals through a mechanism that is β-catenin independent.

In planarians, Evi function is therefore required for the secretion of Wnts that signal through β-catenin-dependent and -independent pathways (Fig. 4F). This suggests that Evi is an ancient factor that had been an important facilitator of Wnt secretion before Wnts functionally diverged. Even though the same Wnts might be able to signal through both canonical and non-canonical pathways,

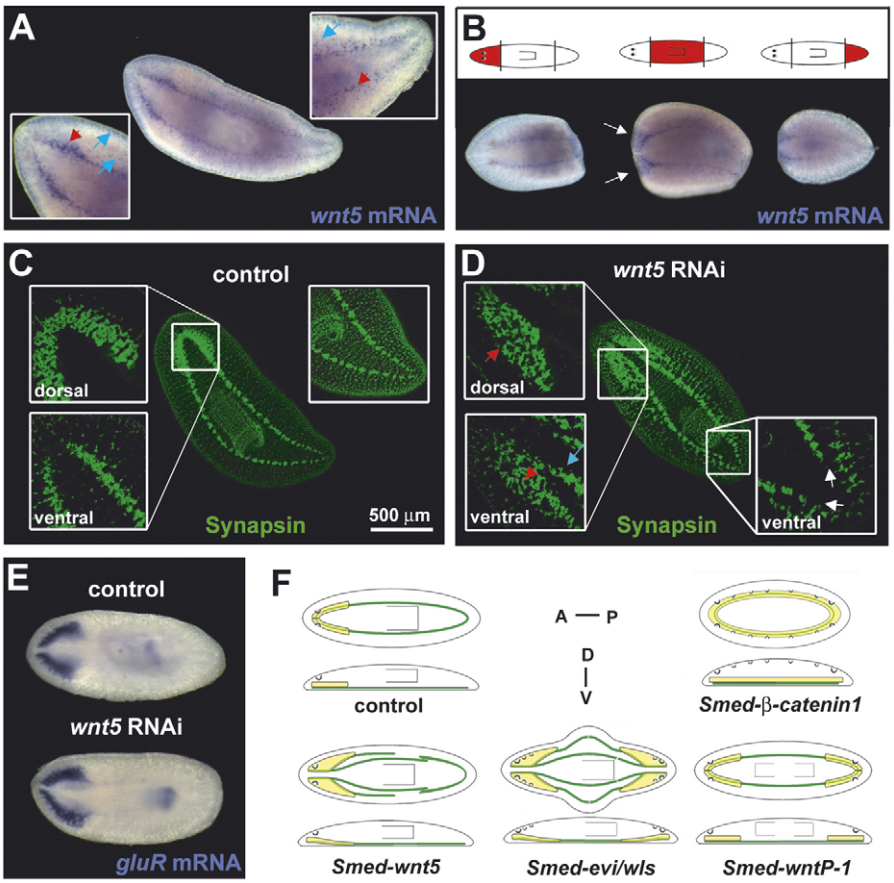



**Fig. 4. *Smed-wnt5* regulates growth and patterning of the planarian regenerating nervous system.** (**A**,**B**) In situ hybridization analysis of *Smed-wnt5* mRNA in intact (A) and regenerating (B) animals. *Smed-wnt5* is expressed in discrete cells along the VNCs and the CG (red arrows), as well as in cells in the periphery and along the DV boundary (blue arrows). It is upregulated in the regenerating CG of the anterior blastema (white arrows in B). (**C**,**D**) Control and *Smed-wnt5* RNAi trunk fragments at day 20 of regeneration, stained with anti-Synapsin. Red arrows point to CG, blue arrows to VNCs. Note the deflection and expansion of regenerating nervous tissue in *Smed-wnt5 RNAi* animals. White arrows indicate the disconnection between old and new nervous tissue. Images are *z*-projections, dorsal and ventral views are single sections. (**E**) In situ hybridization analysis of *gluR* mRNA. No brain tissue is detected in the tail. (**F**) Summary of RNAi phenotypes of regenerating trunk fragments. Shown are dorsal and lateral views. Yellow indicates CG; green, VNCs. A, anterior; P, posterior; D, dorsal; V, ventral.

depending on the receptor context (reviewed by van Amerongen et al., 2008), we demonstrate that Wnts can have distinct canonical or non-canonical functions in planarians. The nature and constellation of planarian Wnt receptors remains the subject of future studies, and might help us to understand how cells translate Wnt signals into diverse cellular responses, both in planarians and in other organisms.

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**Supplementary material**

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/6/905/DC1

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