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Early planarian brain regeneration is independent of blastema polarity mediated by the Wnt/β-catenin pathway

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ABSTRACT

Analysis of anteroposterior (AP) axis specification in regenerating planarian flatworms has shown that Wnt/β-catenin signaling is required for posterior specification and that the FGF-like receptor molecule *noudarake* (ndk) may be involved in restricting brain regeneration to anterior regions. The relationship between re-establishment of AP identity and correct morphogenesis of the brain is, however, still poorly understood. Here we report the characterization of two *axin* paralogs in the planarian *Schmidtea mediterranea.* Although Axins are well known negative regulators of Wnt/β-catenin signaling, no role in AP specification has previously been reported for *axin* genes in planarians. We show that silencing of *Smed-axin* genes by RNA interference (RNAi) results in two-tailed planarians, a phenotype previously reported after silencing of *Smed-APC-1*, another β-catenin inhibitor. More strikingly, we show for the first time that while early brain formation at anterior wounds remains unaffected, subsequent development of the brain is blocked in the two-tailed planarians generated after silencing of *Smed-axin* genes and *Smed-APC-1*. These findings suggest that the mechanisms underlying early brain formation can be uncoupled from the specification of AP identity by the Wnt/β-catenin pathway. Finally, the posterior expansion of the brain observed following *Smed-ndk* RNAi is enhanced by silencing *Smed-APC-1*, revealing an indirect relationship between the FGFR/Ndk and Wnt/β-catenin signaling systems in establishing the posterior limits of brain differentiation.

Introduction

The ability of adult animals to functionally restore missing structures varies in degree across the animal kingdom. One of the most striking examples of regenerative capacity is found in planarian flatworms, which are capable of regenerating a whole organism from a small piece of almost any part of their body. After amputation, planarian neoblasts (adult stem cells) proliferate to give rise to a mass of unpigmented tissue called the blastema, where the missing parts will differentiate. In addition, remodeling of pre-existing tissues (a phenomenon termed morphallaxis, Morgan, 1901) is required to integrate the new and old tissues thereby properly restoring the new body proportions (for reviews see Agata, 2003; Cebrià et al., 2002a; Newmark and Sanchez Alvarado, 2002; Reddien and Sanchez Alvarado, 2004; Saló, 2006; Sanchez Alvarado, 2006). Since TH Morgan's classical works at the beginning of the 20th century, many scientists have sought to understand how the anterior–posterior (AP) axis is re-established during planarian regeneration. After amputation of the head and tail of a planarian, the remaining tissue is able to register which tissue is missing and activate mechanisms to re-establish axial polarity and differentiate head and tail structures at the anterior and posterior wounds respectively.

The canonical Wnt signaling pathway is an evolutionarily conserved mechanism generally used during metazoan development to promote posterior polarized features of the AP axis (Petersen and Reddien, 2009a). Its main function at the level of signal transduction is to regulate the stability of the transcriptional coactivator β-catenin, the key downstream effector. In the absence of Wnt ligand stimulation, cytoplasmic β-catenin is constitutively targeted for degradation by the action of a multiprotein destruction complex containing the scaffolding protein Axin, Adenomatous Polyposis Coli (APC), Glycogen Synthase Kinase 3 and Casein Kinase 1. Wnt ligand inactivation of the β-catenin destruction complex stabilizes β-catenin, which accumulates and translocates to the nucleus where, together with T-cell factor/Lymphoid enhancer factor proteins, it activates target gene transcription (Logan and Nusse, 2004). In planarians, it has been widely demonstrated that the Wnt/β-catenin signaling pathway is required for posterior specification during regeneration and homeostasis (Adell et al., 2009; Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008, 2009b, 2011). Whereas *Smed-β-catenin1* silencing by RNA interference (RNAi) induces a gradual anteriorization of regenerating planarians that ranges from two-

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headed to hypercephalized planarians (Iglesias et al., 2008), RNAi for *Smed-APC-1* results in planarians that regenerate a tail instead of a head (Gurley et al., 2008). Furthermore, loss of function of Wnt11-6 (formerly known as WntA [Gurley et al., 2010]) results in the expansion of the brain towards more posterior regions without further disturbing head-trunk identities (Adell et al., 2009; Kobayashi et al., 2007), a phenotypic trait also observed after silencing of the FGFR-related gene *nou-darake* (*ndk*) (Cebrià et al., 2002a; Felix and Aboobaker, 2010). The relationship between the re-establishment of AP identity and correct morphogenesis of the central nervous system, however, remains poorly understood.

Here we report the characterization of two *axin* paralogs from *Schmidtea mediterranea (Smed-axinA* and *Smed-axinB)*. We show that while both *Smed-axin* genes are required for the re-establishment of AP polarity during planarian regeneration, their effect on blastema polarity does not influence early brain differentiation. However, *Smed-axinA/Smed-axinB* double RNAi (abbreviated as *Smed-axins* RNAi) does prevent the development of a fully formed brain. Remarkably, loss of function of another β-catenin inhibitor, *Smed-APC-1*, phenocopies *Smed-axins* RNAi. Furthermore, we provide evidence of an indirect relationship between the Wnt/β-catenin and FGFR/ndk signaling systems in the control of the posterior limits of brain differentiation. These findings provide clear evidence of independent mechanisms controlling early brain differentiation and subsequent development and provide important insights into the relationship between the specification of AP identity and organogenesis during regeneration.

Material and methods

Organisms

The planarians used in these experiments belong to an asexual biotype of *S. mediterranea* collected from an artificial spring in Montjuïc, Barcelona, Spain. The animals were maintained at 20 °C in a 1:1 (v/v) mixture of distilled water and tap water treated with AquaSafe (TetraAqua, Melle, Germany). Animals were fed with homogenized organic veal liver and starved for at least a week before the experiments. Planarians 2 to 6 mm in length were used for all experiments.

Isolation of S. mediterranea genes

The *S. mediterranea* genome is in the process of being sequenced and assembled (Washington University, St. Louis, USA). Fragments of *Smed-axinA* and *Smed-axinB* were identified from the *S. mediterranea* genomic contigs through a BLAST search with *axin* sequences from other species. The corresponding full-length transcripts were amplified by rapid amplification of cDNA ends (RACE) using the Invitrogen GeneRacer Kit (Invitrogen). The identity of *Smed-axinA* and *Smed-axinB* cDNAs was confirmed by sequencing (ABI Prism 3730 Applied Biosystems/Hitachi, Foster City, USA) and BLASTX analysis. *Smed-Gpas* (G protein α-subunit) was identified from the *S. mediterranea* genomic database using the *Dj-1791hh* homolog from *Dugesia japonica* (Cebrià et al., 2002b). Specific primers were designed to partially isolate the corresponding cDNA sequence.

RNAi analysis

Double-stranded RNAs (dsRNAs) were synthesized by *in vitro* transcription (Roche) as described previously (Boutros et al., 2004; Sanchez Alvarado and Newmark, 1999). dsRNA microinjections were performed as described elsewhere (Sanchez Alvarado and Newmark, 1999) following the standard protocol of a 32 nl injection of dsRNA on three consecutive days before amputation (one round of injection). Control animals were injected with water or a dsRNA corresponding to the GFP sequence. For combinatorial RNAi experiments, the concentration of dsRNA for each target gene was maintained at the same dose as for single RNAi after mixing. For experiments involving low doses of *Smed-β-catenin1* and *Smed-APC-1* RNAi, animals were injected just one day before amputation. In double *Smed-ndk*(−)/*Smed-APC-1*(−) experiments, animals were injected with two consecutive rounds of *Smed-APC-1* dsRNAi with amputation just after the first round, followed by a third round of *Smed-ndk* RNAi injection. The respective *Smed-APC-1*(−) and *Smed-ndk*(−) controls were injected with GFP when appropriate to follow the same protocol of injection and amputation. The following pairs of specific primers were used to generate each dsRNA target gene:

dsRNA against *Smed-axinA*
5′-AGAGATGTCGATTGTTCTCATGTG-3′;
5′-TTGTGAATAAGGAGGTCTATTGTGC-3′
dsRNA against *Smed-axinB*
5′-CGAGTAACTTTGATTCAGGAGTCAG-3′;
5′-TAAGGAACAGGGTCATTTCCTATATAG-3′
dsRNA against *Smed-β-catenin1*
5′-TCAGGGATTGCAGATTCTCATTCG-3′;
5′-GGCTAATGATCAATTCCAGTCC-3′
dsRNA against *Smed-APC-1*
5′-TCTACGGGATCTGCTGCTAC-3′;
5′-CTATCATAGTCATCAGGATACG-3′

Quantitative real-time PCR

Total RNA was extracted from a pool of three head or trunk fragments of RNAi-treated planarians using TRIzol® reagent (invitrogen). RNA samples were DNAse-treated using DNase I (Roche), and cDNA was synthesized using a First-Strand Synthesis System kit from Invitrogen. Real-time PCR was performed using SYBR Green (Applied Biosystems) in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Three samples for each condition were run in parallel. Data were normalized to the expression of the internal control UDP. Statistical analyses were performed with SPSS software. The following sets of specific primers were used:

Smed-axinA mRNA
5′-GTCGGCGAAATAGGAGTG-3′; 5′-CTGAGGCCTGACTTTTACC-3′
Smed-axinB mRNA
5′-ATATTACGCTTGGGCAATTC-3′; 5′-ACTACTCCACAGTCGAATTC-3′
Smed-β-catenin1 mRNA
5′-ATTCTGTCGAATTTGACTTGC-3′; 5′-CTAAATTCCACTCGATAGTCC-3′

Smed-udp mRNA was detected with primers described previously (Molina et al., 2011).

Irradiation

Intact planarians were γ-irradiated at 10 krad as described previously (Handberg-Thorsager and Saló, 2007) and fixed for *in situ* hybridization at 3 and 7 days post-irradiation.

Whole-mount in situ hybridization

Planarians were fixed and then processed in an *In situ* Pro hybridization robot (Abimed/Intavis) as previously described (Molina et al., 2007; Umesono et al., 1997). Hybridizations were carried out at 56 °C for 16 h. The following digoxigenin-labeled riboprobes were synthesized using an *in vitro* transcription kit (Roche): *Smed-axinA*, *Smed-axinB* and *Smed-Gpas* (novel); *Smed-otxA* and *Smed-otxB*

(Almuedo-Castillo et al., 2011); *Smed-otp* (kindly provided by M. Handberg-Thorsager); *Smed-FzA* (kindly provided by M. Sureda-Gómez); *Smed-Wnt11-6* (Adell et al., 2009); *Smed-HoxD* and *Smed-β-catenin1* (Iglesias et al., 2008); *Smed-septin* (Zayas et al., 2005); *Smed-eye53* (Collins et al., 2010); *Smed-sFRP-1* (Gurley et al., 2008; Petersen and Reddien, 2008); *Smedwi-2* (Reddien et al., 2005); and *cintillo* (Oviedo et al., 2003). Samples were observed through Leica MZ16F and Zeiss Stemi SV6 stereomicroscopes and a Zeiss Axiophot microscope; images were captured with a Nikon Coolpix E995 or Leica DFC300FX camera.

Whole-mount immunostaining

Immunostaining was carried out essentially as described previously (Cebrià and Newmark, 2005; Sanchez Alvarado and Newmark, 1999). The following antibodies were used: anti-synapsin (anti-SYNORF1, Developmental Studies Hybridoma Bank) at a 1:50 dilution and anti-Smed-β-catenin2 (Chai et al., 2010) at 1:1000. Highly cross-absorbed Alexa Fluor 488-conjugated goat anti-mouse IgG or Alexa Fluor 568-conjugated goat anti-rabbit IgG secondary antibodies (Molecular Probes) were used at dilutions of 1:400 and 1:1000, respectively. Confocal laser scanning microscopy was performed with a Leica TCS 4D (Leica Lasertechnik, Heidelberg) adapted for an inverted microscope (Leitz DMIRB).

Results and discussion

Graded expression of Smed-axins in differentiated cells and neoblasts

Two *axin* genes were identified and full-length transcripts isolated from the planarian *S. mediterranea* genome sequences (available in NCBI). The predicted Smed-axin proteins contain the two main conserved domains that characterize axins: the RGS (Regulator of G protein signaling) domain near the NH2 terminus and the C-terminal DIX (Dishevelled/Axin homologous) domain, which is necessary for homodimerization (Fig. S1) (Zeng et al., 1997). Phylogenetic analyses of axin homologs from different species showed that the two planarian axins arise from a lineage-specific duplication. We therefore named them *Smed-axinA* and *Smed-axinB* to avoid confusion with the already described vertebrate orthologous genes *axin1* and *axin2* (also *axil*/*conductin*) (Fig. 1A).

In situ hybridization experiments revealed similar expression patterns for the *Smed-axins*. In adult animals, both transcripts were detected in the central nervous system, the pharynx, and in both differentiated cells and neoblasts in the parenchyma (Figs. 1B and S2). Notably, when *in situs* were developed for a shorter time, a posterior to anterior gradient of expression was observed for both genes. Both *Smed-axins* were expressed in the anterior and posterior blastemas early during bipolar regeneration (trunk fragments regenerating a new head and tail), but the timing differed according to the paralog

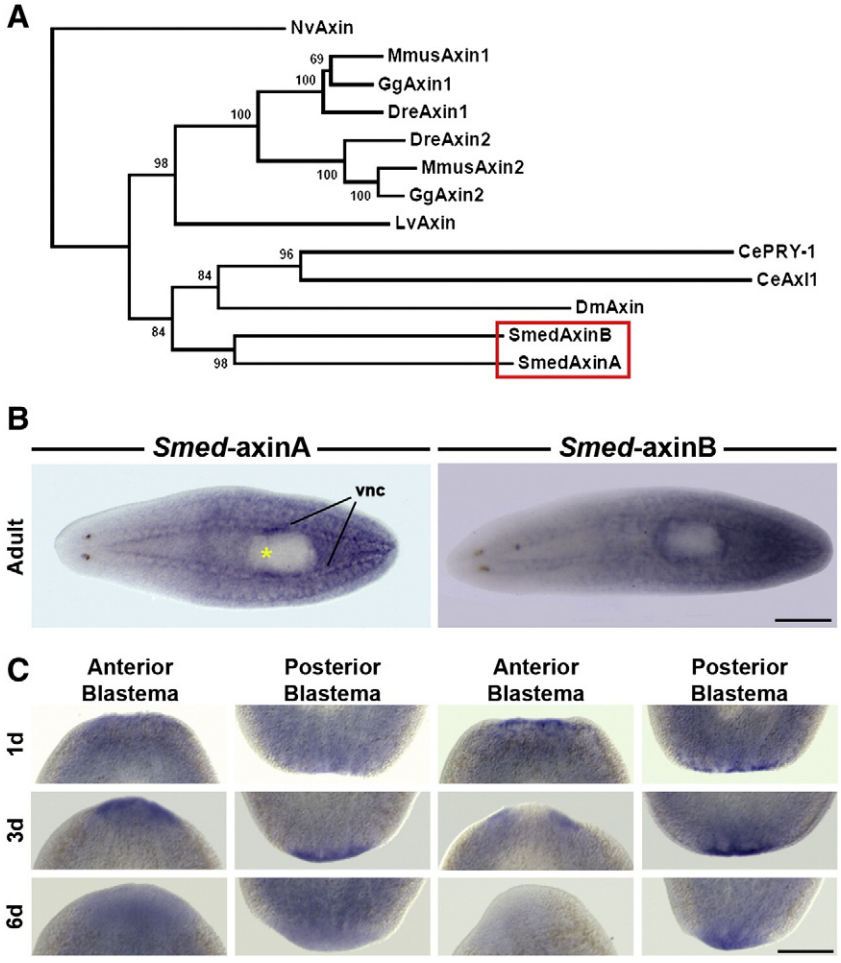

Fig. 1. Expression pattern of *Smed-axin* paralogs. (A) Phylogenetic tree of *Schmidtea mediterranea* Axin proteins showing that the two planarian *Smed-axins* represent a lineage-specific duplication. Nv: *Nematostella vectensis*; MMus: *Mus musculus*; Gg: *Gallus gallus*; Dre: *Danio rerio*; Lv: *Lytechinus variegatus*; Ce: *Caenorhabditis elegans*; DM: *Drosophila melanogaster*; Smed: *Schmidtea mediterranea*. (B) Expression pattern of *Smed-axinA* and *Smed-axinB* in adult and (C) regenerating trunks revealed by whole-mount *in situ* hybridization. Anterior is shown to the left in (B) and to the top in (C); yellow asterisk indicates the pharynx; d, days after amputation; vnc, ventral nerve cords. Scale bars, 500 μm in (B) and 200 μm in (C).

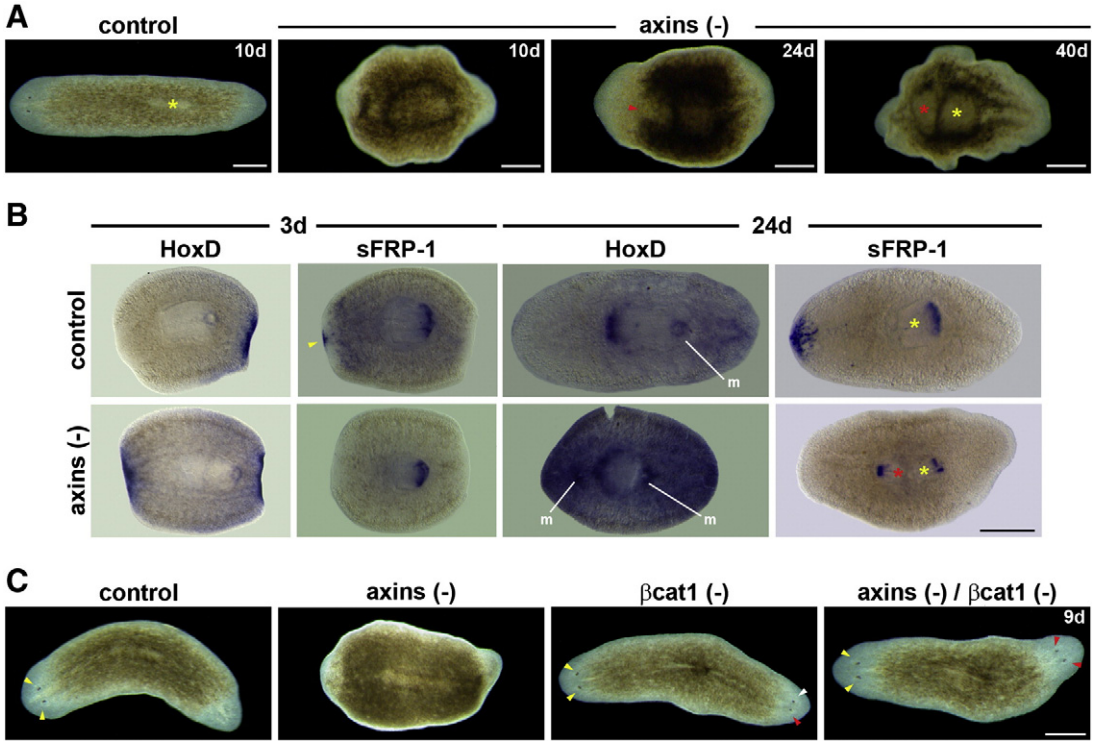

Fig. 2. Ectopic Wnt/β-catenin pathway activation by *Smed-axins* RNAi results in two-tailed regenerated planarians. (A) *Smed-axins* RNAi during trunk regeneration. While control planarians regenerate a head at anterior wounds, *Smed-axins* RNAi animals regenerate a tail and pharynx with opposite polarity. (B) Posteriorization is accompanied by loss of anterior identity in *Smed-axins*-silenced planarians. Analysis with the central-posterior *Smed-HoxD* marker and the anterior *Smed-sFRP-1* marker, which is expressed at the most anterior tip (yellow arrow) and the pharynx, reveals that *Smed-axins* RNAi-treated planarians lose anterior and acquire central-posterior identity during regeneration. Note the differentiation of an ectopic mouth (m) following *Smed-axins* RNAi. (C) Triple RNAi for *Smed-β-catenin1* and *Smed-axins* demonstrates that the posteriorized phenotype after *Smed-axins* RNAi requires the Smed-β-catenin1 gene. All control planarians regenerate normally; *Smed-axins* RNAi animals exhibit a two-tailed phenotype (penetrance = 47/49 [96%]); *Smed-β-catenin1* RNAi results in a two-headed phenotype (penetrance = 39/39 [100%]); and *Smed-axins* plus *Smed-β-catenin1* RNAi leads to a two-headed phenotype similar to that seen with *Smed-β-catenin1* RNAi alone (penetrance = 62/65 [95%]; 5 independent experiments). All images correspond to regenerating trunks fixed at different time points after amputation. Anterior is shown to the left. Yellow/red arrowheads indicate the differentiation of eyes in normal and ectopic positions, respectively. Yellow/red asterisks indicate the normal and ectopic pharynx, respectively. d: days after amputation. Scale bars, 500 μm.

analyzed. *Smed-axinA* was expressed in both blastemas at day 3 of regeneration. As regeneration proceeded, *Smed-axinA* expression decreased and eventually the adult expression pattern was restored (Fig. 1C). In contrast, *Smed-axinB* expression was detected in both blastemas as early as 1 day after amputation. At day 3 of regeneration, *Smed-axinB* expression at anterior blastemas began to decrease and it had disappeared by day 6 after amputation. As regeneration proceeded, the *Smed-axinB* expression pattern observed in adult animals was restored (Fig. 1C). These expression data during regeneration and, in particular, in intact animals suggest that *Smed-axins* might have a role in AP polarity.

Ectopic Wnt/β-catenin pathway activation by Smed-axins RNAi results in two-tailed planarians

To explore the role of *Smed-axins* in AP polarity, we performed RNAi experiments. Planarians were amputated pre- and post-pharyngeally and the resulting fragments were allowed to regenerate. Ten days after cutting, control trunks differentiated a pair of new eyes within the anterior blastema (unpigmented new tissue). In contrast, following *Smed-axinA*/*Smed-axinB* double knockdown (abbreviated as *Smed-axins* RNAi), regenerating trunks did not develop eyes (Fig. 2A). As regeneration proceeded, most *Smed-axins* RNAi planarians had an unpigmented bulge between the old and new anterior tissue (Fig. 2A, red arrowhead at 24 days) that corresponded to an ectopic pharynx with a reversed orientation (Fig. 2A, red asterisk at 40 days; see also Fig. 2B). *Smed-axins* RNAi-regenerated trunks exhibited tail morphology at their anterior wounds, resulting in animals with tails and pharynges at both body ends. We refer to this as a two-tailed phenotype (Fig. 2A). No clear AP defect was detected in regenerating trunks after *Smed-axinA* or *Smed-axinB* single RNAi (Fig. S3A), although the efficiency of RNAi experiments was confirmed by quantitative PCR (Fig. S4). Interestingly, most of the *Smed-axinB* RNAi regenerating tails exhibited a posteriorized phenotype (Fig. S3B), suggesting that *Smed-axin* genes may have undergone some degree of sub-functionalization (see also note in Fig. S6). However, the two paralogs act synergistically to control AP polarity decisions during regeneration since both genes must be knocked down before clear defects in regenerating trunks and two-tailed planarians are observed. We therefore decided to characterize *Smed-axinA*/*Smed-axinB* double knockdowns in greater detail.

To assess whether these external morphological changes were accompanied by a fate switch in anterior blastemas, we used *Smed-HoxD* and *Smed-sFRP-1* as markers of central-posterior and anterior identity, respectively. From early stages of regeneration, *Smed-axins* RNAi regenerating trunks expressed *Smed-HoxD* at both ends, whereas *Smed-sFRP-1* expression was absent. This pattern remained constant throughout the regeneration process (Fig. 2B). Moreover, analyses with these and other markers (see below) revealed that most regenerated trunks from *Smed-axins* RNAi animals developed a new ectopic mouth and a pharynx with an opposing polarity in relation to the existing pharynx. As observed in *Smed-β-catenin1* RNAi (Iglesias et al., 2008), analysis of *Smed-axins* knockdowns with markers of dorsal and ventral structures suggests that the dorsoventral (DV) axis was not affected (Fig. S5).

Axins are well known negative regulators of the Wnt/β-catenin signaling pathway (Zeng et al., 1997), acting as scaffold proteins for β-catenin degradation in the absence of Wnt signaling. To test whether the *Smed-axins* RNAi phenotype depends on *Smed-β-catenin1* function,

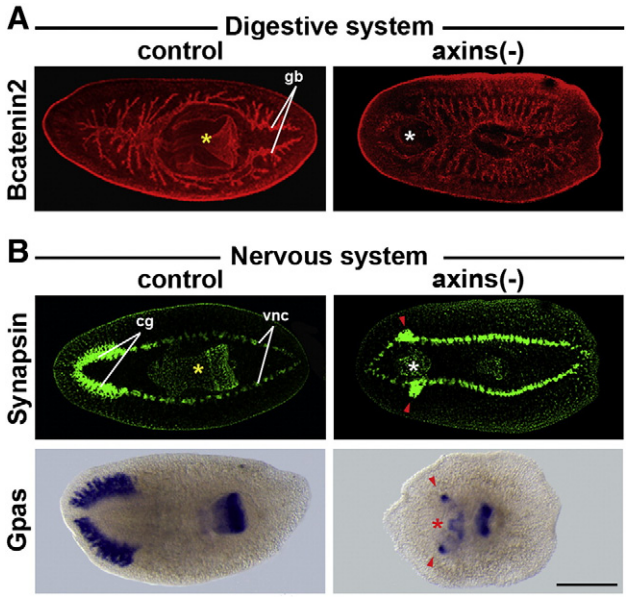

Fig. 3. Nervous and digestive systems in regenerated trunks after *Smed-axins* RNAi. (A) Analysis of the digestive system with anti-*Smed-β-catenin2* antibody reveals the differentiation of two posterior gut branches (gb) at each edge of *Smed-axins* RNAi-treated worms. Note also the differentiation of an ectopic pharynx with opposite polarity at their anterior wounds (white asterisk). (B) Analyses of the central nervous system with the pan-neural marker Synapsin and the marker *Smed-Gpas*, which is specifically expressed in the cephalic branches and the pharynx, reveal that, along with two ventral nerve cords in the anterior tail, *Smed-axins* RNAi-treated animals differentiate brain-like tissues (red arrowheads). All images correspond to 22 to 26-day regenerating trunks. Anterior is shown to the left. Yellow and white/red asterisks indicate the normal and ectopic pharynx, respectively. Synapsin and anti-*Smed-β-catenin2* images correspond to confocal z-projections. cg, cephalic ganglia; vnc, ventral nerve cords. Scale bars, 500 μm.

we performed combinatorial RNAi experiments. The efficiency of the RNAi experiments was confirmed by quantitative PCR for each gene after RNAi (Fig. S6). Triple RNAi knockdowns for *Smed-axins* and *Smed-β-catenin1* resulted in two-headed planarians identical to those of the single *Smed-β-catenin1* RNAi phenotype (Fig. 2C). This finding suggests that the two-tailed phenotype observed in *Smed-axins* RNAi planarians requires the *Smed-β-catenin1* gene.

Although no role in AP axis specification has previously been reported for *axin* genes in planarians (Gurley et al., 2008), the data presented here demonstrate that *Smed-axins* are conserved negative regulators of the Wnt/β-catenin pathway and are required for correct AP polarity re-establishment during planarian regeneration. Loss of function of these genes during regeneration results in the loss of anterior identity and acquisition of a central-posterior identity, resulting in animals with two tails and pharynges at both body ends. In agreement with our observations, the two-tailed phenotype has been also reported in planarians after promoting either the Hedgehog pathway or the Wnt/β-catenin pathway itself by knocking down other negative regulators of the canonical Wnt pathway (Gurley et al., 2008; Petersen and Reddien, 2011; Rink et al., 2009; Yazawa et al., 2009). Notably, Hedgehog signaling influences posterior specification by regulating Wnt/β-catenin signaling (Rink et al., 2009; Yazawa et al., 2009).

Brain differentiation occurs in two-tailed planarians after silencing of either Smed-axins or Smed-APC-1

To address whether the AP polarity of specific organs is affected by *Smed-axins* RNAi, we analyzed the regeneration of the digestive and nervous systems. The planarian digestive system is composed of a pharynx located in the middle of the trunk, from which one anterior and two posterior gut branches extend (Fig. 3A) (Saló, 2006). The central nervous system consists of two anterior cephalic ganglia (brain) situated above two ventral nerve cords (VNCs), which extend along the body and converge in the tail (Fig. 3B) (Agata et al., 1998; Cebrià et al., 2002b). Smed-β-catenin2 immunostaining (which strongly labels the gut and pharynx) showed that trunks from *Smed-axins* RNAi-treated animals regenerated two posterior gut branches at each end of the animal (Fig. 3A). Moreover, most of them differentiated an ectopic pharynx with opposite polarity at their anterior wounds (white/red asterisks in Fig. 3 and Table S1). Surprisingly, however, analyses with the pan-neuronal marker synapsin revealed that, along with two VNCs in the ectopic "anterior" tail, *Smed-axins* RNAi animals differentiated two clusters of cells with brain-like characteristics next to the ectopic pharynx (red arrowheads in Fig. 3B). The brain identity of these cell clusters was further confirmed by analysis of the expression of *Smed-Gpas*, a brain-specific marker that also labels the pharynx (Fig. 3B). Remarkably, 100% of trunks analyzed between 24 and 30 days after amputation differentiated brain tissue in the ectopic "anterior" tail (Table S1). Together with the previous section, these results show that while posterior identity of anterior blastemas is accompanied by the differentiation of a posterior digestive system after *Smed-axins* RNAi, the differentiation of brain tissue is not completely abolished.

Previous studies did not report discernible brain tissue after directly or indirectly promoting the Wnt/β-catenin pathway (Gurley et al., 2008; Petersen and Reddien, 2011; Rink et al., 2009; Yazawa et al., 2009). To test the possibility that a hypomorphic phenotype occurs as a result of *Smed-axins* RNAi, we performed RNAi-dosage experiments. When the dsRNA dose was increased (two rounds of *Smed-axins* dsRNA injections), we observed that brain tissue still differentiated at anterior wounds and its size was the same as that observed after only one round of injections (Fig. S7). This suggests that the appearance of brain tissue after *Smed-axins* RNAi is not an effect of Axin protein persistence. Moreover, the finding that loss of function of another negative regulator of the Wnt/β-catenin pathway, *Smed-APC-1,* phenocopies *Smed-axins* RNAi at both the morphological and molecular level ruled out a pleiotropic effect of *Smed-axins* in brain differentiation (Fig. S8 and Fig. 4).

Overall, these findings show that brain differentiation occurs in two-tailed planarians generated by silencing *Smed-axins* and *Smed-APC-1*. Our data thus supports the idea that the mechanisms that control brain differentiation can be uncoupled from those driven by Wnt/β-catenin that determine AP body polarity (Adell et al., 2010). These findings are consistent with the results obtained after silencing Wnt11-6 and *ndk* genes, which led to the differentiation of ectopic brain tissues along the planarian body without further disturbing AP identities (Adell et al., 2009; Cebrià et al., 2002a; Kobayashi et al., 2007).

Early brain regeneration is independent of blastema polarity mediated by Wnt/β-catenin pathway

To investigate the nature of this brain tissue differentiation after ectopic activation of Wnt/β-catenin pathway, we studied the process of planarian brain regeneration in more detail. A working model for planarian central nervous system regeneration has been suggested (Agata and Umesono, 2008; Cebrià et al., 2002b). Based on this model, the initial stage of brain regeneration is characterized by the formation and subsequent patterning of the brain primordia within the anterior blastema. These brain primordia then grow and re-establish proper connections with the regenerating VNCs in the blastema. Finally, the regenerated central nervous system recovers its functionality.

Regeneration time-course experiments in control animals with the early brain-specific marker *Smed-Gpas* showed that brain primordia in the form of two small cell clusters can be detected as early as 2 days after amputation (Fig. 4A). *Smed-axins* and *Smed-APC-1* RNAi animals

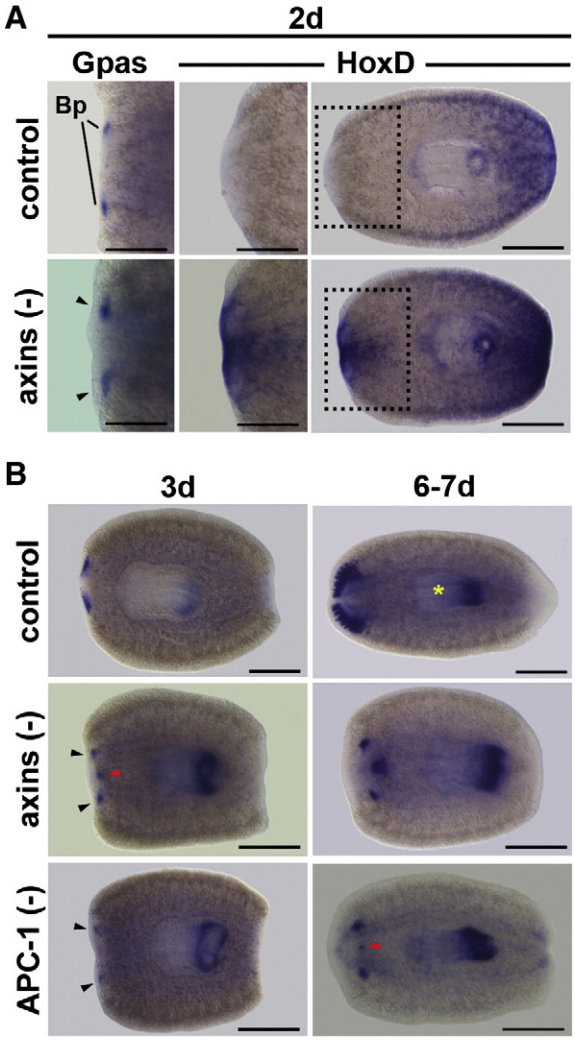

Fig. 4. Early brain regeneration is independent of blastema polarity mediated by the Wnt/β-catenin pathway. (A) Analysis of regenerating *Smed-axins* RNAi trunk fragments with *Smed-Gpas* and *Smed-HoxD* 2 days after cutting. As occurs in control animals, *Smed-axins* RNAi planarians regenerate brain primordia (bp) at anterior wounds soon after amputation (black arrowheads). However, after *Smed-axins* RNAi, the brain primordia differentiate within tissue with a central-posterior identity and in a more posterior region than in control animals. High magnification views of *Smed-HoxD* expression in the anterior blastema (dotted box) are shown to the right. (B) Expression analysis of *Smed-Gpas* during early stages of regeneration. In control animals, brain primordia grow and develop into a well formed brain as regeneration proceeds. By contrast, following *Smed-axins* or *Smed-APC-1* RNAi these primordia (black arrowheads) either fail to develop or disappear as regeneration proceeds. Anterior is shown to the left. d, days after amputation; red arrowhead, ectopic pharynx primordia; yellow asterisk indicates the normal pharynx. Scale bars, 300 μm in high magnification and 500 μm in all other images.

also differentiated brain primordia at anterior wounds, but these primordia either never developed into normal brains or disappeared as regeneration proceeded (Fig. 4B). Interestingly, a detailed view of anterior wounds following *Smed-axins* RNAi revealed that the brain primordia differentiated within tissue with a central-posterior identity (indicated by *Smed-HoxD* expression) but in a more posterior/proximal region as compared to control animals. Whereas the brain primordia differentiated distally within the anterior blastema 2 days after cutting in control animals, they differentiated close to the blastema/post-blastema (old tissue) boundary in *Smed-axins* RNAi planarians (Fig. 4A).

To ascertain whether brain patterning was affected we analyzed the expression of otd/Otx family genes (*OtxA* and *OtxB*) and the homeobox-containing gene *ortopedia* (*Otp*) (Umesono et al., 1997, 1999). As in the control animals, *Smed-OtxA*, *Smed-OtxB* and *Smed-Otp* are expressed sequentially along the medio-lateral axis of the brain in both *Smed-axins* and *Smed-APC-1* RNAi planarians (Fig. 5). With respect to patterning along the AP axis, it has been shown that a Frizzled homolog appears to be mainly expressed in the anterior part of the brain, whereas a Wnt11 homolog is restricted to the most posterior part and along the VNCs (Kobayashi et al., 2007). Consequently, we studied the expression of these two markers in RNAi-treated animals. *Smed-Wnt11-6* and *Smed-FzA* were expressed in the brain primordia of *Smed-axins* and *Smed-APC-1* knockdowns (Fig. 6). However, as at early stages of brain regeneration in control planarians, the compartments defined by these genes in the brain primordia that differentiated after *Smed-axins* or *Smed-APC-1* were less well delimited than for the *Otx/otp* genes since there appears to be overlapping expression in some regions of the brain (Fig. 6 at 3 days). This made it more difficult to unambiguously detect any defect in the specification of *Smed-Wnt11-6* and *Smed-FzA* territories. Based on the currently available markers, our results show that the silencing of *Smed-axins* or *Smed-APC-1* leads to the differentiation of a small round brain primordia that fails to develop into a well-formed brain but appears to be quite well patterned.

In summary, our data show that the silencing of either *Smed-axins* or *Smed-APC-1* results in the transformation of anterior blastemas into posterior ones (Fig. 2 and Fig. S8). In contrast, a posterior to anterior identity switch is observed in the blastemas of *Smed-β-catenin1* RNAi animals (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). Since the posteriorized phenotype observed after *Smed-axins* or *Smed-APC-1* RNAi requires the *Smed-β-catenin1* gene (Fig. 2 and Gurley et al., 2008), blastema identity appears to be controlled by β-catenin activity in planarians (even though no data on the exact localization of β-catenin activation has yet been reported); basically, low levels of β-catenin activity would define anterior identity whereas high levels would induce a posterior one. Surprisingly, brain primordia differentiate

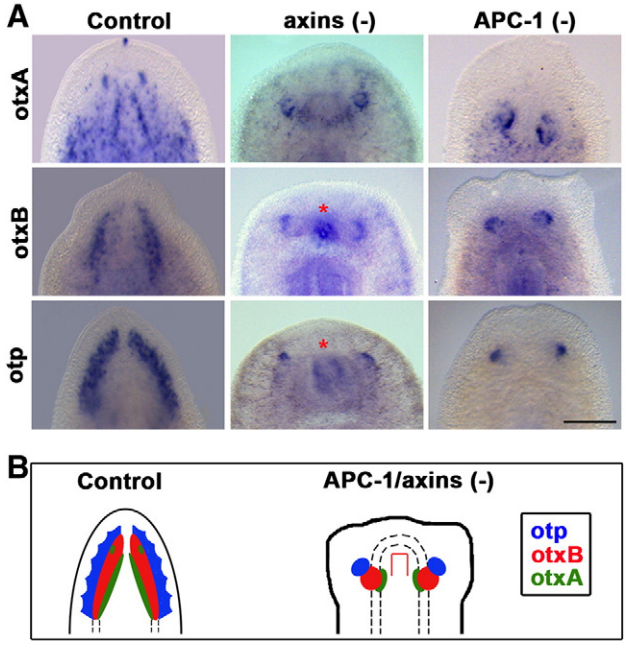

Fig. 5. Medio-lateral patterning of brain primordia is not affected in *Smed-axins* or *Smed-APC-1* RNAi-treated animals. (A) Expression analysis of *Smed-OtxA*, *Smed-OtxB* and *Smed-Otp*. As in control animals, in *Smed-axins* and *Smed-APC-1* RNAi-treated animals, *Smed-OtxA*, *Smed-OtxB* and *Smed-Otp* are expressed in distinct non-overlapping domains along the medio-lateral axis of the brain. All images correspond to high magnifications of anterior regions of 12-day regenerating trunks. Anterior is shown to the top; red asterisk indicates ectopic pharynx. Scale bars, 200 μm. (B) Schematic representation of brain patterning in the different phenotypes described in (A).

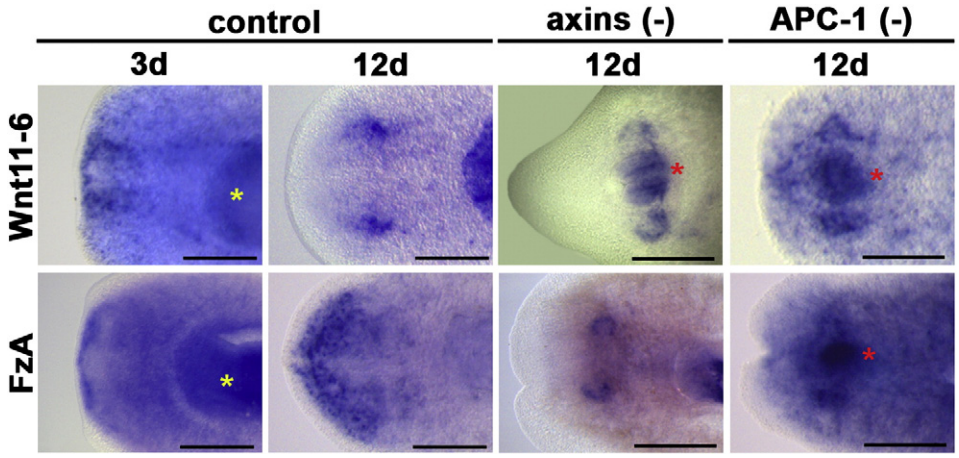

Fig. 6. *Smed-FzA* and *Smed-Wnt11-6* are expressed in brain primordia after *Smed-axins* or *Smed-APC-1* loss of function. In control animals, *Smed-FzA* and *Smed-Wnt11-6* are expressed in the brain at early stages of regeneration (3 d) but their expression only becomes segregated along the anteroposterior axis when the brain primordia grow (12 d). Similarly, *Smed-FzA* and *Smed-Wnt11-6* are also expressed in the small round brain primordia following *Smed-axins* or *Smed-APC-1* RNAi. However, since the growth of these brain primordia is arrested in RNAi-treated animals, it is difficult to unambiguously detect any defect along the anteroposterior axis of the brain. All images correspond to high magnifications of anterior regions. Anterior is shown to the left. d, days after amputation; red asterisk indicates ectopic pharynx. Scale bars, 200 μm.

at the interface of the posterior-fated blastemas and anterior wounds of *Smed-APC-1* or *Smed-axins* RNAi animals (Fig. 4). This suggests that the mechanisms controlling early brain regeneration can be uncoupled from those involved in providing blastema polarity mediated by the Wnt/β-catenin pathway. An important point is that these brain primordia display an overall proper pattern, but do not grow and develop into a fully formed brain within those posterior blastemas. Considering that those blastemas should display a high level of β-catenin activity, the fact that brain primordia do not further develop within them may suggest that low levels of β-catenin activity are required at late stages of brain regeneration for proper brain development. Consistent with this possibility, lower doses of dsRNA against *Smed-APC-1* allow brain primordia to grow to a certain extent (Fig. S9). However, further investigation is needed to ascertain whether the Wnt/β-catenin pathway affects brain development directly or indirectly by promoting posterior identity in regenerating blastemas.

We are currently unable to explain why brain primordia differentiate upon amputation after silencing of *Smed-APC-1* or *Smed-axins*. However, our results suggest that an unknown mechanism is underlying early brain regeneration at anterior wounds despite the silencing of *Smed-axins* or *Smed-APC-1*. Two main scenarios can be considered. One recently proposed hypothesis is that the anterior wound goes through a transitory stage characterized by a low level of β-catenin activity that allows the initial development of brain primordia (Adell et al., 2010). This can also be extrapolated from the findings of Yazawa et al. (2009). The gradual increase in the level of β-catenin activity as a consequence of the silencing of *Smed-APC-1* or *Smed-axins* subsequently blocks further development of a fully formed brain in these, otherwise, posterior blastemas. This scenario implies that brain differentiation is incompatible with high β-catenin activity and that the aforementioned unknown mechanism may operate temporarily at anterior wounds to overcome the effect of *Smed-axins* or *Smed-APC-1* RNAi on β-catenin activity and consequently commit early brain primordia. Consistent with this hypothesis, the silencing of *Smed-β-catenin1* not only induces early regeneration of anterior/brain structures at any wound but also a gradual cephalization/anteriorization of RNAi-treated planarians and eventually a hypercephalized phenotype (Iglesias et al., 2008). An alternative, and less parsimonious, scenario would be that early brain regeneration is compatible with high levels of β-catenin activity whereas subsequent development of the brain is not. Further experiments are needed to clarify how the different levels of β-catenin activity influence not only blastema polarity but also brain differentiation within them.

Relationship between the FGFR/ndk and Wnt/β-catenin signaling pathways in planarian brain regeneration

The existence in planarians of a brain-inducing circuit based on an FGF signaling pathway has been proposed. This hypothesis is based on the study of the ndk RNAi phenotype in planarians (which is characterized by the expansion of the brain outside the head region) and the fact that *ndk* is a FGFR-related gene that negatively regulates FGF signaling in *Xenopus* embryos (Cebrià et al., 2002a). Of particular interest in the observation of the *ndk* RNAi phenotype is that ectopic brain tissues also differentiated *de novo* at posterior wounds close to the blastema/post-blastema boundary (see Fig. S10), but these posterior brain tissues never expanded towards pre-existing tissues or posterior blastemas. This phenotypic trait is strikingly similar to the brain primordia observed at “anterior” wounds in the two-tailed planarians generated after ectopic Wnt/β-catenin activation because, in both cases it takes place at the interface of posterior-fated blastemas and pre-existing tissues. Thus, we reasoned that the FGF/ndk signaling system could be one of the mechanisms postulated above that can overcome the *Smed-axins*/*Smed-APC-1* RNAi effect at anterior wounds and promote brain primordia differentiation despite the posteriorization of the blastema. The ideal way to test this possibility would be to inhibit the brain-inducing signals modulated by ndk at anterior wounds, but no FGF-like ligands (called brain activator/s in the planarian literature) or FGFR-like receptors responsible for anterior brain regeneration in planarians have yet been identified (Agata and Umesono, 2008). Alternatively, by performing combinatorial RNAi experiments, we sought to determine whether silencing *Smed-APC-1* would allow neoblast response to the brain-inducing signals modulated by *Smed-ndk* in pre-existing tissues. In order to ensure the effectiveness of these RNAi experiments we chose *Smed-APC-1* instead of *Smed-axins* since we reasoned that silencing two genes in combination would be easier. Moreover, we carried out two rounds of *Smed-APC*-1 RNAi and amputation followed by a third round of *Smed-ndk* RNAi and amputation to properly down-regulate *Smed-APC-1* in pre-existing tissues. As reported above, following *Smed-ndk* RNAi, not only did the regenerating brain expand towards more posterior regions without further disturbing AP identities, but ectopic brain tissues also differentiated *de novo* at posterior wounds (Figs. 7 and S10). As in *Smed-APC-1* RNAi, double *Smed-ndk*/*Smed-APC-1* RNAi planarians did not develop well-formed brains at anterior wounds, and similarly to *Smed-ndk* RNAi differentiated brain tissues to more posterior regions. Thus, the silencing of *Smed-APC-1* does not impair the response of neoblast to the brain-

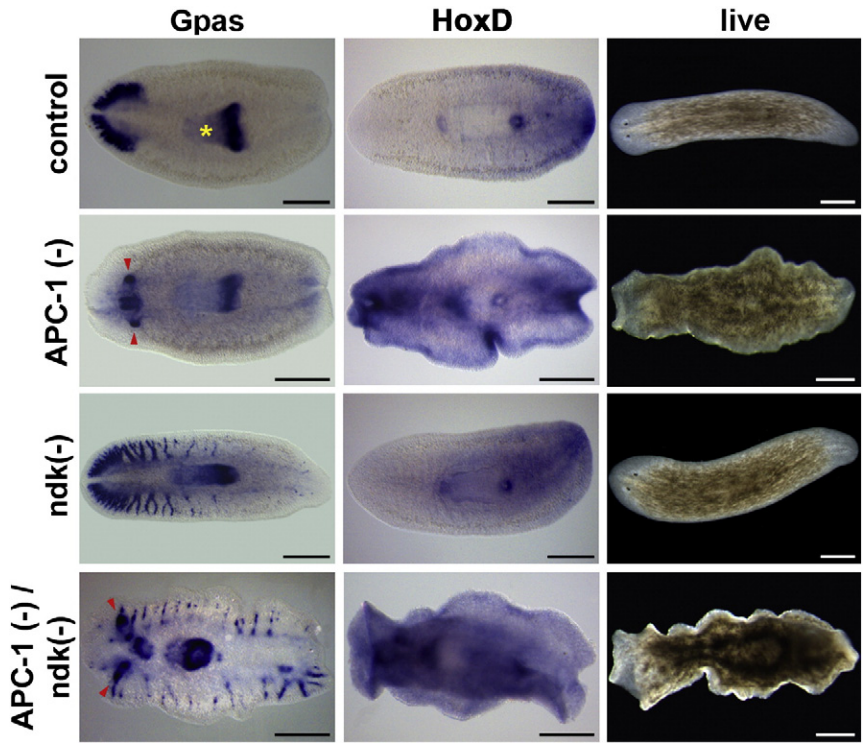

Fig. 7. Relationship between Wnt/β-catenin and Ndk/FGFR pathways in planarian brain regeneration. *Smed-Gpas* expression analyses show expected phenotypes after *Smed-APC-1* and *Smed-ndk* RNAi. By contrast, double *Smed-ndk*/*Smed-APC-1* knockdown results in broader posterior expansion of brain tissues than single *Smed-ndk knockdown.* In addition, note the different morphology of the brain primordia in these double *Smed-ndk*/*Smed-APC-1* knockdowns (red arrowheads). *Smed-HoxD* analyses confirm posteriorization after *Smed-ndk*/*Smed-APC-1* and *Smed-APC-1* silencing, while anteroposterior identity is not affected in *Smed-ndk* RNAi planarians. All images correspond to 12-day regenerating trunks. Anterior is shown to the left. Yellow asterisk indicates the normal pharynx. Scale bars, 500 μm.

inducing signals modulated by *Smed-ndk* in pre-existing tissues. Notably, we observed broader posterior expansion of brain tissues in double *Smed-ndk*/*Smed-APC-1* RNAi planarians than in *Smed-ndk* RNAi planarians (Fig. 7). This unexpected finding revealed that the FGFR/ndk and Wnt/β-catenin signaling systems interact indirectly to establish the posterior limits of brain differentiation. Perhaps a feedback-loop between these two signaling systems is operating during planarian brain regeneration since cross-talk between FGF and Wnt signaling has been reported in many tissues and organisms and, depending on the developmental context, this can trigger synergistic or antagonistic effects (Kim et al., 2006). Remarkably, it has been shown that FGF signaling can specifically inhibit Wnt/β-catenin signaling downstream of the β-catenin destruction complex in which Axin and APC operate (Ambrosetti et al., 2008) and that Wnt signaling can regulate the expression of different FGF ligands during development (Matsunaga et al., 2002). However, further studies are needed to better characterize the FGF/ndk system and determine exactly how these pathways interact during planarian brain regeneration.

Brain tissues form close to the pharynx at late stages of regeneration in two-tailed planarians

Surprisingly, during late stages of regeneration we observed a second mode of brain tissue differentiation after Wnt/β-catenin ectopic activation. In 44% of *Smed-axins* RNAi animals analyzed, one or two additional clusters of cells resembling brain primordia (named brain primordia-like) appeared next to the original pharynx between 18 and 25 days after amputation, probably as a remodeling response. Like the early brain primordia described above, these brain primordia-like structures did not develop into fully formed brains but were homeostatically maintained. The phenotypes observed in regenerated *Smed-axins* RNAi trunks displayed a temporal progression (Table S1). Likewise, *Smed-APC-1* RNAi trunk fragments differentiated brain primordia and brain primordia-like structures at anterior wounds and next to the original pharynx, respectively (Fig. 8). Noteworthy,

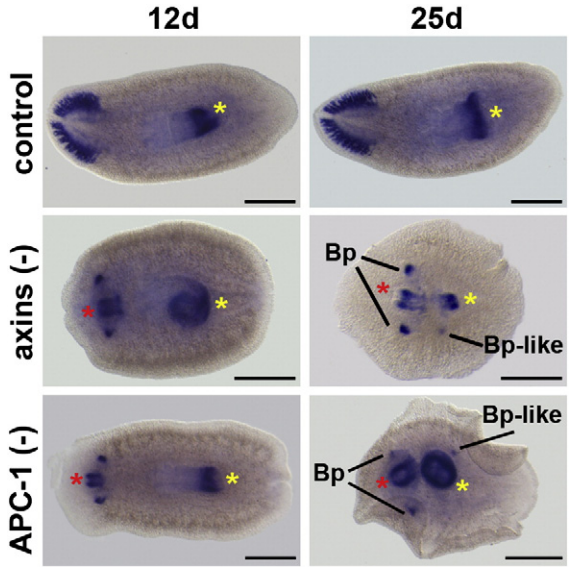

Fig. 8. Brain primordia-like structures differentiate close to the original pharynx at late stages of regeneration in *Smed-axins* and *Smed-APC-1* RNAi-treated animals. Analysis of *Smed-Gpas* expression showing the dynamics of brain tissue differentiation in trunk fragments. Two successive modes of brain tissue differentiation are observed following *Smed-axins* and *Smed-APC-1* RNAi. Firstly, like control animals, RNAi-treated animals regenerate brain primordia (bp) at anterior wounds (see also Fig. 4). Secondly, at late stages of regeneration, brain primordia-like (bp-like) structures differentiate next to the original pharynx. Anterior is shown to the left. d, days after amputation; yellow and red asterisks indicate normal and ectopic pharynx, respectively. Scale bars, 500 μm.

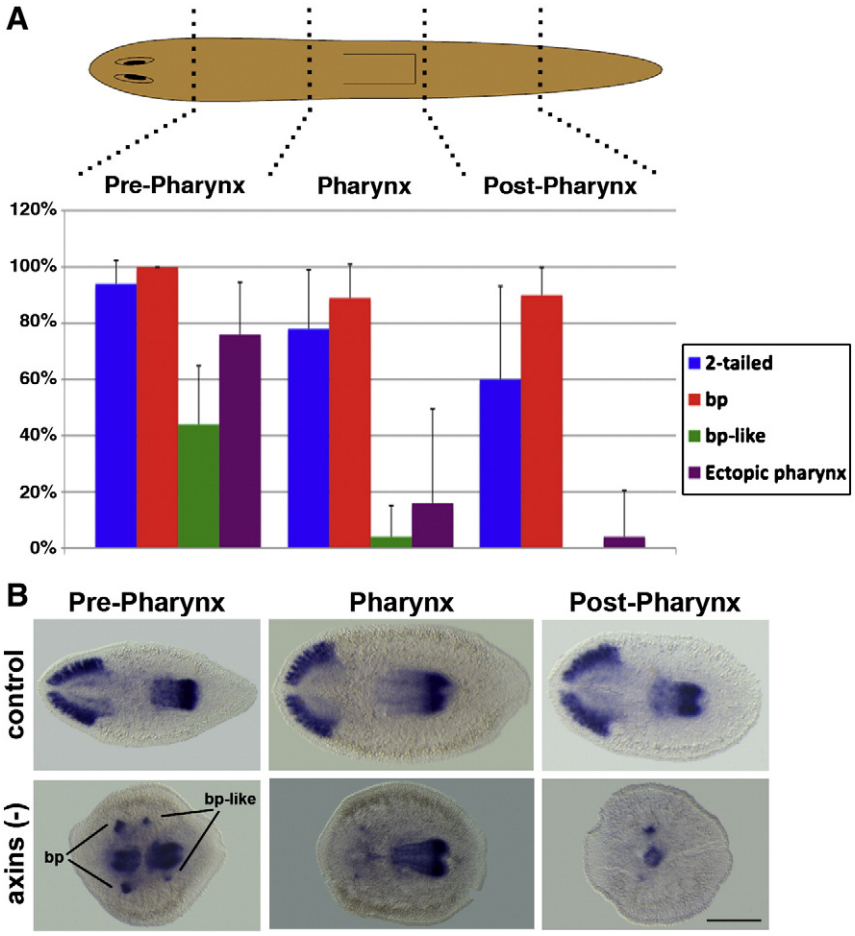

Fig. 9. The effect of *Smed-axins* RNAi on regeneration depends on the level of amputation along the anteroposterior (AP) axis. (A) Schematic representation of transverse amputations at different positions along the AP axis of *Smed-axins* RNAi-treated planarians. The two-tailed phenotype was scored on the basis of morphology whereas the other phenotypic traits were analyzed with the marker *Smed-Gpas*. The resulting bipolar fragments from more anterior locations were more likely to develop the two-tailed phenotype and ectopically differentiate a pharynx and brain primordia-like structures adjacent to the normal/original pharynx. Data show means for at least four experiments (see Table S4) analyzed at late stages of regeneration; bars are standard deviation. Statistically significant differences were observed between the different fragments for the two-tailed, ectopic pharynx and brain primordia-like phenotypic traits when analyzed by Chi square test at a significance level of 0.05. However, the differences between the fragments for the brain primordia phenotypic trait were not statistically significant. (B) Representative *Smed-axins* RNAi phenotypes along the AP axis analyzed with *Smed-Gpas*. All images correspond to 12-day regenerating fragments. Anterior is shown to the left. bp: brain primordia; bp-like: brain primordia-like; Scale bar, 300 μm.

brain primordia-like structures also differentiated next to the newly formed pharynx in regenerating head fragments after both *Smed-axins* RNAi (Fig. S11 and Table S2) and *Smed-APC-1* RNAi (data not shown). The penetrance of this phenotype was directly proportional to the dose of dsRNA injected (Table S3).

Together with previous sections, these results show that, upon amputation, two successive modes of brain tissue differentiation are observed after ectopic activation of the Wnt/β-catenin pathway. The first of these was an initial "default" response, in which brain primordia differentiated early during regeneration at anterior wounds independently of blastema polarity and dose of dsRNA injected (Fig. 4 and Fig. S7). In the second mode, differentiation of brain primordia-like structures occurred close to the original pharynx. This latter effect depended on the time of regeneration and the dose of dsRNA injected (Tables S1 and S3). Thus, the different phenotypes observed after ectopic Wnt/β-catenin pathway activation appear to correspond to different degrees of remodeling of pre-existing tissues (or pharynx) to integrate them into the new body polarity. The differentiation of brain tissues next to both the ectopic and the original pharynx was the most severe phenotype observed (Fig. 8 at 25 days). Thus, it is tempting to speculate that during regeneration the presence of two opposite posterior blastemas leads to organize two opposed body axes composed of tail, pharynx and brain primordium tissues (the most severe phenotype). This is consistent with the idea that canonical Wnt pathway specifies a posterior organizer, which in turns patterns the AP axis during planarian regeneration (Adell et al., 2010; Meinhardt, 2009a, 2009b). Such a mechanism for axial patterning has not only been shown to operate during hydra regeneration, but has also been proposed to represent an ancestral system for patterning the eumetazoan embryonic primary axis (Bode, 2009; Holland, 2002; Kusserow et al., 2005; Lee et al., 2006).

Our results have also uncovered a striking relationship between the pharynx and brain tissues, which always appear close to each other after over-activation of the Wnt/β-catenin pathway. Interestingly, low doses of *Smed-β-catenin1* RNAi result in two-headed planarians with two pharynges located close to each other but with opposite polarities, and the differentiation of brain primordia-like structures is also observed (Fig. S12). Therefore, the appearance of these brain primordia-like structures close to the pharynx is not merely a consequence of the presence of two opposite posterior blastemas. Perhaps, a common feature of perturbing the Wnt/β-catenin pathway would be the remodeling response of the pharynx to two confronting body axes. If so, the data would suggest that the pharynx somehow instructs the position at which brain primordia-like structures will differentiate. Further studies will be necessary to elucidate the role of the pharynx during planarian regeneration. In particular, it would be interesting to ascertain whether the region where the pharynx joins the anterior gut branch (the esophagus)

functions as a signaling center since this is a region in which many signaling factors are expressed (Fraguas et al., 2011; Gurley et al., 2010; Molina et al., 2007; Rink et al., 2009).

The Smed-axins RNAi phenotype depends on the level of amputation along the AP axis

Recently, a gradient of Smed-β-catenin1 activity originating from a posterior organizer has been proposed to underlie positional identity along the AP axis (Adell et al., 2009, 2010; Iglesias et al., 2008; Meinhardt, 2009b). The severity of the phenotype after ectopic Wnt/β-catenin pathway activation could therefore be dependent on a pre-existing morphogenetic gradient along the AP axis of the regenerating animal. To assess this possibility, planarians were amputated at four levels along the AP axis and the regeneration of the resulting bipolar pre-pharynx, pharynx, and post-pharynx fragments were analyzed after silencing *Smed-axins* (Fig. 9). All control bipolar regenerating fragments developed normal anterior blastemas in which a normal brain developed irrespective of the level of amputation. In contrast, after *Smed-axins* RNAi, the penetrance of the two-tailed phenotype gradually increased as the level of amputation was moved towards the anterior end. The highest penetrance was observed in pre-pharynx fragments, which were posteriorized in 94% of cases. In addition, analyses of two-tailed fragments with the marker *Smed-Gpas* also revealed varying penetrance in the differentiation of brain primordia-like structures and ectopic pharynges according to the AP level from which the regenerating fragment originated (Fig. 9 and Fig. S13). Three observations are particularly noteworthy. First, all bipolar regenerating fragments differentiated brain primordia at anterior wounds. Second, differentiation of one or two brain primordia-like structures was observed next to the normal/original pharynx as a remodeling response in 44% and 4% of pre-pharynx and pharynx fragments, respectively (Table S4). Third, the susceptibility of bipolar regenerating fragments to ectopically differentiate a pharynx with opposite polarity increased in more anterior fragments such that the pre-pharynx fragments were most susceptible (76%).

Overall, these data suggest that early brain regeneration at anterior wounds occurs independently of any pre-existing AP morphogenetic gradient controlled by the Wnt/β-catenin pathway. In contrast, the likelihood of developing the most severe *Smed-axins* RNAi phenotype is a function of the position along the AP axis, with more anterior areas being more susceptible. This supports the existence of a Smed-β-catenin activity gradient originating from posterior blastemas since this susceptibility to develop the most severe phenotype could reflect relative differences of Smed-β-catenin1 activity levels between the newly formed posterior blastema (high levels) and the pre-existing AP gradient of the regenerating fragment. However, further analyses will be required to determine whether a posterior organizer established by the Wnt/β-catenin pathway specifies the planarian AP axis through a gradient of Smed-β-catenin1 activity.

Conclusions

Our data demonstrate that *Smed-axins* are conserved negative regulators of the Wnt/β-catenin pathway required for the re-establishment of AP polarity during planarian regeneration. Furthermore, we have shown that the mechanisms controlling early brain differentiation at anterior wounds are independent of those that control blastema polarity via the Wnt/β-catenin pathway. In contrast, however, ectopic Wnt/β-catenin activation by silencing *Smed-axins* or *Smed-APC-1* prevents the development of a fully formed brain, an indication that distinct mechanisms control early and late brain development. It remains to be determined whether β-catenin activity allows only early brain development or whether, upon amputation, unknown mechanisms operate at anterior wounds to overcome temporarily the effect of *Smed-axins* or *Smed-APC-1* RNAi on β-catenin activity and consequently commit early brain primordia. Furthermore, we provide evidence of an indirect relationship between the Wnt/β-catenin and FGFR/ndk signaling systems in the control of the posterior limits of brain differentiation. Future studies will address the possibility that a feedback-loop between Wnt/β-catenin and the FGFR/ndk signaling systems controls AP patterning of the nervous system via effects on β-catenin activity.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.07.013.

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