

Localization of planarian β -CATENIN-1 reveals multiple roles during anterior-posterior regeneration and organogenesis

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ABSTRACT

The β -catenin-dependent Wnt pathway exerts multiple context-dependent roles in embryonic and adult tissues. In planarians, β -catenin-1 is thought to specify posterior identities through the generation of an anteroposterior gradient. However, the existence of such a gradient has not been directly demonstrated. Here, we use a specific polyclonal antibody to demonstrate that nuclear β -CATENIN-1 exists as an anteroposterior gradient from the pre-pharyngeal region to the tail of the planarian *Schmidtea polychroa*. High levels in the posterior region steadily decrease towards the pre-pharyngeal region but then increase again in the head region. During regeneration, β -CATENIN-1 is nuclearized in both anterior and posterior blastemas, but the canonical WNT1 ligand only influences posterior nuclearization. Additionally, β -catenin-1 is required for proper anterior morphogenesis, consistent with the high levels of nuclear β -CATENIN-1 observed in this region. We further demonstrate that β -CATENIN-1 is abundant in developing and differentiated organs, and is particularly required for the specification of the germline. Altogether, our findings provide the first direct evidence of an anteroposterior nuclear β -CATENIN-1 gradient in adult planarians and uncover novel, context-dependent roles for β -catenin-1 during anterior regeneration and organogenesis.

KEY WORDS: β -catenin, Gradient, Wnt, Axial patterning, Organogenesis, Planarians

INTRODUCTION

The canonical (β -catenin-dependent) Wnt pathway is an evolutionarily conserved signaling pathway in animals which controls multiple crucial events during development, adult homeostasis and body regeneration (Clevers et al., 2014; Huelsken and Birchmeier, 2001; Martin and Kimelman, 2009; van Amerongen and Nusse, 2009; Yokoyama et al., 2007). During canonical signaling, secreted Wnt ligands bind to their Frizzled receptors to induce cytoplasmic accumulation and nuclear translocation of β -catenin (Clevers and Nusse, 2012; Mikels and Nusse, 2006), which is the key intracellular component of the transduction cascade. In the nucleus, β -catenin binds to the TCF/LEF transcription factors, triggering changes in chromatin conformation and gene transcription (Komiya and Habas, 2008; Valenta et al., 2012). Thus, β -catenin acts as a master regulatory gene, responsible for the control of multiple processes. During early

animal embryogenesis, β -catenin specifies the site of endoderm internalization and promotes posterior fates in most studied systems (Byrum and Wikramanayake, 2013; Darras et al., 2011; Haegel et al., 1995; Henry et al., 2008; Lee et al., 2006; Logan et al., 1999; Srivastava et al., 2014; Wikramanayake et al., 2003). In later stages of development, the canonical Wnt pathway influences the formation of multiple cell types, tissues and organ systems (Aulehla et al., 2008; Grigoryan et al., 2008; Hari et al., 2002; Holland et al., 2005; Kiecker and Niehrs, 2001; Lewis et al., 2004; Petersen and Reddien, 2009a; Schneider and Bowerman, 2007; Tan et al., 2006; Watanabe et al., 2014), and most prominently, the anteroposterior (AP) patterning of the vertebrate central nervous system (Ciani and Salinas, 2005). In adult organisms β -catenin is essential for tissue homeostasis and its deregulation leads to degenerative diseases and cancers (Clevers and Nusse, 2012). Thus, β -catenin plays multiple, context-dependent roles in development, homeostasis and regeneration.

Planarian flatworms are a powerful model to analyze the multiple roles of β -catenin-dependent Wnt signaling. Their striking regenerative capacity coupled with the continuous remodeling of their tissues to adapt their body size to food availability (Saló, 2006) allows analysis of signaling pathways in the intact organism in multiple situations. The planarians *Schmidtea mediterranea* and *Schmidtea polychroa* have two β -catenin paralogs, namely β -catenin-1 and β -catenin-2 (Gurley et al., 2008; Iglesias et al., 2008; Martín-Durán and Romero, 2011; Martín-Durán et al., 2010). While β -catenin-2 is involved in cell-cell adhesions (Chai et al., 2010), inhibition of planarian β -catenin-1 results in a striking ‘radial-like’ hypercephalized phenotype, revealing a central role for canonical Wnt signaling in the specification of posterior identities during adult regeneration and homeostasis (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). In contrast, inhibition of negative regulators of the canonical Wnt pathway, such as the components of the β -catenin destruction complex, APC and axin, leads to the duplication of posterior territories (i.e. posteriorized phenotype) (Gurley et al., 2008; Iglesias et al., 2011). To explain these phenotypes, it is widely postulated that there is a gradient of increasing β -catenin activity from anterior to posterior that specifies and maintains AP axial identity in planarians (Adell et al., 2010). This model, which departs from classic regeneration experiments (Child, 1911, 1941; Morgan, 1904, 1905), is further supported by the range of phenotypes generated after β -catenin-1 inhibition, in which anterior and posterior structures are gradually affected according to the dose and time of inhibition (Adell et al., 2010; Iglesias et al., 2008). These phenotypes vary from loss of the most posterior structures (‘tail-less’ phenotype) to ‘radial-like’ hypercephalization with no posterior or central identity (Adell et al., 2010; Iglesias et al., 2008). Moreover, it is known that several Wnt ligands are expressed posteriorly, whereas inhibitors (e.g. *notum* and *sFRP*) localize to the anterior pole, and their inhibition leads to anteriorized or posteriorized phenotypes, respectively (Adell et al.,

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2009; Gurley et al., 2010; Lander and Petersen, 2016; Petersen and Reddien, 2009b, 2011; Scimone et al., 2016; Sureda-Gómez et al., 2015). Interestingly, recent reports have suggested that *β-catenin-1* exerts additional roles in eye and brain patterning (Hill and Petersen, 2015; Owen et al., 2015), which is consistent with the ubiquitous mRNA expression of *β-catenin-1* (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). To date, however, no studies have described the localization of β-CATENIN-1 protein either as an AP gradient or in specific developing tissues.

Since β-catenin is regulated at the protein level (Clevers, 2006), analysis of its different roles in intact and regenerating planarians requires tools to visualize the localization of β-catenin protein. In this study, we analyze the role of β-catenin using a polyclonal antibody generated against the N-terminal region of planarian β-CATENIN-1. Immunohistochemistry demonstrates the existence of a gradient of nuclear β-CATENIN-1 along the AP axis, with the lowest levels in the pre-pharyngeal region and the highest levels at the tip of the tail. β-CATENIN-1 levels are also high in the head region. We demonstrate that the WNT1 ligand is specifically required for the nuclear localization of β-CATENIN-1 in posterior regions. β-CATENIN-1 is also abundant in the anterior pole, where it controls proper brain and head regeneration. Finally, immunodetection of β-CATENIN-1 also reveals high levels of nuclear protein in specific organs during adult homeostasis and regeneration, as well as during embryogenesis. In this context, we demonstrate an essential function of β-CATENIN-1 in female and male germline development. Altogether, our findings validate the main assumptions of the current model for AP specification in planarian flatworms, and uncover new roles for the canonical Wnt pathway in embryonic and adult organogenesis.

RESULTS

β-CATENIN-1 displays a nuclear gradient along the AP axis, from the pre-pharyngeal region to the tail

To analyze the localization of β-CATENIN-1 in planarians, we generated a polyclonal antibody against the N-terminal region of the *Schmidtea mediterranea* β-CATENIN-1 protein (anti-βCAT-1) (Fig. S1A). Western blot analysis on protein extracts of adult specimens of *S. mediterranea* showed a specific band of the expected size (107 kDa) (Fig. S1B). Importantly, anti-βCAT-1 also recognizes the β-CATENIN-1 protein from *Schmidtea polychroa* (Fig. S1B), which is the sister species of *S. mediterranea* (Alvarez-Presas et al., 2008). The region used for raising the antibody exhibits 98% amino acid identity between both species (Fig. S1A). We found that the antibody produced a more reliable signal for immunohistochemistry in *S. polychroa*. Since *S. polychroa* is a sexual species, which enables the study of the reproductive system and the embryogenesis, we therefore focused all subsequent analyses on this species.

To confirm that anti-βCAT-1 specifically recognizes β-CATENIN-1, we performed western blot analysis on protein extracts from *β-catenin-1(RNAi)* and *APC(RNAi)* animals after 5 weeks of treatment. In *S. polychroa*, these animals did not yet show any AP morphological transformation (Fig. S2A). As expected, inhibition of *β-catenin-1* transcription resulted in a reduction of β-CATENIN-1 levels, and inhibition of *APC*, which downregulates β-catenin (Clevers, 2006), produced an increase in β-CATENIN-1 levels (Fig. S2B). qPCR analysis of *β-catenin-1* mRNA levels in those samples showed that *β-catenin-1* was downregulated after *β-catenin-1 RNAi* but was not upregulated after *APC RNAi* (Fig. S2C). This observation further supports the specificity of the antibody, since *APC* induces the destruction of β-CATENIN-1 protein but does not

act at the transcriptional level (Clevers, 2006). We further validated the specificity of the anti-βCAT-1 antibody by quantifying its immunoreactivity in anterior and posterior regions in control, *β-catenin-1(RNAi)* and *APC(RNAi)* intact animals after 2 weeks of treatment. Consistent with the previous western blot results, immunoreactivity decreases in *β-catenin-1(RNAi)* animals and increases in *APC(RNAi)* planarians compared with controls (Fig. S3).

To examine the subcellular localization of β-CATENIN-1, we performed immunohistochemistry on paraffin sections of uninjured animals. β-CATENIN-1 was mostly located in the nuclei in both anterior and posterior regions (Fig. 1A). Although we cannot rule out residual localization in the cytoplasm of scattered cells, β-CATENIN-1 was never observed in the cell membrane, consistent with the functional specialization of the planarian β-catenin genes (Chai et al., 2010). We next analyzed distribution of β-CATENIN-1 along the AP axis of uninjured planarians in sagittal sections (Fig. 1B; Fig. S4A). Immunoreactivity for β-CATENIN-1 was abundant in the mesenchyme, mostly posteriorly, and in the brain and pharynx (Fig. 1B; Fig. S4A). A heat map of the localization of β-CATENIN-1 demonstrated that the highest levels were found in the brain, the distal part of the pharynx and the posterior end of the animal (Fig. 1B; Fig. S4A). We next quantified the signal of nuclear β-CATENIN-1 along the AP axis to detect differences in any region of the body (Fig. 1C; Fig. S4C). Although β-CATENIN-1 was almost always located in the nucleus, we applied a nuclear mask to specifically quantify the nuclear signal (Fig. S4B). For this analysis, we considered sagittal sections corresponding only to the region around the midline, and we excluded the brain and pharyngeal signal, which we assumed was related to organogenesis rather than to axial positional information (Fig. 1C). We quantified nuclear β-CATENIN-1 levels in eight consecutive regions along the AP axis of the animal, which demonstrated that the highest levels of nuclear β-CATENIN-1 occurred in the most posterior region of the animal, whereas the lowest levels were observed in the pre-pharyngeal area (Fig. 1C). Remarkably, we detected higher levels of nuclear β-CATENIN-1 in the head than in the pre-pharyngeal region. Importantly, the gradient distribution of β-CATENIN-1 along the AP axis was statistically significant (Fig. 1C; Fig. S4C; Table S1). Although we cannot specify the cell types that contribute to the gradient, we observed nuclear localization of β-CATENIN-1 in sub-epidermal muscular cells, which are the source of positional information cues (Witchley et al., 2013) and in proliferating cells, which correspond to neoblast and early progeny stem cells (Reddien, 2013) (Fig. S4D). Therefore, our findings demonstrate that the nuclear localization of β-CATENIN-1 exhibits a gradient along the AP axis, with the highest levels posteriorly and the lowest levels in the pre-pharynx. High levels of nuclear localization are also observed in the head.

wnt1 controls β-CATENIN-1 localization in posterior blastemas

Because *β-catenin-1* mRNA is expressed in both anterior and posterior blastemas from the first hours after amputation (Fig. S5A) (Iglesias et al., 2008), we analyzed β-CATENIN-1 localization in anterior and posterior blastemas at different time points during regeneration on sagittal sections (Fig. 2A; Fig. S5B). At 6 h post amputation (hpa), β-CATENIN-1 localized to the nucleus of cells located in the tip of anterior and posterior wounds (Fig. S5B). With the formation of the blastema at 12 hpa and 1 dpa, we observed accumulation of β-CATENIN-1 in both anterior and posterior blastemas compared with the adjacent pre-existing tissue (Fig. 2A; Fig. S5B,C). This situation was apparently maintained 3 and 5 days

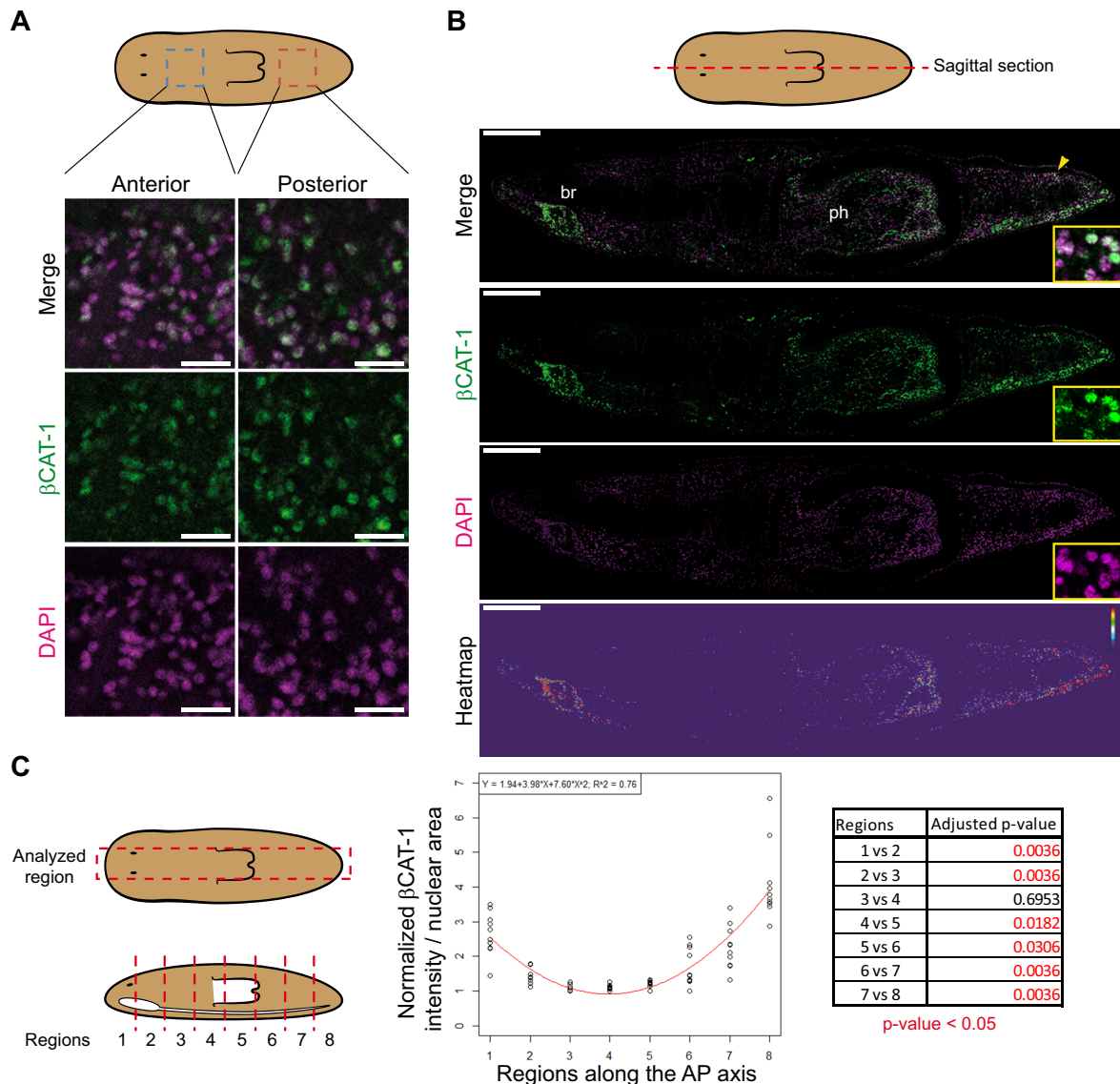


Fig. 1. β -CATENIN-1 is expressed as a nuclear gradient from the prepharyngeal region to the tail. (A) Immunostaining on paraffin sections against β -CATENIN-1 (green) and nuclear staining with DAPI (magenta) in uninjured planarians. β -CATENIN-1 is localized in the nucleus both in anterior and posterior regions. (B) Immunostaining on a sagittal section corresponding to the region around the midline against β -CATENIN-1 (green) and nuclear staining with DAPI (magenta) in uninjured animals. The images shown correspond to a mosaic of sub-images. The rainbow heat map of β -CATENIN-1 (bottom) illustrates the intensity of the signal (red, maximum). Dorsal, top. Arrowhead indicates areas shown at higher magnification in insets. (C) Quantification of nuclear β -CATENIN-1 intensity on sagittal sections corresponding to the region around the midline normalized with the nuclear area of each section (red lines indicate the different regions analyzed along the AP axis), excluding the brain and pharynx (white areas) and relative to the lower value ($n=10$ sections corresponding to four different animals). Values are represented using a polynomial regression and were analyzed using the Wilcoxon signed-rank test. Table shows the P -values obtained comparing the different regions. P -values in red are significant. br, brain; ph, pharynx. Scale bars: 25 μ m (A), 200 μ m (B). In all images, anterior is left.

post amputation (dpa), although β -CATENIN-1 became more abundant in the ventral side of the anterior blastema as the brain formed (Fig. 2A; Fig. S5B). Therefore, nuclear localization of β -CATENIN-1 during regeneration does not depend on the identity of the blastema.

The observation that *wnt1* is expressed in a few cells of the posterior dorsal midline (Fig. S5A) and that *wnt1* silencing phenocopies β -catenin-1(RNAi) (Fig. S6A) suggests that *wnt1* specifies posterior identity via activation of β -catenin-1 (Adell et al., 2009; Petersen and Reddien, 2009b). However, *wnt1* is expressed in anterior and posterior regenerating regions at early stages of regeneration, from 12 hpa to 1 dpa (Fig. S5A) (Gurley et al., 2010; Petersen and Reddien, 2009b). We therefore analyzed whether *wnt1* is responsible for β -CATENIN-1 localization in anterior and

posterior blastemas during the earliest stages of regeneration. Western blots of protein extracts from 1 dpa anterior and posterior wounds showed that β -CATENIN-1 levels only decayed in posterior blastemas in *wnt1*(RNAi) animals (Fig. 2B). We confirmed this specific decrease by immunohistochemistry on anterior and posterior 1 day regenerating blastemas (Fig. 2C; Fig. S6B). Altogether, our results indicate that *wnt1* positively controls β -CATENIN-1 nuclear levels in posterior blastemas at 1 dpa, but it does not have a significant role in maintaining β -CATENIN-1 levels in anterior regenerating regions.

β -CATENIN-1 is required for proper anterior regeneration

The high levels of nuclear β -CATENIN-1 in anterior blastemas suggest a role for β -catenin-1 in anterior regeneration and

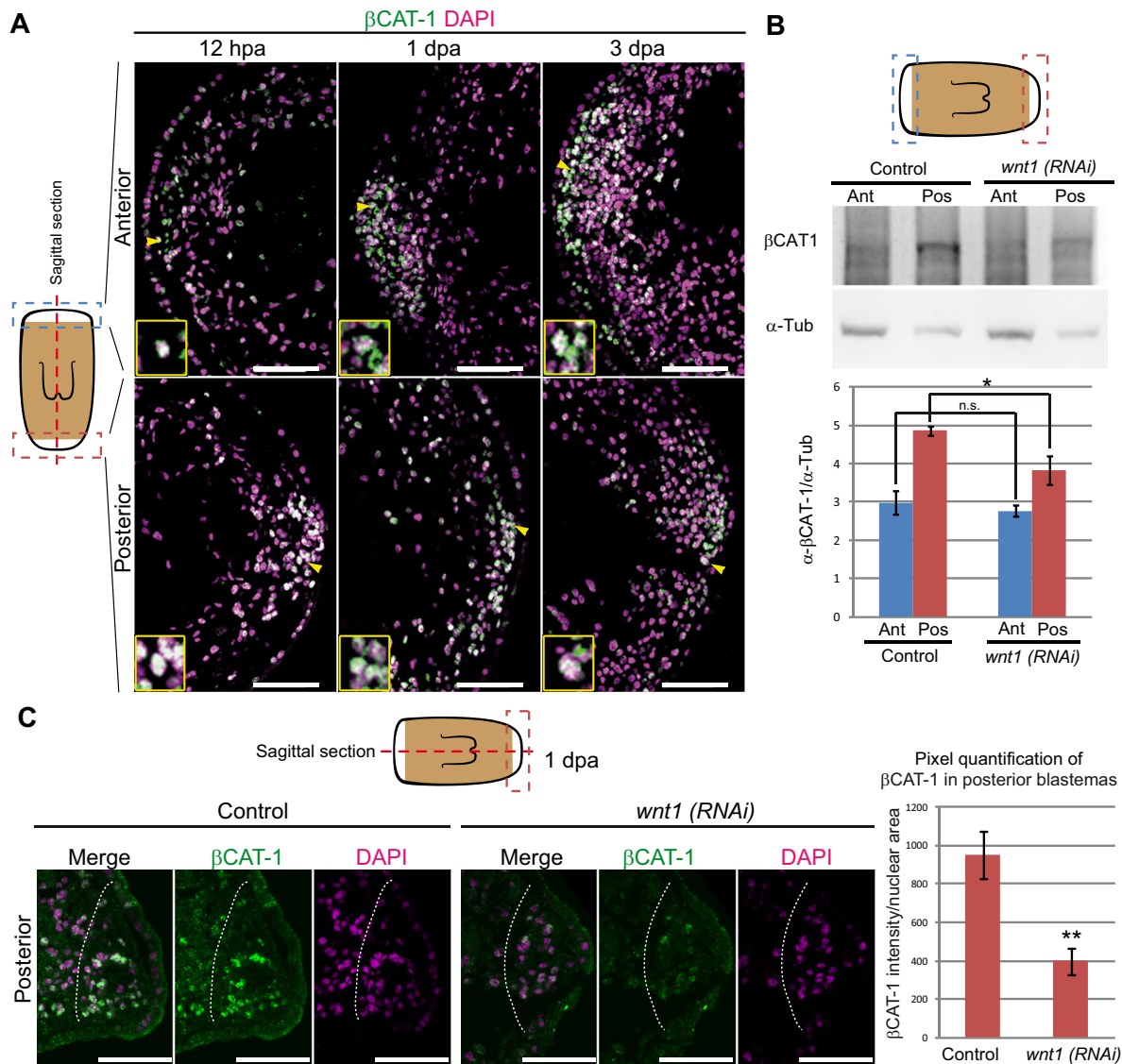


Fig. 2. β-CATENIN-1 is localized in the nucleus of anterior and posterior blastemas but only posterior β-CATENIN-1 depends on WNT1.

(A) Immunoreactive pattern of the βCAT-1 antibody (green) on sagittal sections and nuclear staining with DAPI (magenta) in 12 h, 1 day and 3 day bipolar regenerating trunks. A nuclear mask was applied to show nuclear signal (original images are shown in Fig. S5B). Dorsal, top; anterior, left. Arrowheads indicate areas shown at higher magnification in insets. (B) Western blot with anti-βCAT-1 antibody of protein extracts from anterior and posterior regenerating regions of 1 day regenerating control and *wnt1(RNAi)* animals. α-Tubulin antibody was used as a loading control. Bar chart shows the quantification of β-CATENIN-1 signal with respect to α-tubulin, normalized with respect to the control anterior region value ($n=5$). β-CATENIN-1 signal decreases after *wnt1(RNAi)* in posterior but not in anterior regions. Error bars indicate s.d., * $P<0.05$; n.s., not significant. (C) Immunostaining on sagittal sections with anti-βCAT-1 antibody (green) and DAPI (magenta) of control and *wnt1(RNAi)* posterior regenerating regions 1 day after amputation. Bar chart shows the quantification of β-CATENIN-1 signal in the blastema region (delimited by the white dotted line). Error bars indicate s.d., ** $P<0.005$. *wnt1(RNAi)* animals show a decrease in posterior nuclear β-CATENIN-1 signal compared with controls ($n=6$). Dorsal, top; anterior, left. Scale bars: 50 μm (A,C).

homeostasis. Analysis of β-CATENIN-1 in the anterior region revealed nuclear localization of the protein in neural cells of the cephalic ganglia and in the head margin (α-tubulin⁺), as well as in non-neural cells (α-tubulin⁻) located in the most anterior dorsal midline (Fig. 3A). Interestingly, this region corresponds to the expression domain of the Wnt inhibitor *notum*, which induces the regeneration of anterior tails when silenced (Petersen and Reddien, 2011). A similar distribution was observed in 3 dpa anterior blastemas, where β-CATENIN-1 localized to dorsal scattered cells at the dorsal midline in addition to neural cells of the brain primordia (Fig. 3B). In order to study the role of β-CATENIN-1 in anterior regeneration, we analyzed the morphology and patterning of anterior blastemas in *β-catenin-1(RNAi)* animals. We first

observed that the eyes formed closer to the pre-existing tissue in *β-catenin-1(RNAi)* planarians compared with controls (Fig. S7A). Quantitative analysis of the expression of a dopaminergic (*th-1*⁺) and an octopaminergic (*tbh*⁺) neuronal marker in anterior blastemas revealed a significant decrease in both cell populations after 3 and 8 dpa (Fig. 3C). Similarly, the number of mechanosensory cells (*cintillo*⁺) in the head margin was also significantly reduced after *β-catenin-1(RNAi)* (Fig. S7B) (Hill and Petersen, 2015; Owen et al., 2015). Quantification of the brain area by DAPI staining and analysis of the distance from the anterior tip of the brain to the anterior tip of the head revealed that the brain ganglia in *β-catenin-1(RNAi)* animals were smaller and formed closer to the head margins than in control planarians (Fig. 3C). In addition, the midline furrow

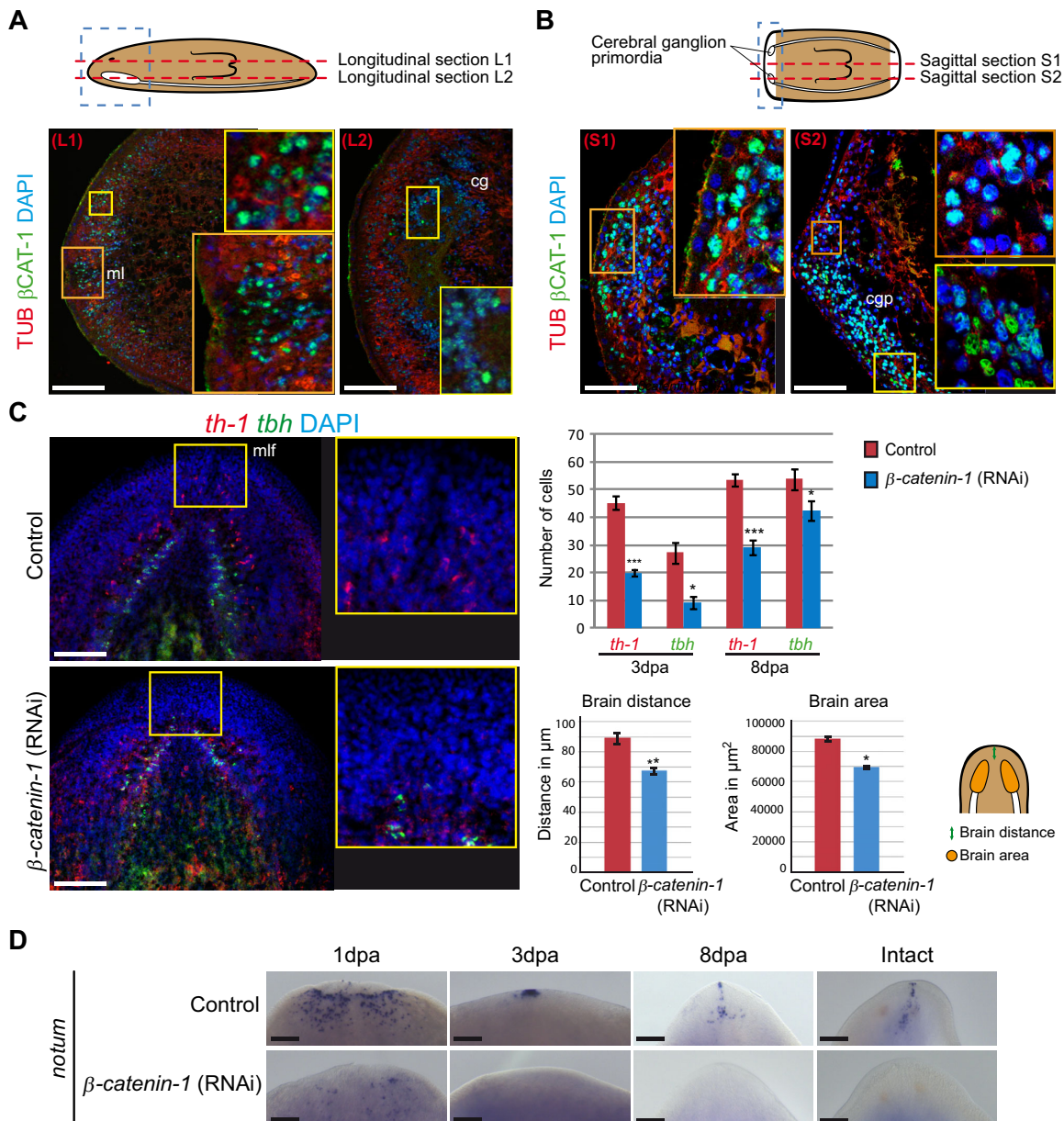


Fig. 3. β -catenin-1 is required for proper anterior regeneration. (A) Immunostaining on longitudinal sections of uninjured planaria with anti- β CAT-1 (green) and anti- α -tubulin (red). Nuclei are labeled with DAPI (blue). Yellow and orange squares indicate regions corresponding to neural and non-neural cells, respectively, which are shown in higher magnification. Anterior, left. (B) Immunostaining on sagittal sections with anti- β CAT-1 (green) and anti- α -tubulin (red) of 3 dpa anterior regions. Nuclei are labeled with DAPI (blue). Yellow and orange squares indicate regions corresponding to ventral and dorsal areas, respectively, which are shown in higher magnification. Anterior, left. (C) Dopaminergic (*th-1*, red) and serotonergic (*tbh*, green) neurons visualized by whole-mount *in situ* hybridization in control and *β-catenin-1*(RNAi) animals fixed 8 dpa. DAPI (blue) labels the nucleus. Yellow squares indicate regions corresponding to the midline ventral furrow (mf) shown in higher magnification. Quantification of *th-1*⁺ and *tbh*⁺ cells showing a decrease of both neural populations in *β-catenin-1*(RNAi) planarians. Quantification of the distance from the tip of the cephalic ganglia to the anterior tip of the animal and of the total brain area, both visualized by DAPI nuclear staining, shows a decrease of both measures in *β-catenin-1*(RNAi) planarians. Error bars indicate s.d., * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ (two-tailed *t*-test); $n = 5$. Anterior, top. (D) Whole-mount *in situ* hybridization showing *notum* expression in control and *β-catenin-1*(RNAi) planarians at 1, 3 and 8 days of regeneration and in uninjured animals. *notum* expression disappears in *β-catenin-1*(RNAi) animals. Anterior, top. cg, cephalic ganglia; ml, midline furrow. Scale bars: 100 μm (A,C,D), 50 μm (B).

between the brain ganglia was not correctly patterned in *β-catenin-1* (RNAi) planarians (Fig. 3C), suggesting a problem in restoration of the anterior tip. We then analyzed whether these morphological defects affected the domain of expression of *notum* in regenerating and intact *β-catenin-1*(RNAi) animals. As early as 1 dpa, the expression of *notum* decreased after *β-catenin-1*(RNAi) and subsequently disappeared in regenerating and uninjured 14-day-old dsRNA-injected animals (Fig. 3D) (Hill and Petersen, 2015;

Petersen and Reddien, 2011; Scimone et al., 2014). Both the tip- and brain-related domains of *notum* expression were affected by *β-catenin-1*(RNAi) treatment. Altogether, these results indicate that β -CATENIN-1 is required for the proper patterning and morphogenesis of the brain and head margin structures. Further work will be required to determine which specific anterior defects are caused by the function of β -CATENIN-1 as an element of the anterior organizing region (e.g. as a *notum* regulator) and which are

due to its probable role in central nervous system (CNS) patterning and morphogenesis.

β -CATENIN-1 is stabilized in the nucleus during adult and embryonic organogenesis

In addition to the strong β -CATENIN-1 immunoreactivity in the brain observed with our anti- β CAT-1 antibody, we also observed

signal in other mature organs (Fig. 4A; Fig. S8A). In the nervous system, β -CATENIN-1 localized preferentially to the external part of the brain ganglia and the brain branches, and in scattered cells in the ventral nerve cords and apparently in the eye (Fig. 4A; Fig. S8A). β -CATENIN-1 was also localized in the nucleus of the digestive system and the pharynx, where it was found to be more abundant in

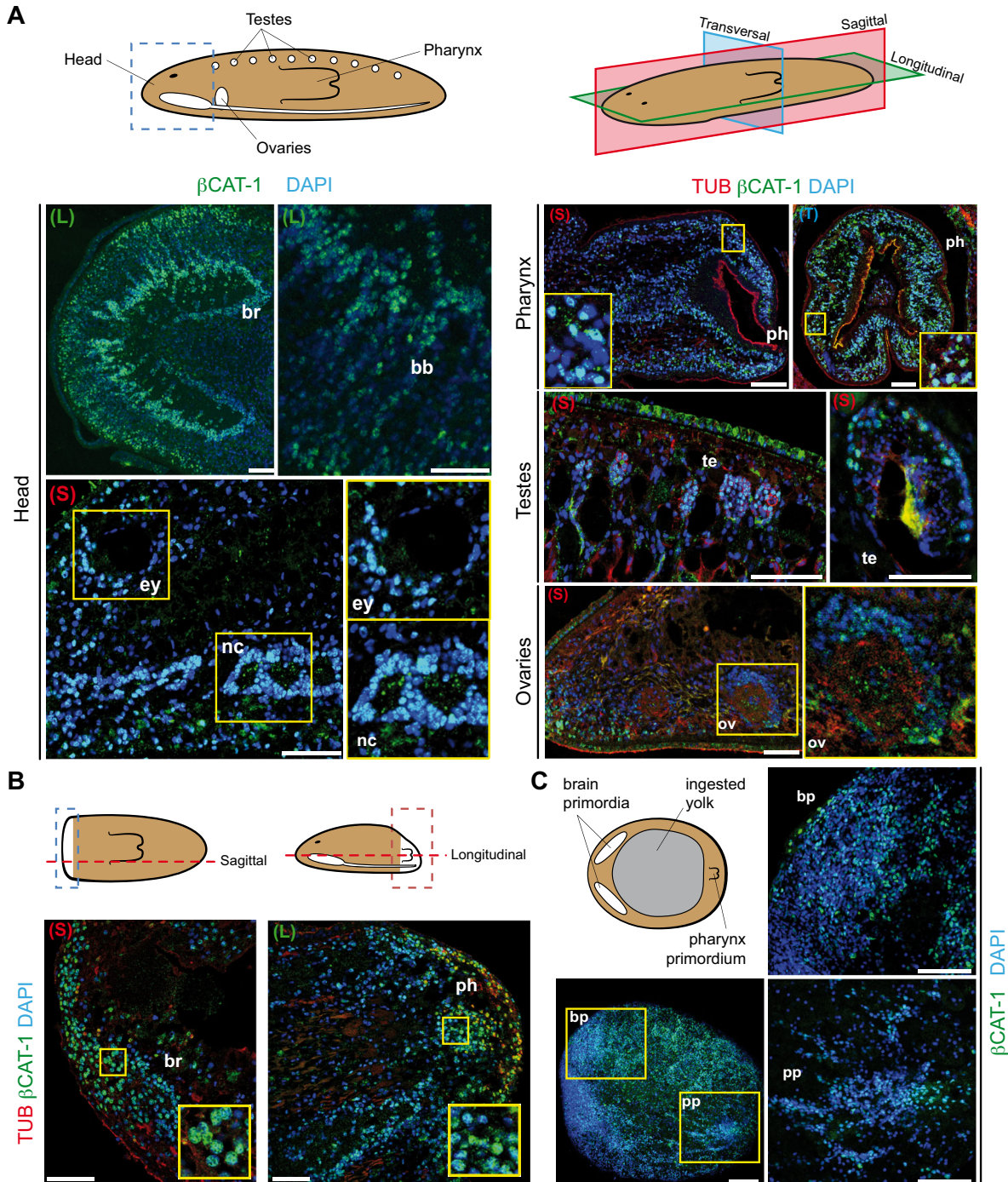


Fig. 4. Nuclear β -CATENIN-1 is upregulated in specific organs during maintenance and *de novo* formation. (A) Immunostaining on sections with anti- β CAT-1 (green) and anti- α -tubulin (red) of uninjured animals. Nuclei are labeled with DAPI (blue). Yellow squares indicate regions shown at higher magnification. Analyzed regions are indicated in the scheme at the top left and planes are indicated in the top right scheme. (B) Immunostaining on sections (planes are indicated in scheme at top) with anti- β CAT-1 (green) and anti- α -tubulin (red) of regenerating animals. Nuclei are labeled with DAPI (blue). Yellow squares indicate images shown at higher magnification. (C) Immunostaining on whole-mount of a 6-day-old embryo (stage 5) with anti- β CAT-1 (green) and anti- α -tubulin (red). Nuclei are labeled with DAPI (blue). Yellow squares indicate images shown at higher magnification. br, brain; bb, brain branches; nc, nerve cord; ph, pharynx; te, testes; ov, ovaries; bp, brain primordium; pp, pharynx primordium. Scale bars: 50 μ m (A,B), 200 μ m (C).

the most distal part (Fig. 4A; Fig. S8A), consistent with the β -catenin-1 expression pattern (Fig. S5A) (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). Remarkably, we detected high levels of nuclear β -CATENIN-1 in the male gonads (the testis), particularly in the germ cells and in the nucleus of the cells around the ovaries, where female germ cells are found (Hoshi et al., 2003) (Fig. 4A). We also observed signal for β -CATENIN-1 in the copulatory apparatus (Fig. S8A). We next analyzed the distribution of β -CATENIN-1 in these organs during regeneration, between 3 and 5 dpa. We observed an increase in the levels of β -CATENIN-1 in the nuclei of the primordial cells of each organ, compared with the surrounding cells (Fig. 4B; Fig. S8B). To further investigate the distribution of β -CATENIN-1 during organogenesis, we characterized its localization in stage 5 embryos, which corresponds to the onset of *de novo* cell differentiation and early organogenesis during planarian embryonic development (Martín-Durán and Romero, 2011; Monjo and Romero, 2015). We detected a widespread distribution of β -CATENIN-1 throughout the entire embryo, and in particular in the presumptive anlagen of the definitive pharynx and brain, which at this stage appear as an anterior bilobed cluster of cells that are already positive for neural markers (Martín-Durán and Romero, 2011; Monjo and Romero, 2015) (Fig. 4C; Fig. S8C). Therefore, in addition to its nuclear localization in the main organs of planarians during homeostasis, β -CATENIN-1 is also dynamically regulated during regeneration and embryogenesis, consistent with a role for β -catenin-1 during planarian organogenesis.

β -CATENIN-1 is required for germline specification

To further elucidate the role of β -catenin-1 in the development and regeneration of the mature organ systems of planarians, we decided to focus on the possible role of β -catenin-1 in germline specification and gonad maturation, since β -CATENIN-1 was strongly detected in male and female gonads (Fig. 4A). β -catenin-1 dsRNA was injected in 1- to 3-day-old hatchlings, prior to expression of *nanos* (*nos*), a germline marker required for proper germline specification (Fig. S9A) (Wang et al., 2007). After 4 weeks of RNAi treatment, control animals had normally developing gonads, with *nos* expression in small clusters along two dorsolateral lines, corresponding to the testis and in two larger ventral clusters behind the brain, corresponding to the ovaries (Fig. 5A). In contrast,

β -catenin-1(RNAi) animals did not show any *nos* expression (Fig. 5A). The same result was obtained using *germlinal histone H4* (*gh4*) as a marker of male germline specification (Wang et al., 2007) (Fig. S9B). Our results thus show that β -catenin-1 is required for germ cell specification and gonad development, uncovering a role for this multi-functional protein in controlling planarian organogenesis, in addition to its well-established axial role.

DISCUSSION

Planarians are an ideal system in which to understand the complex context-dependent roles of β -catenin, since it is possible to study multiple cellular and genetic processes in the whole organism during embryonic development, regeneration and adult homeostasis. To date, however, a major challenge for planarian biology has been to analyze protein expression with specific antibodies. In this study, we successfully generated a specific anti- β -CATENIN-1 antibody that allowed analysis of the functional behavior of β -CATENIN-1 in the whole planarian and in different developmental stages.

Our study provides the first direct confirmation of the hypothesized AP gradient of nuclear β -CATENIN-1. Interestingly, however, we found that there are two regions with high levels of nuclear β -CATENIN-1, one in the tail and one in the head, with the lowest levels of nuclear localization of β -CATENIN-1 found in the pre-pharyngeal region. During regeneration, β -CATENIN-1 is actively nuclearized in posterior wounds in a *wnt1*-dependent manner. Similar nuclear accumulation was observed in anterior blastemas and in the organ primordia, highlighting an additional role of β -catenin-1 in head patterning and organogenesis, and in particular for germline development.

Importantly, β -CATENIN-1 signal was mostly found in the nucleus and never in the membrane. This result contrasts with the results reported from other systems, mainly in adult stages, where β -catenin is only occasionally detected in the nucleus, and at much lower levels in comparison to its membrane localization (Anderson et al., 2002). This is explained by the functional specialization of planarian β -catenin paralogs, where β -catenin-1 is dedicated to nuclear signaling and β -catenin-2 to cell-cell adhesion (Chai et al., 2010). Moreover, the significant levels of β -CATENIN-1 found in the nucleus are striking, and indicate the degree of Wnt signaling activation required in this unique species in the adult stage. Since nuclear localization of β -catenin is considered to be a hallmark of Wnt activation, the fact that planarians have evolved separate nuclear- and membrane-localized β -catenin proteins facilitate the analysis of specific context-dependent functions for nuclear β -catenin. In addition, the high expression levels of nuclear β -catenin in planarians improve visualization of the protein at different levels. Thus, analysis of nuclear β -catenin function in planarians may shed light on potential roles that were previously overlooked in other systems.

A gradient of β -CATENIN-1 along the AP axis

The hypothesis that a dynamic morphogenetic gradient of axial potential underlies the re-establishment of the AP polarity during planarian regeneration is rooted in classic cutting experiments performed more than a century ago (Child, 1911, 1941; Lewis et al., 1977; Meinhardt, 1978; Morgan, 1904, 1905). In recent years, functional characterization of the key elements of the canonical Wnt pathway suggested that the morphogenetic role of the *wnt* ligands secreted by a posterior organizing center could generate a gradient of nuclear β -CATENIN-1 responsible for the specification and maintenance of the planarian AP axis (Adell et al., 2009, 2010; Almuedo-Castillo et al., 2012; Lander and Petersen, 2016; Petersen

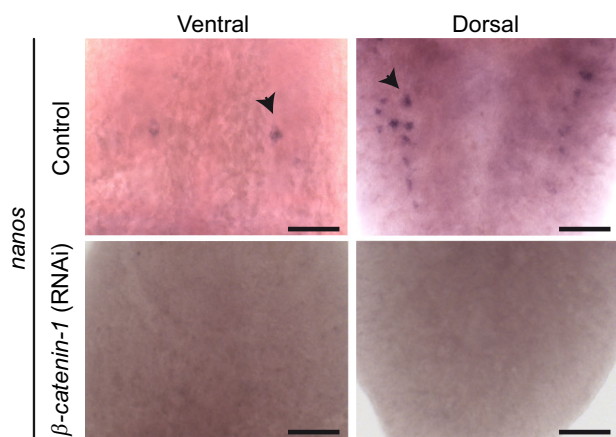


Fig. 5. β -CATENIN-1 is required for germline specification. Analysis of expression of *nanos* by whole-mount *in situ* hybridization in control and β -catenin-1 (RNAi) 4-week-old hatchling planarians ($n=16$). β -catenin-1(RNAi) (12/16) animals lack expression of *nanos* in ovaries and testes compared with controls (16/16). Black arrows indicate testes and ovaries. Anterior, up. Scale bars: 200 μ m.

and Reddien, 2009b; Scimone et al., 2016; Sureda-Gómez et al., 2015). The nested expression of several Wnt ligands (*wnt1*, *wnt11-1*, *wnt11-2* and *wnt11-5*) in the pharyngeal and tail region of the planarian body, and the expression of Wnt inhibitors (*notum* and *sFRP*) in the anterior tip of the head gave support to the gradient hypothesis (Adell et al., 2010; Gurley et al., 2010; Lander and Petersen, 2016; Petersen and Reddien, 2011; Scimone et al., 2016; Sureda-Gómez et al., 2015). Additionally, inhibition of these posterior Wnt ligands leads to ‘tail-less’ or ‘two-headed’ planarians, as does the inhibition of β -catenin-1 itself (Adell et al., 2009; Gurley et al., 2010; Petersen and Reddien, 2009b; Sureda-Gómez et al., 2015). However, silencing of *notum* and *APC* results in the opposite phenotype, characterized by the regeneration of ‘two-tailed’ planarians (Gurley et al., 2008; Iglesias et al., 2011; Petersen and Reddien, 2011). The fact that the expansion of anterior fates after β -catenin-1(RNAi) depends on the time and dose of inhibition (Adell et al., 2010; Iglesias et al., 2008) and that the cutting position along the AP axis influences the severity of the phenotype after *APC* (RNAi) (Iglesias et al., 2011) are also in agreement with a gradient hypothesis. Although β -CATENIN-1 is widely present in specific organs of adult planarians, our findings robustly demonstrate the existence of a nuclear gradient of β -CATENIN-1 (Fig. 1; Fig. S4; Fig. 6A). The highest levels of nuclear β -CATENIN-1 occur in posterior territories, but strikingly, the lowest levels are not found in the anterior pole, as proposed in the gradient hypothesis, but rather in the pre-pharyngeal region. We found differentiated (e.g. muscle) and proliferating cell types endowing nuclear β -CATENIN-1, recognizing that the specific cell types that contribute to this gradient deserve further studies. Although the observation of the nuclear β -CATENIN-1 gradient confirms the main assumption of the gradient hypothesis, it also suggests that the patterning of the head region involves more complex epistatic interactions between different signaling molecules (see below). In this context, the role of the anterior Wnt inhibitors (*notum* and *sFRPs*) might not merely result in complete inhibition of β -CATENIN-1, but rather in its fine regulation.

During bi-polar regeneration, β -catenin-1 is expressed in both blastemas (Iglesias et al., 2008). The secreted elements *wnt1* and *notum* are first expressed in all wounds, and later become restricted to the posterior and anterior blastemas, respectively (Petersen and Reddien, 2009b, 2011; Wenemoser et al., 2012). Our results demonstrate that there is abundant nuclear β -CATENIN-1 in both anterior and posterior blastemas at any stage (Fig. 2A). However, 1 day after amputation, when the axial identities of the blastemas are established, nuclear localization of β -CATENIN-1 depends on *wnt1* activity only in posterior blastemas (Fig. 2B,C; Fig. 6B). This observation supports the hypothesis that the *wnt1* ligand, secreted by the newly formed posterior organizer, is the morphogen that sustains nuclear translocation of β -CATENIN-1 at the posterior pole during regeneration (Adell et al., 2009; Petersen and Reddien, 2009b). Later on, the nested expression of the other posterior Wnt ligands would control the re-establishment and maintenance of the gradient (Hayashi et al., 2011; Lander and Petersen, 2016; März et al., 2013; Scimone et al., 2016; Sureda-Gómez et al., 2015) (Fig. 6A). Therefore, the signaling network that specifies the AP axial identity behaves differently according to the regenerating context. However, our findings indicate that β -CATENIN-1 could be a component of both the anterior and the posterior organizers, and that it is the fine-tuning of β -CATENIN-1 that is essential for the organizing function in each pole. This is further supported by the observation that β -catenin-1 inhibition restores the ability to regenerate a head in anterior wounds in planarian species that cannot

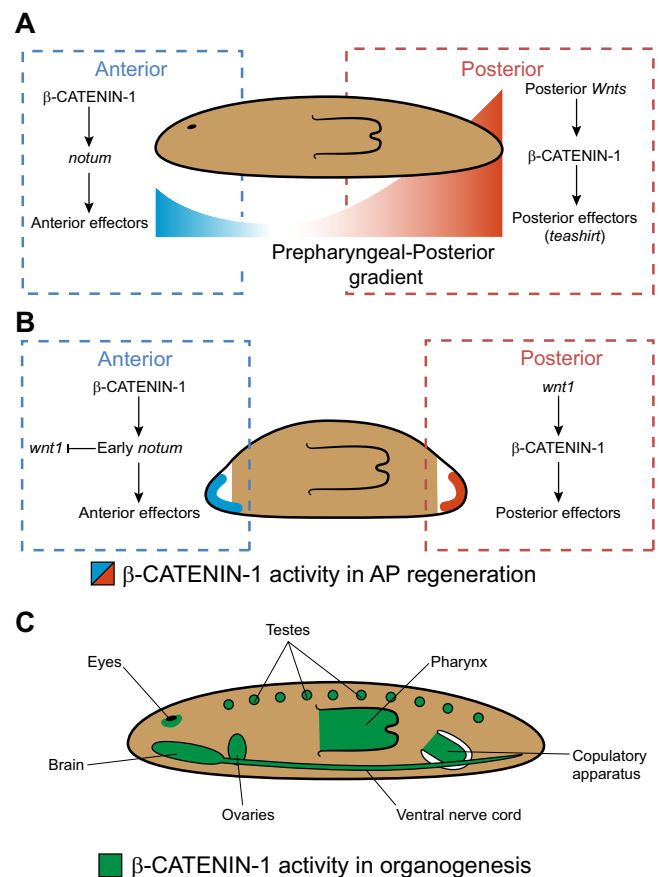


Fig. 6. Summary of β -CATENIN-1 function in planarians. (A) β -CATENIN-1 displays a gradient from the prepharyngeal region to the tail. Nuclear localization of β -CATENIN-1, which is responsible for activation of effectors of posterior identity, like *teashirt* (Reuter et al., 2015), would depend on the action of the posterior Wnts (WNT1, WNT11-1, WNT11-2, WNT11-5). In the anterior region, β -CATENIN-1 is required for expression of *notum* among other effectors of anterior morphogenesis. (B) During bi-polar regeneration, β -CATENIN-1 is localized in both anterior and posterior blastemas. Posterior β -CATENIN-1, which is responsible for conferring posterior identity, would be specifically activated by *wnt1*. Anterior β -CATENIN-1 is also required for *notum* expression during regeneration. Early *notum* could be the signal which inhibits the action of WNT1 in anterior-facing wounds. It remains unknown which signal triggers anterior β -CATENIN-1 activation during regeneration and homeostasis. (C) β -CATENIN-1 is localized in the nucleus of several planarian organs. It controls embryonic and adult organogenesis and is specifically required for brain patterning and for normal development of the spermatogonia and oogonia.

regenerate anteriorly (Liu et al., 2013; Sikes and Newmark, 2013; Umesono et al., 2013). Similarly, β -CATENIN-1 stabilization also restores the ability to regenerate anterior wounds in these animals, even though this experiment causes the expected polarity reversal and the planarians develop a two-tailed phenotype (Liu et al., 2013; Sikes and Newmark, 2013; Umesono et al., 2013). Nevertheless, further work will be necessary to determine which specific upstream regulators generate this context-specific behavior and enable the differential roles of nuclear β -CATENIN-1 in anterior and posterior blastemas.

β -CATENIN-1 controls anterior patterning

In addition to its role in posterior development, the canonical Wnt pathway appears to be implicated in the regeneration of the planarian head and brain (Fraguas et al., 2014; Hill and Petersen,

2015; Iglesias et al., 2011; Owen et al., 2015). In this respect, β -CATENIN-1 localizes abundantly to the head region, both in the cephalic ganglia and in the mesenchymal cells (Figs 1, 3 and 4). A closer examination revealed that these mesenchymal cells with active β -CATENIN-1 include neural and non-neural cell types. The latter are mostly located in the dorsal-anterior tip of the animal, where *notum* is expressed (Petersen and Reddien, 2011). As in intact animals, β -CATENIN-1 is active both ventrally, where the brain primordium forms, and dorsally, in anterior regenerating blastemas. In accordance with this complex pattern of activity, our findings show that *β -catenin-1* is required to regenerate a normal sized brain, with the correct number of neural cell populations, as well as a normal head sensory margin (Hill and Petersen, 2015; Owen et al., 2015). Importantly, the midline ventral furrow and *notum* expression in the anterior midline also disappears in *β -catenin-1 (RNAi)* animals, which suggest a problem in anterior tip patterning and the establishment of the anterior organizing region. Considering that not only the tip-related expression of *notum* but also the brain-related expression completely disappears after *β -catenin-1 RNAi*, *notum* may be directly regulated by *β -catenin-1*, generating a regulatory loop to control *β -catenin-1* levels in the anterior region of planarians (Hill and Petersen, 2015; Petersen and Reddien, 2011; Scimone et al., 2014). Our findings thus support three major roles of *β -catenin-1* in anterior regeneration: (1) the specification and maintenance of the anterior signaling center, defined by the tip-related expression of *notum* (Petersen and Reddien, 2011) and other genes (Chen et al., 2013; Gaviño et al., 2013; Roberts-Galbraith and Newmark, 2013; Scimone et al., 2014; Vásquez-Doorman and Petersen, 2014; Vogg et al., 2014) (Fig. 6A,B); (2) the regeneration of the brain, through the regulation of the brain-related expression of *notum* (Hill and Petersen, 2015); and (3) the specification of different neuronal cell types, as described in most animal models (Ciani and Salinas, 2005; Hari et al., 2002; Lewis et al., 2004; Machon et al., 2003; Watanabe et al., 2014; Yu and Malenka, 2003; Zechner et al., 2003).

Further experiments will be required in order to determine the exact nature of the signaling network that enables the specific roles of β -CATENIN-1 in anterior patterning and the mechanisms that activate the different pools of β -CATENIN-1 in the head region. Of the two Wnt genes expressed in the head (*wnt2* and *wnt11-6*), a function has only been reported for *wnt11-6* (Adell et al., 2009; Kobayashi et al., 2007). Hill and Petersen (2015) demonstrate that *wnt11-6* regulates the brain-related expression of *notum* through β -CATENIN-1 and that a *wnt11-6/notum* system would control the size of the brain. However, *wnt11-6* does not control the expression of *notum* in the anterior pole. Consequently, the mechanism through which β -CATENIN-1 is activated in this specific area remains unclear. Since no Wnt has been found to be expressed in this region, it is possible that β -CATENIN-1 is activated in a Wnt-independent manner, for instance, through WNK (with no Lysine) kinases (Serysheva et al., 2013) or via growth factors such as platelet-derived growth factor (PDGF) or basic fibroblast growth factor (bFGF) (Couffignal et al., 2006).

β -CATENIN-1 controls embryonic and adult organogenesis

Besides its role in axial patterning, the involvement of β -catenin in organogenesis has been broadly reported in many developmental systems, such as mouse, zebrafish and *Drosophila* (Clevers, 2006; Huelsken and Birchmeier, 2001; Lewis et al., 2004; Orsulic and Peifer, 1996; Tan et al., 2006). Our knowledge of this additional role of the canonical Wnt pathway during planarian regeneration is still poor, primarily because of the strong axial phenotypes obtained

after *β -catenin-1 (RNAi)* treatment that can mask other more subtle defects. Our findings demonstrate that there is nuclear β -CATENIN-1 in the brain, ventral nerve cords, pharynx, eyes, copulatory apparatus, and the male and female gonads (summarized in Fig. 6C). Moreover, we observed strong nuclear signal during the regeneration of the organ primordia and during embryogenesis. Although the role of the canonical Wnt pathway in all these organs (e.g. in the pharynx and the eyes) is still unclear, *β -catenin-1* appears to be required for brain patterning (see above) (Hill and Petersen, 2015), as well as for the proper specification and development of the spermatogonia and oogonia (Fig. 5). This latter observation contrasts with the fact that *β -catenin-1 (RNAi)* treatment does not produce any apparent neoblast-related phenotype. The germline and the neoblasts (adult stem cells) are the only two cell types with the ability to self-maintain in adult planarians. Therefore, our results uncover different requirements for *β -catenin-1* in the two dividing cell populations of planarians, indicating that β -catenin does not confer a universal stemness property to cells (Clevers and Nusse, 2012). In our model, the canonical Wnt pathway might be conferring particular cell identities, rather than maintaining stemness.

Altogether, our findings demonstrate that the canonical Wnt pathway, and in particular β -CATENIN-1, is a multifaceted signaling network during planarian regeneration and homeostasis. It defines the posterior identity through an activity gradient, participates in the morphogenesis of anterior structures by controlling anterior gene expression and promotes normal organogenesis, for instance, of the brain and reproductive organs. The analysis and visualization of β -CATENIN-1 protein distribution fills a key practical and theoretical gap in the study of planarian biology and more generally in the understanding of the multiple context-dependent roles of β -catenin. Our study thus delivers an important tool that will improve future investigations on the molecular basis of planarian regeneration and homeostasis, as well as on the multiple roles of β -catenin-dependent Wnt signaling.

MATERIALS AND METHODS

Planarian culture

An asexual clonal strain population of *S. mediterranea* BCN-10 biotype was maintained as previously described (Fernández-Taboada et al., 2010). A sexual population of *S. polychroa* from Sant Celoni (Barcelona, Spain) was maintained in the lab as described elsewhere (Martín-Durán et al., 2008). Animals were starved for 1 week before conducting any experiment. Egg capsules were collected and fixed as described previously (Cardona et al., 2005a).

Generation of the anti- β CAT-1 polyclonal antibody

The cDNA region of the first 201 N-terminal amino acids of *Smed*- β -CATENIN-1 was cloned into the pET-His tagged vector (Novagen) and 200 μ g of the recombinant protein was used as immunogen to produce polyclonal IgGs in three rabbits (Innoprot, Spain). Three more immunizations were performed using 100 μ g of the recombinant protein. The post-immunization serum was precipitated in ammonium sulfate and stored at 4°C. Before use in immunohistochemistry, IgGs were purified using a Protein A Antibody Purification Kit (Sigma-Aldrich).

Western blot assays

Intact planarians and regenerating blastemas were dissociated in lysis buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS). Protein extracts were denatured for 15 min at 95°C in lysis buffer, run on 8% SDS-PAGE gels and transferred to western blot membranes. After incubation with anti- β CAT-1 (1:30,000) and anti- α -tubulin (Sigma, T9026; 1:10,000) antibodies, signal was developed using the Clarity Western ECL Substrate (Bio-Rad) or the Amersham ECL Prime Western Blotting Detection Reagent and chemiluminescence was detected using a C-DiGit Chemiluminescent

Western Blot Scanner (LI-COR) or LAS 4000 (Fujifilm). Quantification was performed with Image Studio Lite and Multi Gauge software and normalized to anti- α -tubulin signal.

Immunohistochemistry

Whole-mount samples

Embryos were dissected as previously described (Cardona et al., 2005a). After blocking in 5% fat-free powdered milk in 0.1% Triton X-100 PBS (PBSTx) for 2 h at room temperature (RT), samples were incubated in anti- β CAT-1 (rabbit, diluted 1:200) and/or anti-FMRamide (DiaSorin, 2009; 1:500) for at least 16 h. After thorough PBSTx washes, samples were incubated with an anti-rabbit HRP-conjugated secondary antibody (Pierce, 1:500) and an anti-rabbit Alexa Fluor 568-conjugated secondary antibody (Molecular Probes, 1:1000). HRP signal was developed with a tyramide signal amplification kit following the manufacturer's recommendations (PerkinElmer). Nuclei were counterstained with DAPI (Sigma, 1:5000).

Paraffin sections

Animals were killed and fixed as for whole-mount immunohistochemistry. Paraffin embedding and sectioning were conducted as previously described (Cardona et al., 2005b). Slides were de-waxed and re-hydrated through a graded xylene-ethanol series and washed for 5 min in PBS. Antigen retrieval was performed using 0.4% pepsin in 0.1 M HCl, pH 5, for 20 min at 37°C in a humidified chamber. Sections were blocked in 5% fat-free powdered milk in PBS for 1 h at RT and then incubated with the primary antibodies diluted in blocking solution [anti- β CAT-1 antibody, 1:200; anti- α -tubulin AA4.3, Developmental Studies Hybridoma Bank, mouse, 1:20; anti-MHC-A 6G10, DSHB, mouse, 1:20; rat monoclonal anti-phosphohistone H3 (clone HTA28), Active Motif, 1:250] for 16 h at 4°C in a humidified chamber. Subsequently, sections were washed in PBS and incubated with secondary antibodies (anti-rabbit HRP-conjugated antibody, Pierce, 1:500; anti-mouse and anti-rat Alexa Fluor 568-conjugated antibody, both from Molecular Probes and both used at 1:1000) in blocking solution for 14 h at 4°C in a humidified chamber. HRP signal was developed with a tyramide signal amplification kit following the manufacturer's recommendations (PerkinElmer). Nuclei were counterstained with DAPI (Sigma, 1:5000) and slides were mounted and stored in 70% glycerol.

Gene expression analysis

For colorimetric whole-mount *in situ* hybridization (WISH) analyses, animals were processed using an *In situ* Pro hybridization robot (Abimed/Intavis), as previously described (Molina et al., 2007; Umesono et al., 1997). For fluorescent whole-mount *in situ* hybridization (FISH), planarians were fixed and stained as previously described (Pearson et al., 2009). Riboprobes were detected with enzyme-conjugated antibodies diluted in MABT supplemented with 10% horse serum (anti-DIG-POD and anti-FITC-POD, both 1:100, Roche; or anti-DIG-AP, 1:2000, Roche). For double FISH, peroxidase activity was quenched using 1% hydrogen peroxide for 45 min at RT. Nuclear staining was performed with DAPI (Sigma, 1:5000).

RNAi experiments

Double-stranded RNA (dsRNA) was synthesized by *in vitro* transcription (Roche), diluted to 1 μ g/ μ l in milliQ water, and then microinjected into planarians using a Femtojet microinjector (Drummond). For regeneration experiments, dsRNA was injected three times over three consecutive days. For homeostasis experiments, animals were injected three times over three consecutive days each week of treatment. Negative control animals were injected with dsRNA of the green fluorescent protein (GFP) gene.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from a pool of three intact control and RNAi planarians, and cDNA was synthesized as previously described (Almuedo-Castillo et al., 2014). Data was normalized to the housekeeping genes *EF2* and *UDP*, obtaining similar results. The primers for qPCR were:

Spo1- β -catenin-1 mRNA, 5'-GGATGATTCCCAAAATGGT-3', 5'-CG GTTGATGATTGAAAGCTA-3'; *Spo1-EF2* mRNA, 5'-GCGAGCCAGA-AGATTTGTAT-3', 5'-TGAATATCTCCCATAGGTCCA-3'; *Spo1-UDP*

mRNA, 5'-GTTCTTTTCCGCAAGTTTA-3', 5'-ATTTGAACAAGCAT-GTGGTC-3'

Image analysis

Imaging

WISH samples were observed under a Leica MZ16F stereomicroscope (Leica Microsystems, Mannheim, BW, Germany) and images were captured with a ProgRes C3 camera from Jenoptik (Jena, TH, Germany). An Axiophot microscope (Zeiss, Jena, TH, Germany) was used to capture higher-magnification images of the WISH of *nanos* mRNA with a Leica DFC300 FX camera (Leica Microsystems, Heerbrugg, CH, Switzerland). FISH images were obtained under a Leica TCS-SP2 confocal laser-scanning microscope (Leica Lasertchnik, Heidelberg, BW, Germany) adapted for an inverted microscope. Immunostaining images were obtained under a Leica TCS-SP5 confocal laser-scanning microscope (Leica Lasertchnik, Heidelberg, BW, Germany).

Cell counting and organ measurements

Populations of *th-1*⁺, *tbh*⁺ and *cintillo*⁺ cells were counted manually using Fiji software (Schindelin et al., 2012). The relative brain area was measured from the most posterior to the most anterior region of the brain ganglion as visualized by DAPI nuclear staining. The distance between the eyes and the pigmented region was measured in images of live animals, and the distance between the anterior tip of the brain ganglion to the tip of the head in DAPI counterstained images. Results from each animal were averaged and significant differences determined by two-tailed Student *t*-tests.

Signal quantification of β -CATENIN-1 levels

Images were processed using Fiji software. Three to four planes were used to build the *z*-projection. Nuclear-stained (DAPI) images were transformed into a mask using the threshold method Yen. The mask was used to obtain the nuclear signal of anti- β CAT-1 antibody images with the Image calculator process. The nuclear signal obtained from the resulting image was measured to obtain the raw integrated density (RID). The nuclear area was obtained from the mask. Then, we normalized the RID with the respective nuclear area. Results from each animal were averaged and significant differences determined by two-tailed Student's *t*-tests, in the cases of Fig. 2C, Fig. S2C, Fig. S5C and Fig. S6B. We followed the same procedure to build the *z*-projection to study β -CATENIN-1 levels along the AP axis. The whole sagittal section and nuclear masks were reconstructed from partial *z*-projections using the Stitching 3D plug-in. We then divided the masked images into eight areas, avoiding the signal from the brain and pharynx. To reconstruct the β -CATENIN-1 levels of the whole sagittal section, the lowest normalized RID among the eight subareas was used to normalize the rest. This process was made for different sagittal images corresponding to different positions of the central region of four planarians. All the values were represented as a polynomial regression and statistically analyzed using the Wilcoxon signed-rank test (Table S1). The detailed analysis of β -CATENIN-1 levels from the pre-pharyngeal to tail region was performed subdividing regions 3–8 of the same images into seven regions and we followed the same strategy to obtain the graph of normalized β -CATENIN-1 expression as described above.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.S.-G., J.M.M.-D., T.A.; Formal analysis and investigation: M.S.-G., J.M.M.-D.; Writing - original draft preparation and review and editing: M.S.-G., J.M.M.-D., T.A.; Funding acquisition: T.A.; Supervision: T.A.

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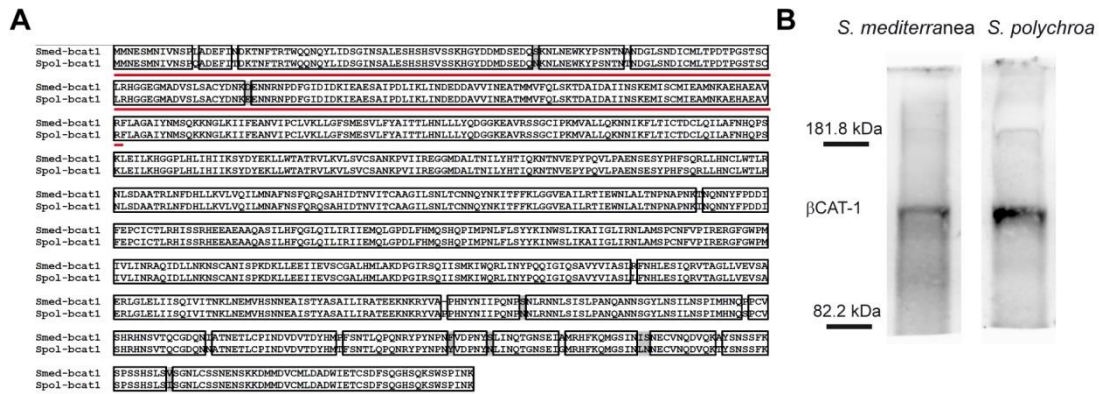
Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.135152.supplemental>

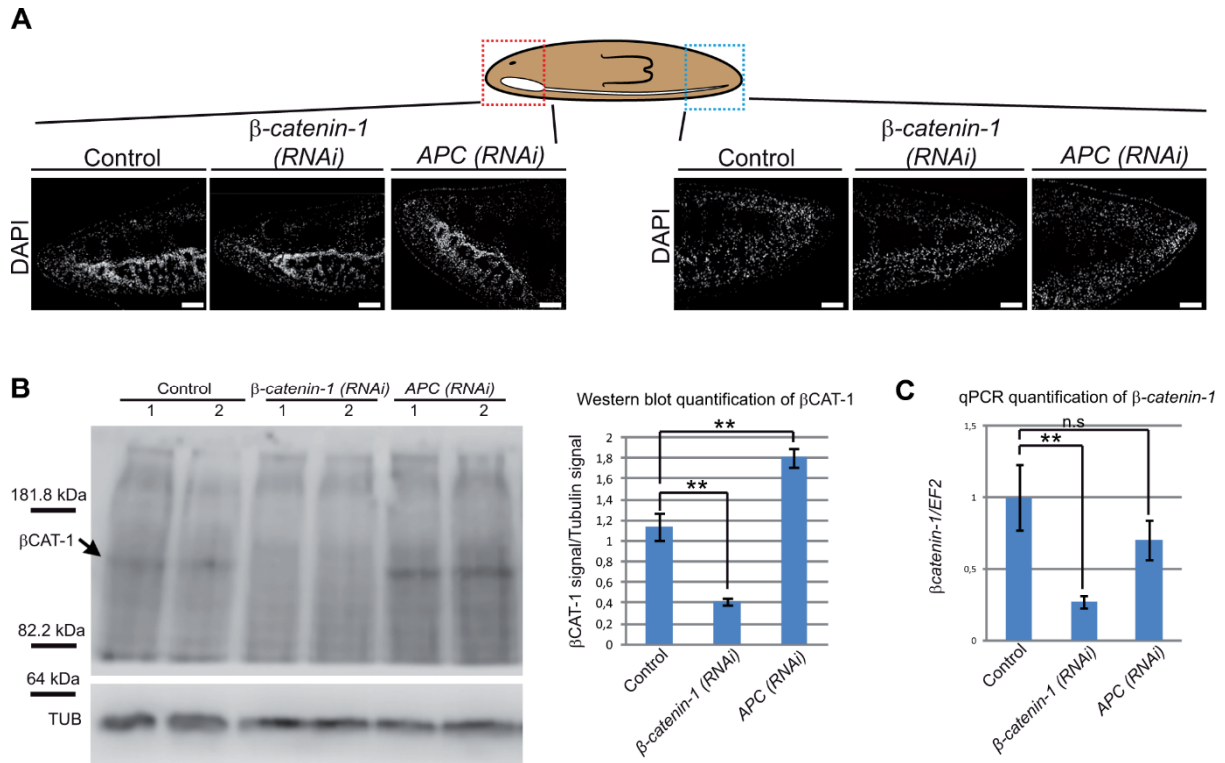
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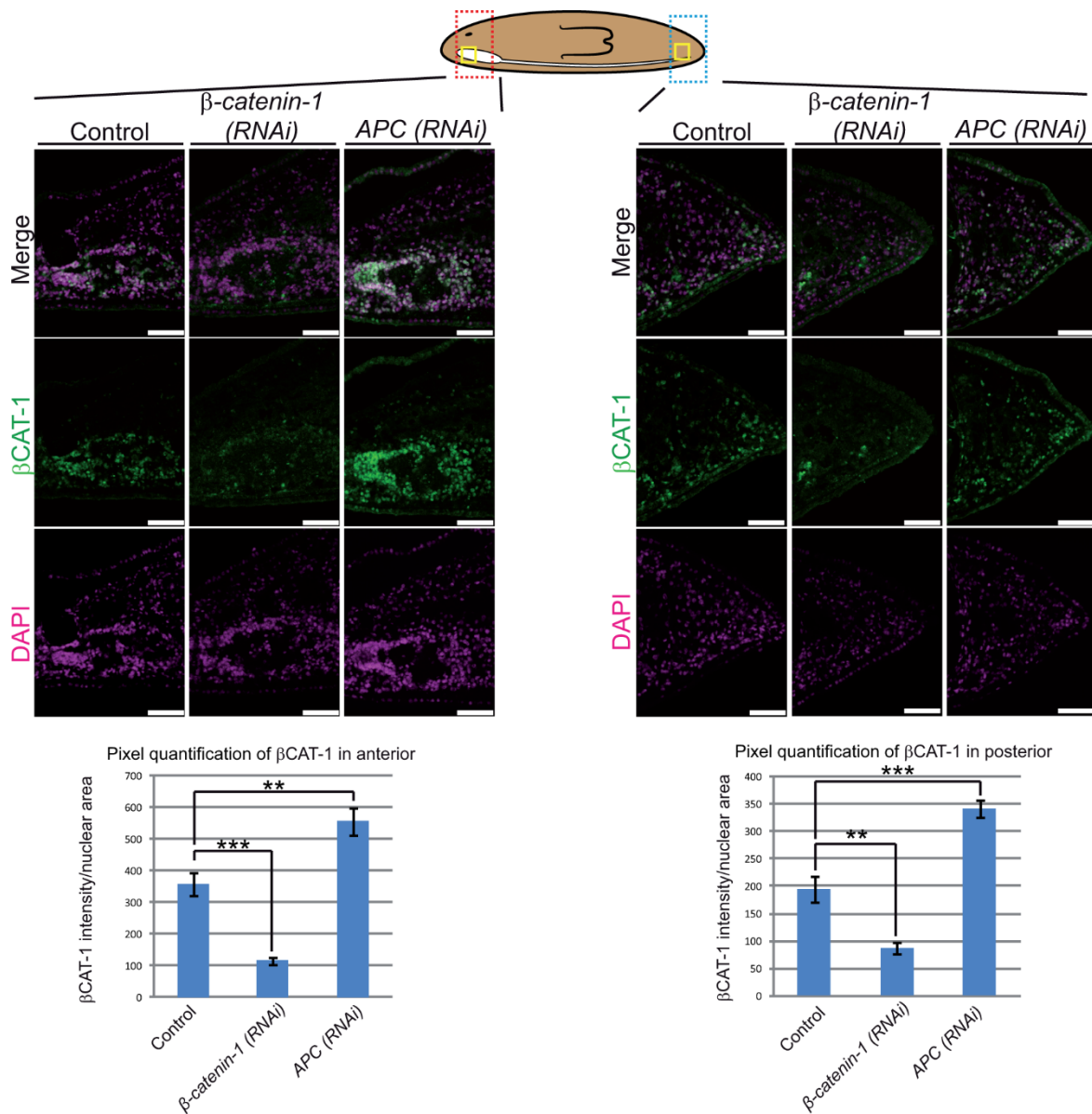


Supplementary figure 1. Immunoreactivity of the anti- β CAT-1 antibody. (A) *S. mediterranea* and *S. polychroa* β -CATENIN-1 protein sequence alignment. Boxed sequences indicate 100% similarity. Red line indicates the region used to produce the polyclonal anti- β CAT-1 antibody. (B) Western blot of protein extracts from uninjured planarians corresponding to *S. mediterranea* and *S. polychroa*, immunoblotted with the anti- β CAT-1 antibody (n=3). A band of the expected size (107 kDa) was detected in extracts of both species.



Supplementary figure 2. Specificity of the anti- β CAT-1 antibody by Western Blot

(A) DAPI immunostaining (white) of sagittal sections corresponding to the anterior and posterior region of β -catenin-1 (RNAi), APC (RNAi) and control uninjured planarians after 5 weeks of RNAi treatment. (B) Western blot of control, β -catenin-1 (RNAi) and APC (RNAi) planarian protein extracts immunoblotted with the anti- β CAT-1 antibody. Extracts correspond to uninjured planarians after 5 weeks of RNAi treatment. Bar chart shows β CATENIN-1 signal quantification relative to control sample 1 and normalized to α -Tubulin levels. Two replicates per condition (n=3). (C) Real-time PCR quantification of β -catenin-1 mRNA levels in samples analyzed in C, relative to control sample and normalized by *Elongation Factor 2* (EF2). Dorsal, top; Anterior, left. Scale bar – 50 μ m.



Supplementary figure 3. Specificity of the anti- β CAT-1 antibody by

immunostaining. Anti- β CAT-1 immunostaining (green) of sagittal sections

corresponding to the anterior and posterior region of β -catenin-1 (RNAi), APC (RNAi)

and control uninjured planarians. Nuclei are stained with DAPI (magenta). β -catenin-1

(RNAi) animals show a decrease of β -CATENIN-1 signal compared with controls

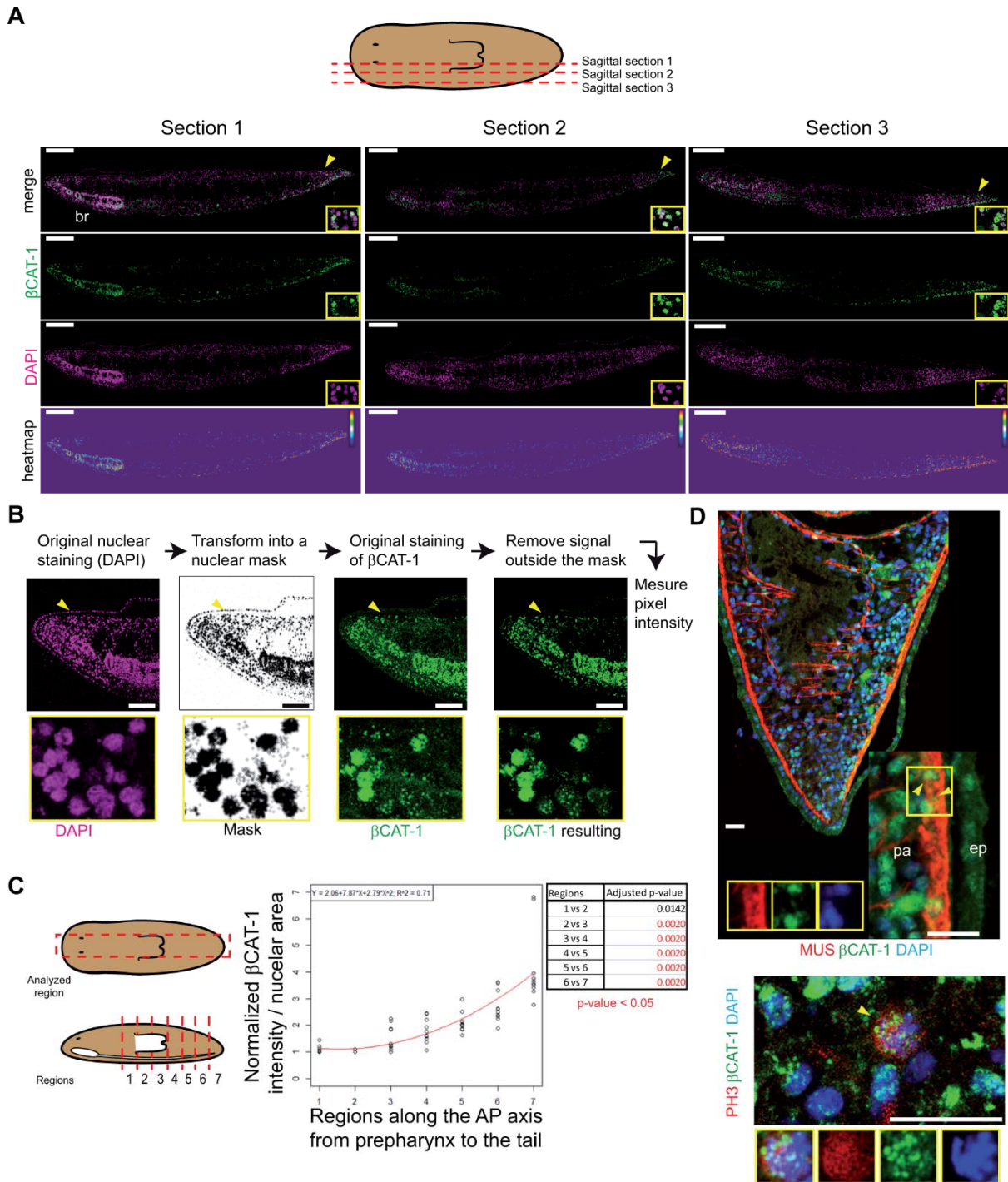
whereas APC (RNAi) animals show an increase. Dotted square in the picture indicates

the area shown in the images. Bar charts show the quantification of β CATENIN-1

signal (yellow squares in the picture indicate the area used to quantify the signal) (n=6).

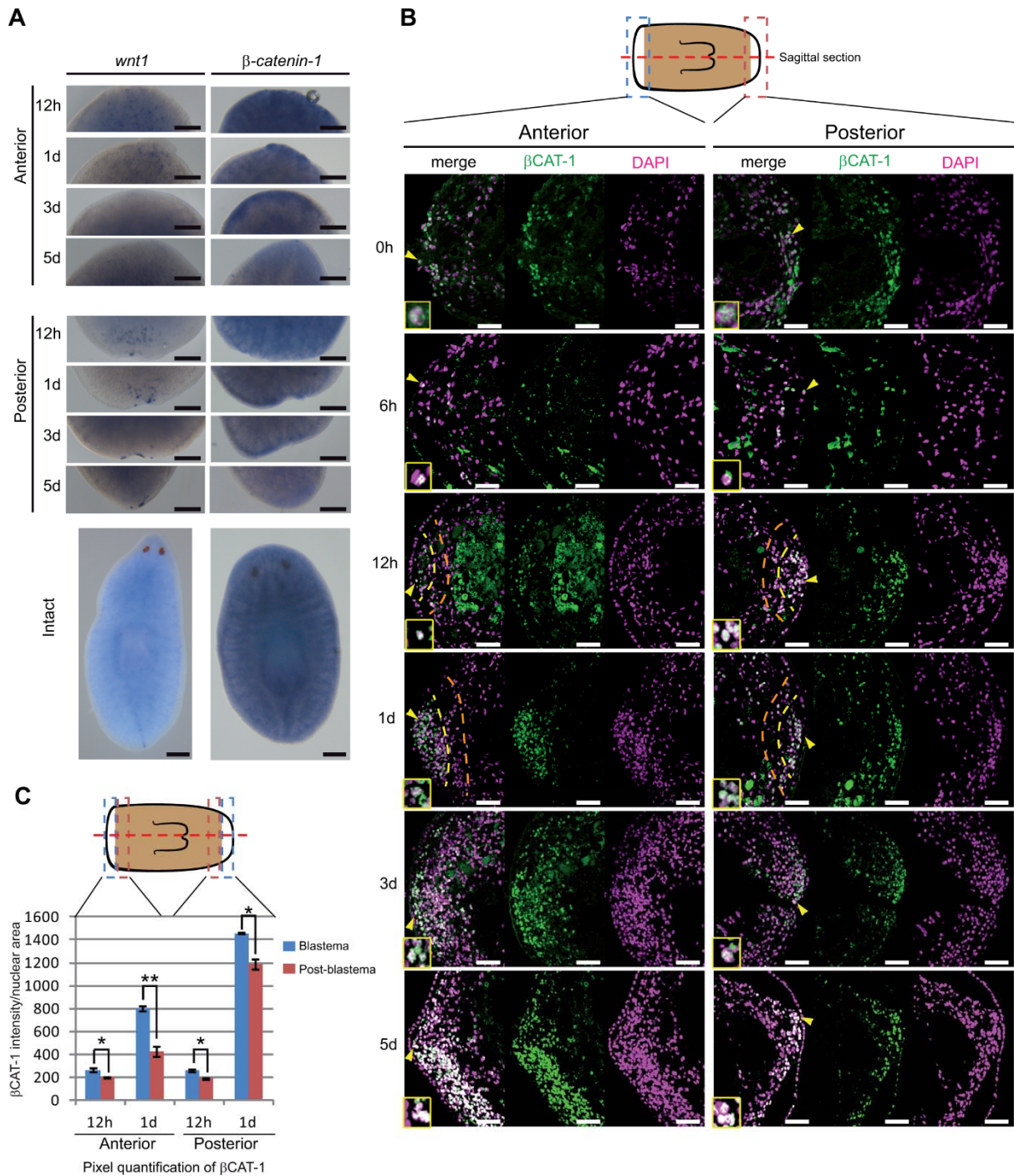
Error bars indicate standard deviations, **p<0.005, ***p<0.0005. Dorsal, top; Anterior,

left. Scale bar – 50 μm .



Supplementary figure 4. Immunostaining of sagittal sections of uninjured planarians with the anti- β CAT-1 antibody and treatment of confocal images to quantify nuclear β -CATENIN-1 signal. (A) anti- β CAT-1 immunostaining (green) of sagittal sections corresponding to the lateral region of uninjured planarians. Nuclei are stained with DAPI (magenta). A rainbow heatmap of anti- β CAT-1 signal illustrates the intensity of the signal (red, maximum). Anterior, left. Arrowheads indicate areas shown

at higher magnification (B) Scheme of the procedure applied to quantify the nuclear signal obtained with the anti- β CAT-1 antibody. Arrowheads indicate areas shown at higher magnification. (C) Quantification of parenchymal nuclear β -CATENIN-1 intensity on sagittal sections corresponding to the region around the midline normalized with the nuclear area of each section (red lines in the picture indicate the different regions analyzed along the AP axis, from the prepharynx region to the tail), excluding the pharynx (white area in the picture) and relative to the lower value (n=10 sections corresponding to 4 different animals). Values are represented using a polynomial regression and were analyzed using the Wilcoxon-signed-rank test. Table shows the p-values obtained comparing the different regions. p-values in red are significant. (D) Immunostaining on sagittal sections of uninjured animals labeled with anti- β CAT-1 (green), anti-MHC-A 6G10 (red) and DAPI (blue) [upper image] and anti- β CAT-1 (green), anti-PH3 (red) and DAPI (blue) [lower image]. A magnification of a muscular cell showing nuclear β CATENIN-1 is showed. Arrowheads indicate areas shown at higher magnification. br, brain; pa, parenquima; ep, epidermis. Scale bar – 200 μ m (A), 50 μ m (B), 20 μ m (D).



Supplementary figure 5. β -CATENIN-1 is localized to the nucleus in both anterior and posterior blastemas during regeneration. (A) *wnt1* and *β -catenin-1* detected by whole-mount *in situ* hybridization in regenerating trunks and uninjured animals.

Animals labeled with *wnt1* are shown dorsally and animals labeled with *β -catenin-1* are shown ventrally. Anterior, top. (B) Trunk fragments were fixed at 0, 6 and 12 hpa, and

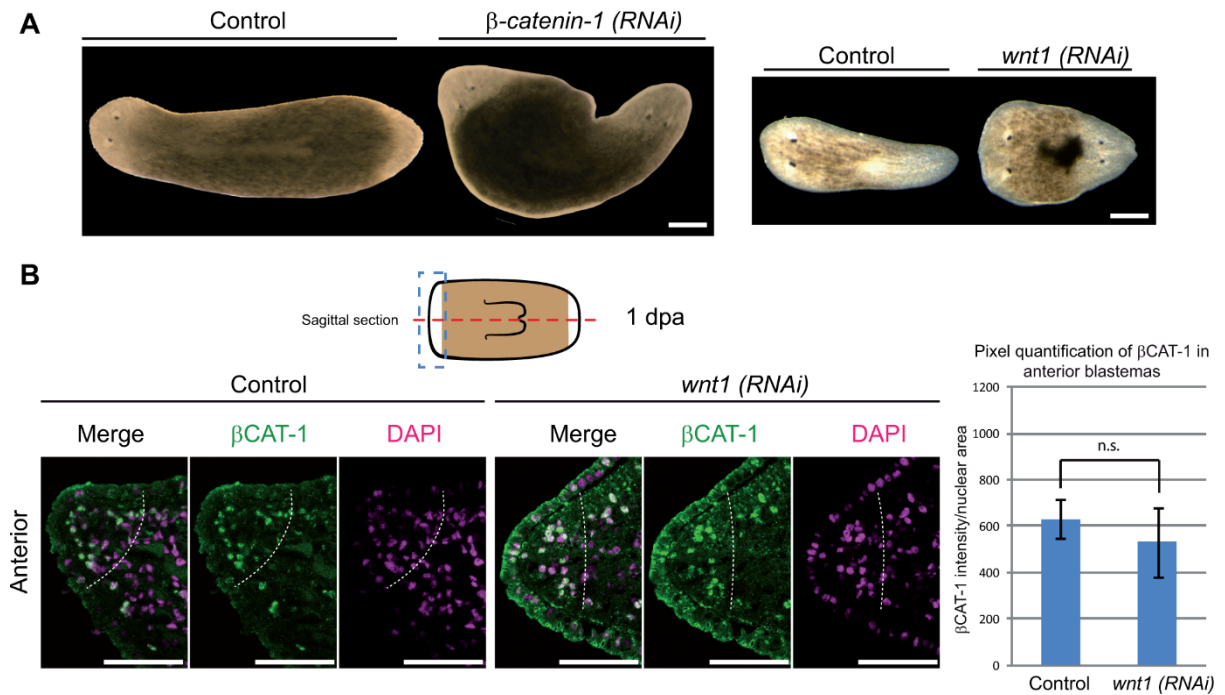
1, 3 and 5 dpa, labeled with anti- β CAT-1 antibody (green) and DAPI (magenta).

Sagittal sections corresponding to the regenerating regions are shown. Anterior, left; Dorsal, top. Arrowheads indicate the magnified regions. Yellow and orange dotted lines delimitate the blastema and adjacent pre-existent region analyzed in the bar chart on C.

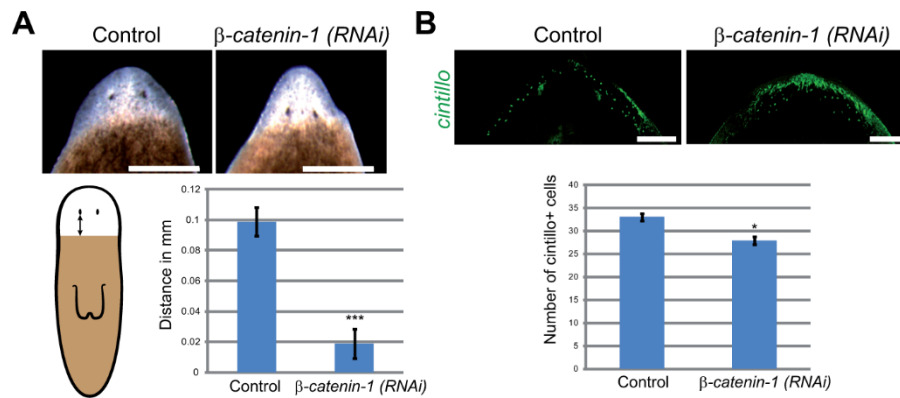
(C) Bar chart shows the quantification of β -CATENIN-1 signal in blastema and adjacent pre-existent region at 12 hours and 1 day after amputation in anterior and posterior wounds. The blastema and adjacent pre-existent region measured areas are equivalent.

Blastema region is delimited by the yellow dotted line in B and adjacent pre-existent region is delimited by the yellow and orange dotted lines. * $p < 0.05$, ** $p < 0.005$ (n=3).

Blastema region shows a preferential accumulation of β -CATENIN-1 signal compared to the adjacent pre-existent region. Scale bar – 200 μ m (A), 50 μ m (B).

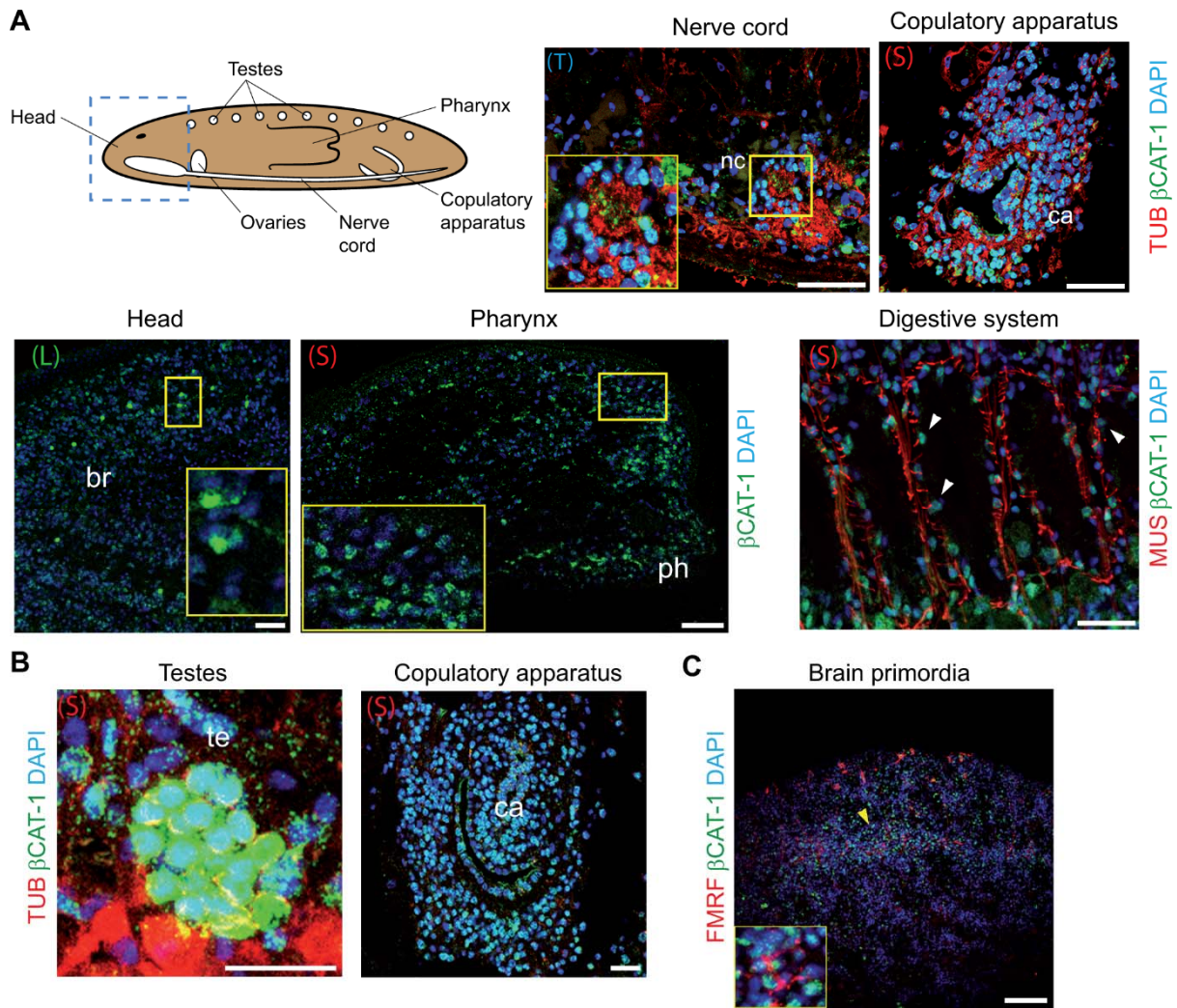


Supplementary figure 6. *wnt1 (RNAi)* phenocopies β catenin-1 (RNAi) and does not affect the β -CATENIN-1 nuclear localization in anterior blastemas during regeneration. (A) Phenotype generated after β -catenin-1 and *wnt1* RNAi silencing in *S. polychroa*. (B) Immunostaining on sagittal sections with anti- β CAT-1 antibody (green) and DAPI (magenta) of control and *wnt1 (RNAi)* posterior regenerating regions 1 day after amputation. Bar chart shows the quantification of β -CATENIN-1 signal in the blastema region (delimited by the white dotted line). n.s. - no significance. *wnt1 (RNAi)* animals do not show differences in nuclear β -CATENIN-1 signal compared with controls (n=6). Dorsal, top; Anterior, left. Scale bar – 200 μ m (A), 50 μ m (B).



Supplementary figure 7. Anterior defects in β -catenin-1 (RNAi) regenerating

planarians. (A) In vivo images of 8-day anterior regenerating control and β -catenin-1 (RNAi) trunks. Cartoon shows the measured distance (arrows) from the eye to the pre-existing pigmented region. Bar chart shows the quantification (n=7 per condition). (B) FISH to detect expression of *cintillo* (green) in 8-day anterior regenerating control and β -catenin-1 (RNAi) trunks. Bar chart shows the quantification of cintillo-positive cells (n=5 per condition). Error bars indicate standard deviations, *p<0.05, ***p<0.0005, two-tailed *t* test. Anterior, top. Scale bar – 500 μ m (A), 200 μ m (B)



Supplementary figure 8. β -CATENIN-1 localization in different organs of

uninjured and regenerating planarians (A) Immunostaining on sections of uninjured

animals labeled with anti- β CAT-1 (green), anti- α -Tubulin (red) or anti-MHC-A 6G10

(red) and DAPI (blue). Yellow squares indicate images shown at higher magnification.

Arrowhead indicates β -CATENIN-1 positive cells on the digestive system. (B)

Immunostaining on sections of 3-day regenerating animals labeled with anti- β CAT-1

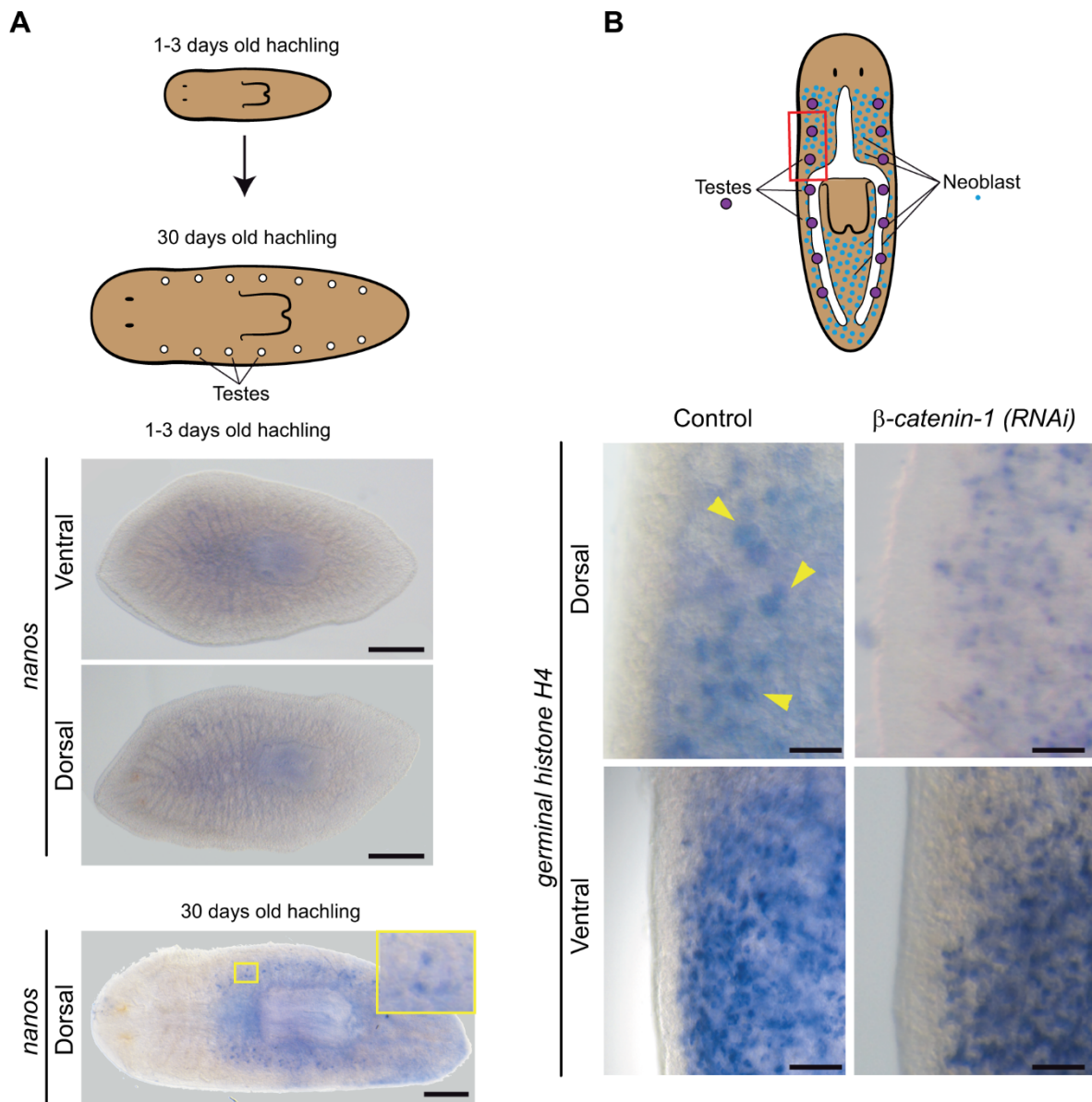
(green), anti- α -Tubulin (red) and DAPI (blue). (C) Immunostaining on whole-mount of

a 6 day-old embryo (stage 5) with anti- β CAT-1 (green) and anti-FMRFamide (red).

Nuclei are labeled with DAPI (blue). Yellow squares indicate images shown at higher

magnification. br, brain; nc, nerve cord; ph, pharynx; te, testes; ca, copulatory

apparatus. Scale bar – 50 μ m (A), 20 μ m (B), 100 μ m (C).



Supplementary figure 9. *nanos* expression in 1-3 day to 30 days old hatchlings and germinal histone H4 expression in controls and β -catenin-1 (RNAi). (A) *In situ* hybridization of *nanos* showing that 1-3 day old hatchlings do not express this marker. 30 days old hatchlings show *nanos* in the dorsal part labeling the germ cells. Yellow square shows a magnification. Anterior, left. (B) Scheme representing the *germinal histone H4* expression pattern: it is expressed in testes (dorsal clusters, in purple) and neoblast (isolated cells in the parenchyma, in blue). Red square indicates the analyzed

region. Analysis of the expression of *germinal histone H4* by whole-mount *in situ* hybridization in control and *β -catenin-1 (RNAi)* 4 week-old hatchling planarians shows that control animals present some clusters of testis labeled with *germinal histone H4* (yellow arrowhead) in the dorsal part (7/10) and neoblast labeling in the ventral part (10/10) while in *β -catenin-1 (RNAi)* animals *germinal histone H4* does not label testis clusters dorsally and only labels neoblasts in the dorsal and ventral part (10/10). Anterior, up. Scale bar – 200 μ m (A), 50 μ m (B).

Table S1. p-values corresponding to the analysis of the gradient using the Wilcoxon-signed-rank test.

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