Development 135, 1215-1221 (2008) doi:10.1242/dev.020289

Silencing of *Smed-βcatenin1* generates radial-like hypercephalized planarians

Marta Iglesias[1], Jose Luis Gomez-Skarmeta[2], Emili Saló[1] and Teresa Adell[1,*]

Little is known about the molecular mechanisms responsible for axis establishment during non-embryonic processes such as regeneration and homeostasis. To address this issue, we set out to analyze the role of the canonical Wnt pathway in planarians, flatworms renowned for their extraordinary morphological plasticity. Canonical Wnt signalling is an evolutionarily conserved mechanism to confer polarity during embryonic development, specifying the anteroposterior (AP) axis in most bilaterians and the dorsoventral (DV) axis in early vertebrate embryos. β-Catenin is a key element in this pathway, although it is a bifunctional protein that is also involved in cell-cell adhesion. Here, we report the characterization of two β-catenin homologs from *Schmidtea mediterranea* (*Smed-βcatenin1/2*). Loss of function of *Smed-βcatenin1*, but not *Smed-βcatenin2,* in both regenerating and intact planarians, generates radial-like hypercephalized planarians in which the AP axis disappears but the DV axis remains unaffected, representing a unique example of a striking body symmetry transformation. The radial-like hypercephalized phenotype demonstrates the requirement for *Smed-βcatenin1* in AP axis re-establishment and maintenance, and supports a conserved role for canonical Wnt signalling in AP axis specification, whereas the role of β-catenin in DV axis establishment would be a vertebrate innovation. When considered alongside the protein domains present in each *S. mediterranea* β-catenin and the results of functional assays in *Xenopus* embryos demonstrating nuclear accumulation and axis induction with Smed-βcatenin1, but not Smed-βcatenin2, these data suggest that *S. mediterranea* β-catenins could be functionally specialized and that only *Smed-βcatenin1* is involved in Wnt signalling.

KEY WORDS: β-catenin, Planarians, Anteroposterior axis, Regeneration

INTRODUCTION

Planarians show a striking morphological plasticity that becomes evident during regeneration and normal tissue homeostasis. They are able to regenerate a whole organism from a piece of almost any part of their body, and, furthermore, they have the ability to grow and degrow according to culture conditions (Morgan, 1898; Saló, 2006). These properties rely on the neoblasts, multipotent stem cells present in adult organisms that are able to differentiate into any planarian cell type (Saló, 2006).

The canonical Wnt signalling pathway has a common role in establishing the anteroposterior (AP) axis during development in several species, including mouse (Marikawa, 2006), chick (Nordstrom et al., 2002), zebrafish (Schier and Talbot, 2005), *Xenopus* (Kiecker and Niehrs, 2001), amphioxus (Holland, 2002), *C. elegans* (Huang et al., 2007) and *Platynereis* (Schneider and Bowerman, 2007). In early vertebrate embryos it is also required for dorsoventral (DV) polarity (De Robertis and Kuroda, 2004). In cnidarians, it specifies the oral-aboral embryonic axis, and it also has a reported role in axial patterning during regeneration (Lee et al., 2006). However, in classical models of regeneration, such as fish or amphibians, canonical Wnt signalling has only been demonstrated to be involved in the regenerative capacity (Kawakami et al., 2006; Yokoyama et al., 2007). β-Catenin is the key intracellular effector of the canonical Wnt signalling pathway, although it is a bi-functional protein that also regulates cell adhesion as a component of adherens junctions (Schneider et al., 2003).

[1]Departament de Genètica, Universitat de Barcelona, 08028 Barcelona, Spain. [2]Centro Andaluz de Biología del Desarrollo, Universidad Pablo de Olavide-CSIC, 41013 Sevilla, Spain.

*Author for correspondence (e-mail: tadellc@ub.edu)

Accepted 4 February 2008

Here, we report the characterization of two β-catenin homologs in the planarian species *Schmidtea mediterranea* (*Smed-βcatenin1* and *Smed-βcatenin2*). Silencing of *Smed-βcatenin1* in regenerating and intact planarians induces a gradual anteriorization of the animals that finally leads to a radial-like hypercephalized phenotype, demonstrating the requirement for Smed-βcatenin1 in AP axis re-establishment and maintenance. Analysis of the protein domains of the *S. mediterranea* β-catenins, and functional assays using *Xenopus* embryos, demonstrate the involvement of *Smed-βcatenin1* but not *Smed-βcatenin2* in Wnt signalling, suggesting a functional specialization of *S. mediterranea* β-catenins.

MATERIALS AND METHODS

Organisms

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

Identification and cloning of *S. mediterranea* genes

Fragments of *Smed-βcatenin1* and *Smed-βcatenin2* were identified from the *S. mediterranea* genomic database through a BLAST search. The corresponding full-length transcripts were amplified by rapid amplification of cDNA ends (RACE) using the Invitrogen GeneRacer Kit (Invitrogen). *Smed-HoxD*, *Smed-AbdBa* and *Smed-TCEN49* were identified from the *S. mediterranea* genomic database using homologs from other planarian species (Orii et al., 1999; Garcia-Fernandez et al., 1993; Nogi and Watanabe, 2001; Bueno et al., 1996). Specific primers were designed to isolate the corresponding full-length cDNA sequences.

Accession numbers

Smed-TCEN49, EU082822; *Smed-AbdBa*, EU082823; *Smed-HoxD*, EU082824; *Smed-βcatenin1*, EU082826; *Smed-βcatenin2*, EU082825.

RNAi silencing

RNAi analyses were performed by feeding planarians with bacteria expressing double-stranded RNA (dsRNA) or by dsRNA microinjection, as described by Newmark et al. (Newmark et al., 2003) and Sánchez Alvarado

and Newmark (Sánchez Alvarado and Newmark, 1999), respectively. The corresponding cDNA for *Smed-βcatenin1* and *Smed-βcatenin2* subcloned into the pPR242 vector was used for feeding. Control animals were fed bacteria containing the vector alone. When dsRNA microinjection was used, *Smed-βcatenin1* and *Smed-βcatenin2* dsRNA was synthesized by in vitro transcription (Roche). Control animals were injected with water. For regeneration experiments, treated planarians were amputated pre- and postpharyngeally, and the trunk pieces allowed to regenerate. Planarians were processed for whole-mount in situ hybridization or whole-mount immunostaining between 3 and 30 days after amputation or last injection (intact animals).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out essentially as described previously (Nogi and Levin, 2005; Umesono et al., 1999). Digoxigenin-labelled riboprobes for *Smed-βcatenin1*, *Smed-βcatenin2*, *Smed-HoxD*, *Smed-AbdBa*, *Smed-TCEN49*, *Smed-Opsin* (K. Eckelt), *H.10.2f* (Sánchez Alvarado et al., 2002), *cintillo* (Oviedo et al., 2003), *Smed-GluR* (F. Cebrià and P. Newmark), *septin* (Zayas et al., 2005) and *eye53* (Zayas et al., 2005; Molina et al., 2007) were synthesized using an in vitro transcription kit (Roche).

Whole-mount immunostaining

Immunostaining was carried out essentially as described previously (Cebrià and Newmark, 2005; Sánchez Alvarado and Newmark, 1999). The following monoclonal antibodies were used: anti-arrestin (VC-1) (Sakai et al., 2000) at a 1:15,000 dilution; anti-synapsin (anti-SYNORF1, Developmental Studies Hybridoma Bank) at 1:25; and P-Tyr-100 (Cell Signalling Technology) at 1:500. Highly cross-absorbed Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes) was used at a 1:400 dilution.

Xenopus microinjection of mRNA and in situ hybridization

The entire coding regions from *S. mediterranea* β-catenins genes were amplified by PCR and inserted into pCS2+ (Turner and Weintraub, 1994). To generate GFP-tagged constructs, a DNA fragment from the 5′ region of each cDNA, which includes unique sites within the open reading frame, was PCR-amplified. The 5′ primers contained an *Eco*RI site to clone the fragments in frame within the pCS2-GFP plasmid. The PCR fragments were cloned in pGEM-T Easy vector and sequenced prior to fusion with their corresponding cDNA. For mRNA preparation, the DNAs were linearized and transcribed with SP6 RNA polymerases as described (Harland and Weintraub, 1985), with GTP cap analog (New England Biolabs). *Xenopus* embryos were injected at the two-cell stage, into one blastomere at the prospective ventral marginal region, with 500-1000 pg of each mRNA, and fixed at the tailbud stage. Antisense RNA probes were prepared from *Otx2*, *Krox20* and *Cad3* cDNAs and labelled with digoxigenin (Roche). *Xenopus* specimens were hybridized as described (Harland, 1991). Antibody staining was performed as described (Gómez-Skarmeta et al., 2001), using the monoclonal antibody 12/101 (Developmental Studies Hybridoma Bank, developed by J. P. Brockes) and rabbit anti-GFP (Molecular Probes).

RESULTS AND DISCUSSION

Smed-βcatenin1 inhibition induces a gradual anteriorization of regenerating planarians

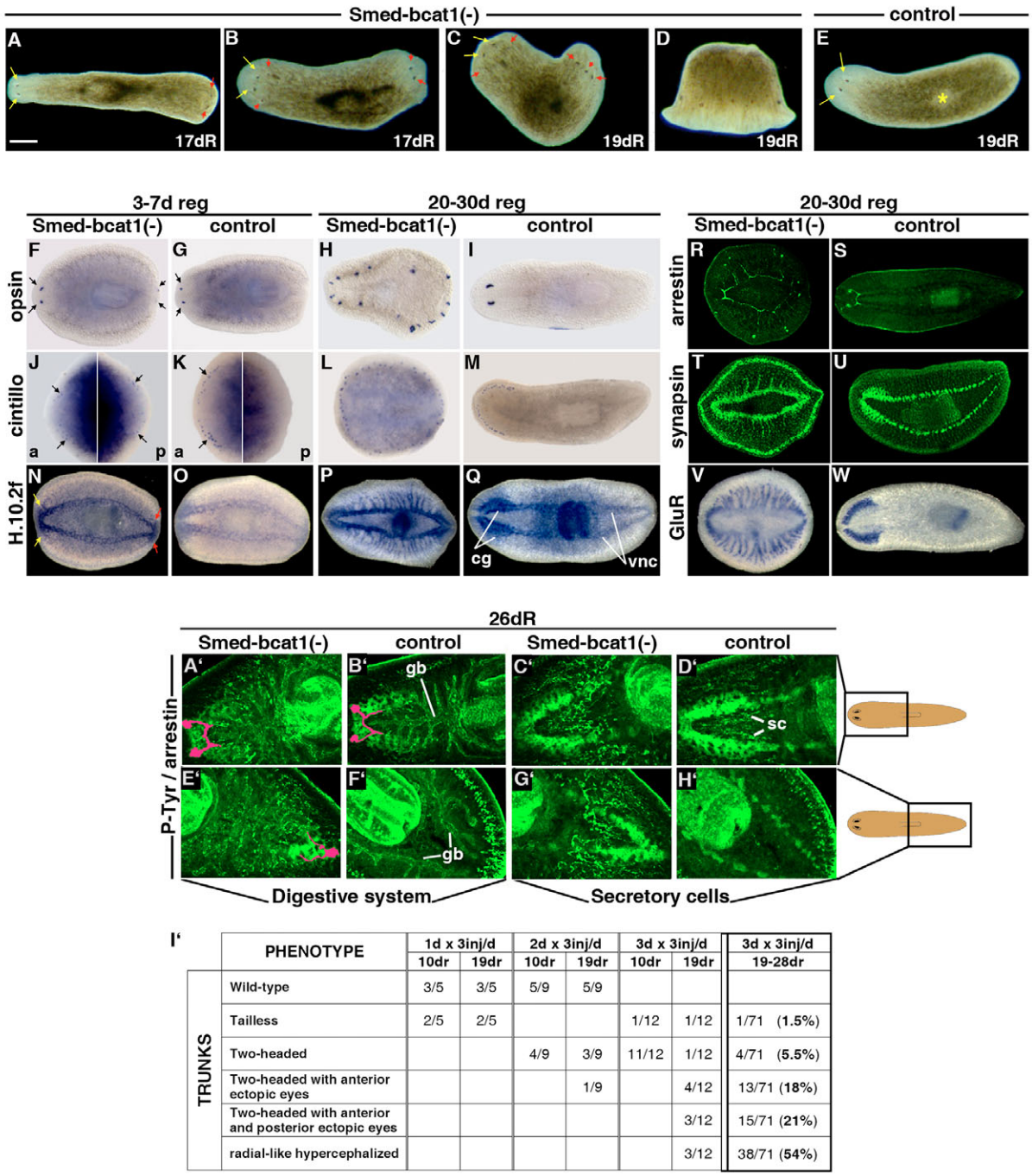
A search for β-catenin homologs in the *S. mediterranea* genome database yielded two genes, which we called *Smed-βcatenin1* and *Smed-βcatenin2*. In situ hybridization experiments revealed different expression patterns for the *S. mediterranea* β-catenins: *Smed-βcatenin1* was expressed ubiquitously but more strongly in the central nervous system (CNS), whereas *Smed-βcatenin2* mRNA was mostly located in the digestive system (see Fig. S1 in the supplementary material). During the process of regeneration, expression of both *S. mediterranea* β-catenin genes was detected in anterior and posterior blastemas (see Fig. S2 in the supplementary material). To assess their potential role in axis re-establishment during regeneration, we carried out RNAi experiments. In situ hybridization for each gene demonstrated that RNAi effectively silenced its expression (see Fig. S3 in the supplementary material). Following *Smed-βcatenin1* silencing, 100% of the regenerating trunk pieces exhibited AP polarity defects, although with varying degrees of transformation: tailless planarians; two-headed planarians (Fig. 1A); two-headed planarians with ectopic eyes next to the normal anterior eyes (Fig. 1B); two-headed planarians with ectopic eyes next to the anterior and posterior ones (Fig. 1C); and, the most severe phenotype, animals displaying apparently radial symmetry with eyes all around the periphery of their body (Fig. 1D; see also Movie 1 in the supplementary material). This last phenotype was referred to as radial-like hypercephalized planarians. The different phenotypes obtained correspond to the degree of severity of the transformation, which was dependent on the time of regeneration and also on the dose of inhibition (Fig. 1I′). After *Smed-βcatenin2* silencing, regenerating planarians did not show morphological defects or altered expression of tissue markers (data not shown).

Several markers were used to characterize *Smed-βcatenin1*-silenced animals. The planarian CNS is composed of two anterior cephalic ganglia (CG) located at one end of two ventral nerve cords (VNCs), which extend along the body and converge in the tail (Fig. 1Q) (Agata et al., 1998). From the two dorsally located eyes, visual projections extend to the CG (Okamoto et al., 2005). During the first regeneration stages (3-7 days) after *Smed-βcatenin1* silencing, in situ hybridization for *opsin* and *cintillo* revealed the ectopic differentiation of photoreceptors and mechanoreceptors, respectively, in the posterior blastema (Fig. 1F,J). Analysis of the pan-neuronal marker H.10.2f revealed differentiation of ectopic CG in the posterior blastema (Fig. 1N). Analysis of radial-like hypercephalized planarians (after 20-30 days of regeneration) showed that ectopic photoreceptors and mechanoreceptors expanded from both ends of the regenerating animal to surround the planarian body (Fig. 1H,L). Synapsin immunostaining, which labels neuronal synapses, and in situ hybridization for H.10.2f and *Smed-GluR* (which is specifically expressed in the lateral branches of the brain), revealed that the CNS of radial-like hypercephalized planarians appears as a thick ring from which cephalic branches ectopically differentiate all around (Fig. 1P,T,V). Note that all photoreceptor cells appeared to be connected through their visual axonal projections to the circular brain, as seen with an anti-arrestin antibody (Fig. 1R). The digestive system of planarians is composed of a pharynx located in the middle of the trunk, from which one anterior and two posterior branches extend (Saló, 2006). The pharynx evaginates through the mouth, which is located ventrally in the middle part of the planarian body. Staining with an anti-P-Tyr antibody revealed that the two posterior branches converge to form a single branch in *Smed-βcatenin1*-silenced planarians (Fig. 1E′). Use of the same marker also showed that secretory cells located in the prepharyngeal mesenchyme of the wild-type planarian were ectopically differentiated in the posterior part of *Smed-βcatenin1*-silenced planarians (Fig. 1G′).

Altogether, these results show that both neural and non-neural structures acquire anterior identity throughout the body of *Smed-βcatenin1*-silenced animals, demonstrating a role for Smed-βcatenin1 in AP axis re-establishment during planarian regeneration.

Trunk and tail identities are lost in *Smed-βcatenin1*-silenced planarians

To assess whether the differentiation of anterior tissues in central and posterior regions was accompanied by the loss of trunk and tail identities, we analyzed central and posterior markers in *Smed-βcatenin1*-silenced planarians. *Smed-HoxD* and *Smed-AbdbA*,



I'

	PHENOTYPE	1d x 3inj/d 10dr	1d x 3inj/d 19dr	2d x 3inj/d 10dr	2d x 3inj/d 19dr	3d x 3inj/d 10dr	3d x 3inj/d 19dr	3d x 3inj/d 19-28dr
TRUNKS	Wild-type	3/5	3/5	5/9	5/9			
	Tailless	2/5	2/5			1/12	1/12	1/71 (**1.5%**)
	Two-headed			4/9	3/9	11/12	1/12	4/71 (**5.5%**)
	Two-headed with anterior ectopic eyes				1/9		4/12	13/71 (**18%**)
	Two-headed with anterior and posterior ectopic eyes						3/12	15/71 (**21%**)
	radial-like hypercephalized						3/12	38/71 (**54%**)

Fig. 1. *Smed-βcatenin1* silencing induces a gradual anteriorization of regenerating planarians. (**A-E**) Stereomicroscope views of regenerating trunk pieces showing the different phenotypes. (**F-W**,**A'-H'**) Several markers were used at different regeneration stages to characterize the phenotype. Analysis of *opsin* (F-I), *cintillo* (J-M) and *H.10.2f* (N-Q) revealed the differentiation of ectopic anterior structures in the posterior blastema, which subsequently expanded throughout the periphery of the planarian body. Anti-arrestin immunostaining (R,S) showed that, in radial-like hypercephalized planarians, all photoreceptors were connected through ectopic visual axons. Synapsin immunostaining (T,U) and in situ hybridization for *GluR* (V,W) and *H.10.2f* (P,Q) demonstrated that the cephalic ganglia (cg) and ventral nerve cords (vnc) are transformed into a thick ring from which cephalic branches ectopically differentiate all around the body of radial-like hypercephalized planarians. (A'-H') Defects in the pattering of gut and secretory cells were visualized with anti-P-Tyr (green) and anti-arrestin (pink pseudocolour) antibodies. A control planarian viewed at the level of the gut has one anterior and two posterior gut branches (gb; B',F'), along with more dorsal secretory cells (sc) in the pre-pharyngeal but not the post-pharyngeal region (D',H'). The two posterior gut branches converge into one in *Smed-bcatenin1*-silenced planarians (E'), and secretory cells ectopically differentiate in the post-pharyngeal region (G'). (**I'**) The dose dependence of the phenotype and a quantification of the different degrees of anteriorization (1, 2 or 3d x 3inj/d indicates that planarians were injected three times during 1, 2 or 3 consecutive days, respectively). Anterior is shown to the left in those cases where an anteroposterior axis is present. (R-U,A'-H') Confocal *z*-projections. Yellow asterisk indicates the pharynx. Yellow/black arrows and red arrows indicate the differentiation of anterior structures in normal and ectopic positions, respectively. d, days after wounding; a, anterior; p, posterior. Scale bar in A: 270 μm in A-C,E,F,N; 180 μm in D; 250 μm in H,L,P,R,T,W; 395 μm in J; 437 μm in K; 300 μm in all other images.

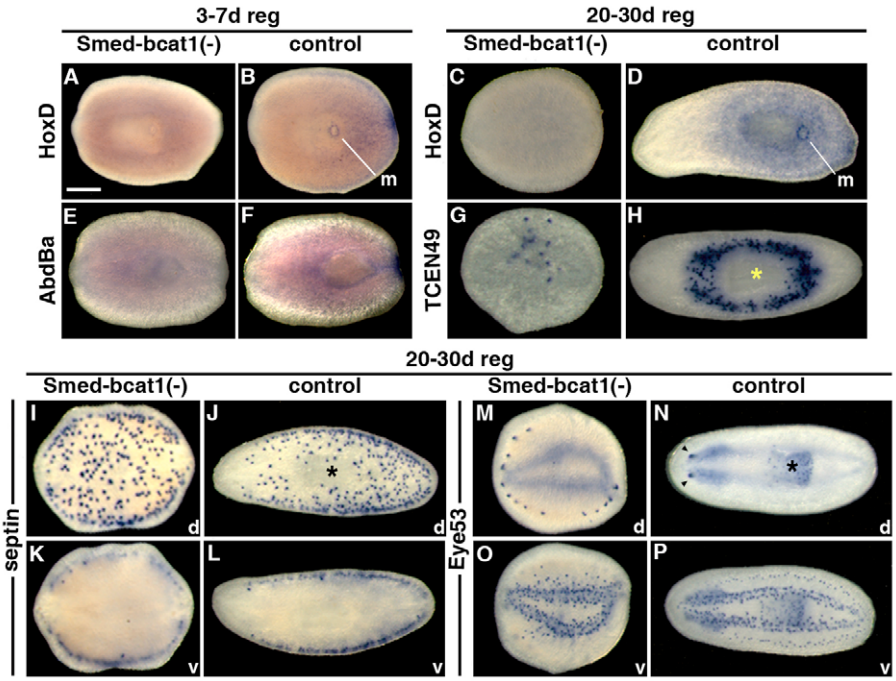


Fig. 2. Trunk and tail identities are lost in *Smed-bcatenin1*-silenced planarians, whereas the dorsoventral axis remains unaffected. (**A-F**) Analysis of *Smed-HoxD* (A-D) and *Smed-AbdBa* (E,F) reveals the disappearance of tail and trunk identities, including the mouth (m). (**G**,**H**) Expression of *Smed-TCEN49* almost disappears in radial-like hypercephalized planarians. (**I-P**) Analysis of *Septin* (I-L) and *Eye53* (M-P) expression demonstrates that the dorsoventral axis is not affected. *Eye53* is also expressed in the eyes (black arrowheads in the control, N), revealing the ectopic eyes around the treated animal (M). Anterior is shown to the left in those cases where an anteroposterior axis is present. Yellow/black asterisks indicate the pharynx. d, dorsal view; v, ventral view. Scale bar in A: 270 μm in A,E; 250 μm in C,G,I,K,M,O; 300 μm in all other images.

central-posterior and posterior Hox genes, respectively, were not expressed at 3 days of regeneration, the earliest stage at which they are detected in wild-type animals (Fig. 2A,E). *Smed-HoxD* continued not to be expressed throughout regeneration (Fig. 2C). Note that the absence of this marker indicated the disappearance of the mouth. Expression of *Smed-TCEN49*, a central marker associated with the pharynx, had almost disappeared at the latest stage (Fig. 2G), and disappearance of the pharynx was also evident when analyzed with other markers (Fig. 1T,V and Fig. 2I,M). These results demonstrate that the anteriorization of *Smed-βcatenin1*-silenced planarians is accompanied by the disappearance of trunk and tail identities. A possible role for Hox genes as targets of *Smed-βcatenin1* needs further investigation.

The DV axis is unaffected in *Smed-βcatenin1*-silenced planarians

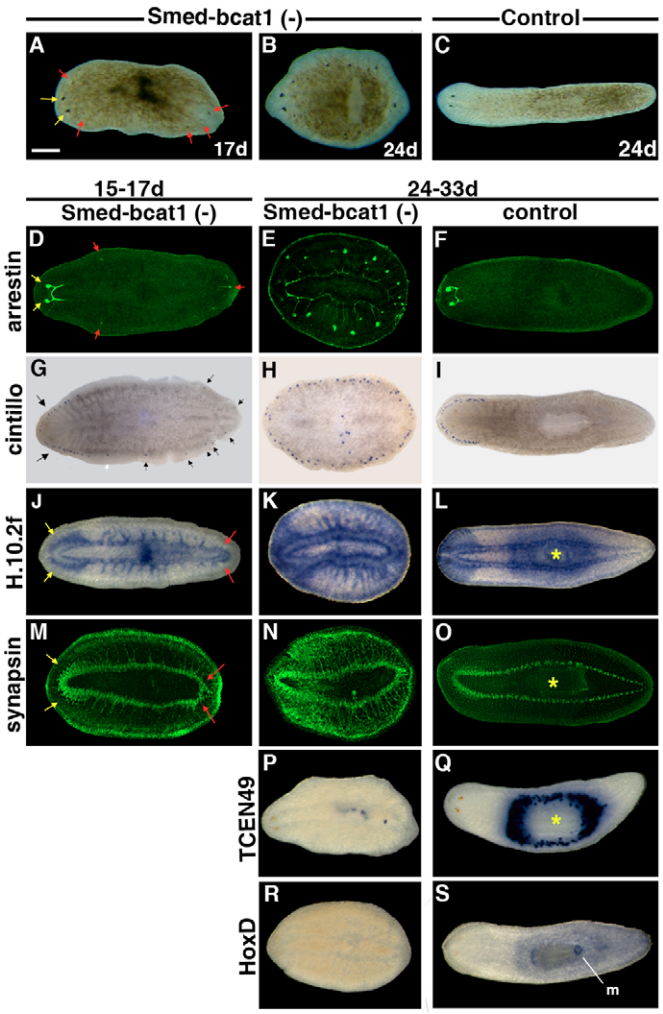
As the AP axis is extensively transformed, we analyzed whether the DV axis was affected after *Smed-βcatenin1* inhibition. Analysis of *septin* and *eye53* expression (dorsal and ventral markers, respectively) revealed that the DV axis of radial-like hypercephalized planarians was not affected (Fig. 2I-P).

Taken together, these results demonstrate that in regenerating *Smed-βcatenin1*-silenced animals the posterior blastema acquires anterior identity and the trunk region is anteriorized, ultimately generating radial-like hypercephalized animals in which the AP axis is lost but the DV axis remains unaffected. Although the canonical Wnt signalling pathway has a common role in establishing the AP axis during development in several species, from cnidarians to vertebrates (Holland, 2002; Kiecker and Niehrs, 2001; Nordstrom et al., 2002; Schneider and Bowerman, 2007), in vertebrates, at earlier developmental stages, nuclear accumulation of β-catenin leads to dorsalization of the embryo (De Robertis and Kuroda, 2004). Our results support a conserved role for the canonical Wnt signal in AP axis specification throughout evolution, and provide further confirmation that its role in DV axis establishment is a vertebrate innovation.

In vertebrate models of regeneration, the Wnt pathway has a reported role in cell proliferation and regenerative outgrowth, but not in axis re-establishment (Kawakami et al., 2006; Yokoyama et al., 2007). Cnidarians are the only species in which this pathway has a demonstrated axial patterning role during regeneration (Lee et al., 2006), although a direct functional study of cnidarian β-catenin has not been reported. Our results, together with two recent reports also in planarians (Gurley et al., 2008; Petersen and Reddien, 2008), are the first direct demonstration of the requirement of β-catenin in AP patterning during the process of regeneration. In contrast to other models, such as *Xenopus* and mouse, in which the inhibition of β-catenin during development produces complex defects and non-viable embryos due to the inhibition of organizer formation and the impairment of gastrulation (Heasman et al., 2000; Huelsken et al., 2000), the planarian model allows in vivo analysis of mutant phenotypes for essential developmental genes.

Smed-βcatenin1 is required for AP axis maintenance during homeostasis

To address whether Smed-βcatenin1 activity could also be required for maintenance of the AP axis, we silenced *Smed-βcatenin1* in intact planarians. At 10-17 days after *Smed-βcatenin1* silencing, ectopic eyes began to differentiate first in the tail region and afterwards anteriorly, adjacent to the original eyes (Fig. 3A,D). Ectopic mechanoreceptor cells appeared in the tail and lateral regions (Fig. 3G), and ectopic brain differentiated along the length of the VNCs (Fig. 3J,M). Around 30 days after *Smed-βcatenin1* silencing, radial-like hypercephalized organisms that were indistinguishable from *Smed-βcatenin1*-silenced regenerating animals were observed. These animals showed ectopic eyes (Fig. 3B,E) that were all connected to the brain by their visual axons, ectopic mechanoreceptor cells all around the periphery of the body (Fig. 3H), and ectopic branching of the CNS all around the body (Fig. 3K,N). Analysis of central and posterior markers revealed the disappearance of trunk and tail identities (Fig. 3P,R). These data demonstrate that Smed-βcatenin1 activity is required not only



T	PHENOTYPE	14d	25d
INTACT	Ectopic anterior eyes	1/15 (**6.7%**)	
	Two-headed	13/15 (**86.7%**)	
	Two-headed with anterior ectopic eyes	1/15 (**6.7%**)	3/15 (**20%**)
	Two-headed with anterior and posterior ectopic eyes		7/15 (**46.7%**)
	radial-like hypercephalized		5/15 (**33.3%**)

Fig. 3. *Smed-βcatenin1*-silenced intact planarians transform to radial-like hypercephalized planarians. (**A-C**) Stereomicroscope views showing two different stages. (**D-S**) Several markers were used at different days after *Smed-βcatenin1* silencing to characterize the phenotype. Anti-arrestin (D-F), *cintillo* (G-I), *H.10.2f* (J-L), *synapsin* (M-O), *Smed-TCEN49* (P,Q) and *Smed-HoxD* (R,S). (**T**) Quantification of the different phenotypes observed after *Smed-bcatenin1* silencing, showing that the anteriorization started in most of the animals in the posterior region. Anterior is to the left in those cases where an anteroposterior axis is present. Confocal *z*-projections (D-F,M-O). Yellow asterisks indicate the pharynx. Yellow/black arrows and red arrows indicate the differentiation of anterior structures in normal and ectopic positions, respectively. d, days after silencing. Scale bar in A: 200 μm in B,E,H,K,N,P,R; 300 μm in all other images.

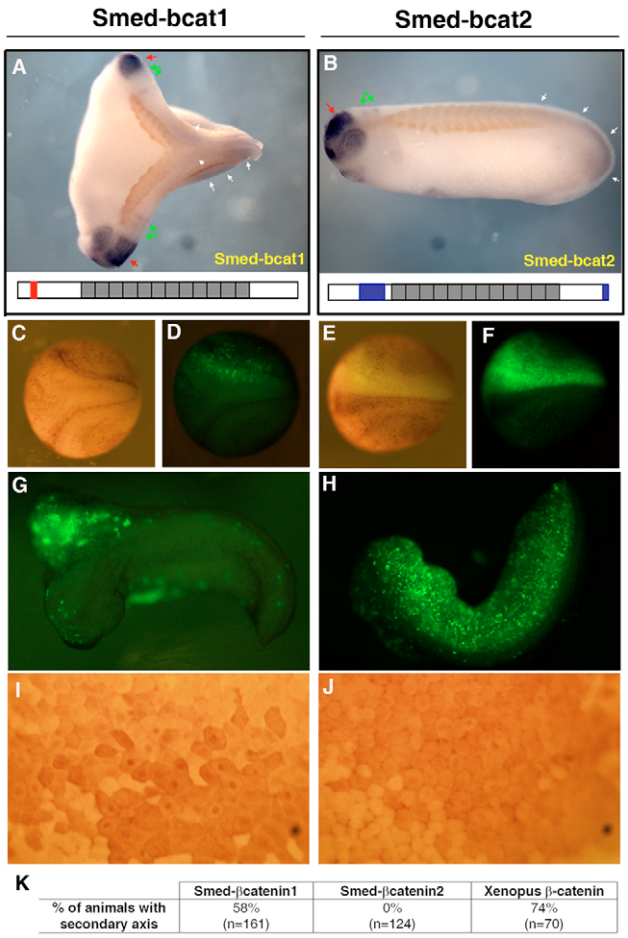


Fig. 4. mRNA injection of *Smed-βcatenin1* but not *Smed-βcatenin2* induces a secondary axis in *Xenopus* embryos. (**A**,**B**) *Xenopus* embryos injected with *Smed-βcatenin1* (A) or *Smed-βcat2* RNA (B), showing *Otx2* (forebrain, red arrow), *Krox20* (rhombomeres 3 and 5, green arrows) and *Cad3* (spinal cord, white arrows) expression, along with muscle staining with the antibody 12/101 (brown signal). A schematic representation of the structure of each β-catenin is shown under the corresponding image. GSK3-binding domain and adhesion domains are shown with red and blue boxes, respectively. (**C-H**) Neurula (C-F) and tailbud (G,H) stage embryos injected with GFP-tagged constructs for both *Schmidtea mediterranea* β-catenins. (**I**,**J**) Smed-βcatenin1 but not Smed-βcatenin2 is found preferentially in the cell nucleus. (**K**) Quantification of the secondary axis obtained after each Smed-βcatenin injection (*Xenopus* β-catenin was used as a control).

during regeneration, but also during normal tissue homeostasis to maintain AP axis polarity in planarians. Interestingly, our data show that the anteriorization process in intact animals follows the same pattern as in regenerating trunks: it starts mainly in the tail region and later expands to the rest of the body (see Fig. 3T for quantification). Further studies are required to elucidate whether this is a direct consequence of the suppression of Smed-βcatenin1 activity or an indirect effect due to, for example, a higher rate of cell turnover in the tail.

The extreme radial-like phenotype obtained after Smed-βcatenin1 inhibition suggests that, in adult planarians, Smed-βcatenin1 activity is required to confer posterior identity, and its inhibition is required to acquire head fate. The differential Smed-βcatenin1 activity along the AP axis in planarians could be a response to a morphogenetic Wnt gradient, as has been described for the patterning of the CNS in *Xenopus* embryos (Kiecker and Niehrs, 2001). Accordingly, during

Xenopus development, repression of Wnt signalling is a characteristic of the head organizer (Niehrs, 1999; Niehrs, 2004). However, the effect could also be explained by a mechanism in which β-catenin regulates sister-cell asymmetry following cell division, as occurs along the animal-vegetal axis during *Platynereis* and *C. elegans* embryogenesis (Schneider and Bowerman, 2007; Kaletta et al., 1997; Lin et al., 1998). The classical janus-headed phenotype (Morgan, 1898), in which two-headed planarians are produced after cutting extremely thin fragments, is a well-known model that supports the gradient hypothesis (Child, 1911), because the phenotype may be explained by the inability of such a small field to support a Smed-βcatenin1 activity gradient. The analysis of Smed-βcatenin1 activity, by analysis of its subcellular localization, for instance, will help to resolve this question.

Functional characterization of *S. mediterranea* β-catenins in *Xenopus* embryos

Sequence analysis of *S. mediterranea* β-catenins demonstrated that the GSK3-binding domain, required for β-catenin degradation during Wnt signalling, is present in Smed-βcatenin1 but not in Smed-βcatenin2. By contrast, residues involved in cell-cell adhesion were conserved in Smed-βcatenin2 but not in Smed-βcatenin1 (for details, see Fig. S4 in the supplementary material). To test whether *S. mediterranea* β-catenins could have undergone a functional specialization, we assayed their ability to induce a secondary axis in *Xenopus* embryos (McMahon and Moon, 1989). A secondary axis was induced after *Smed-βcatenin1* injection but not after *Smed-βcatenin2* injection (Fig. 4A,B,K). A GFP-tagged construct of each *S. mediterranea* β-catenin was injected to quantify the amount of translated protein in each embryo, demonstrating that even when twice the amount of Smed-βcatenin2 was present, it could never induce a secondary axis (Fig. 4C-H). Moreover, anti-GFP staining demonstrated that Smed-βcatenin1, but not Smed-βcatenin2, accumulates in the nucleus (Fig. 4I,J).

Altogether, these results demonstrate the functional conservation of *Smed-βcatenin1* in Wnt signalling, and point to a functional specialization of *S. mediterranea* β-catenins, such that Smed-βcatenin1 would be involved in signalling and Smed-bcatenin2 would be involved in the membrane cell-cell contacts. All metazoans studied to date have a single β-catenin gene encoding a protein containing both kinds of functional domains, involved in signaling and in cell-cell adhesion (Schneider et al., 2003), with the exception of *C. elegans*, which is the only species in which β-catenin gene duplication and functional specialization have been reported (Korswagen et al., 2000). A phylogenetic analysis using β-catenin homologs from several species demonstrates that the duplication in *S. mediterranea* occurred independently from the one in *C. elegans* (see Fig. S5 in the supplementary material). Molecular studies of other species belonging to different phyla will clarify the significance of these duplications.

We thank F. Cebrià, P. Martinez, J. Garcia-Fernandez, M. Irimia, J. Baguñà and members of the laboratory for discussion and suggestions; F. Cebrià and P. Newmark for providing *H.10.2f*, *cintillo*, *Smed-GluR*, *septin* and *Eye53* clones; K. Eckelt for the *Smed-opsin* clone; H. Orii and K. Watanabe for providing anti-VC-1; M. Andreazzoli, A. Fainsod, N. Papalopulu and D. Turner for reagents; and I. Patten for editorial advice. This work was supported by grants from the Ministerio de Educación y Ciencia, Spain, from AGAUR (Generalitat de Catalunya, Spain), and from La Junta de Andalucía, Spain.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/7/1215/DC1

References

Agata, K., Soejima, Y., Kato, K., Kobayashi, C., Umesono Y. and Watanabe, K. (1998). Structure of the planarian nervous system (CNS) revealed by neuronal cell markers. *Zool. Sci.* **15**, 433-440.

Bueno, D., Baguna, J. and Romero, R. (1996). A central body region defined by a position-specific molecule in the planarian Dugesia (Girardia) tigrina: spatial and temporal variations during regeneration. *Dev. Biol.* **178**, 446-458.

Cebrià, F. and Newmark, P. A. (2005). Planarian homologs of netrin and netrin receptorare required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development* **132**, 3691-3703.

Child, C. M. (1911). Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. I. The axial gradient in planaria dorotocephala as a limiting factor in regulation. *J. Exp. Zool.* **10**, 265-320.

De Robertis, E. M. and Kuroda, H. (2004). Dorsal-ventral patterning and neural induction in Xenopus embryos. *Annu. Rev. Cell Dev. Biol.* **20**, 285-308.

Garcia-Fernandez, J., Baguna, J. and Salo, E. (1993). Genomic organization and expression of the planarian homeobox genes Dth-1 and Dth-2. *Development* **118**, 241-253.

Gómez-Skarmeta, J. L., de la Calle-Mustienes, E. and Modolell, J. (2001). The Wnt-activated Xiro1 gene encodes a repressor that is essential for neural development and downregulates BMP4. *Development* **128**, 551-560.

Gurley, K. A., Rink, J. C. and Sánchez Alvarado, A. (2008). Beta-catenin defines head versus tail identity during planarian regeneration and homeostasis. *Science* **319**, 323-327.

Harland, R. (1991). In situ hybridization: an improved whole mount method for Xenopus embryos. *Methods Cell Biol.* **36**, 685-695.

Harland, R. and Weintraub, H. (1985). Translation of mRNA injected into Xenopus oocytes is specifically inhibited by antisense RNA. *J. Cell Biol.* **101**, 1094-1099.

Heasman, J., Kofron, M. and Wylie, C. (2000). Beta-catenin signalling activity dissected in the early Xenopus embryo: a novel antisense approach. *Dev. Biol.* **222**, 124-134.

Holland, L. Z. (2002). Heads or tails? Amphioxus and the evolution of anterior-posterior patterning in deuterostomes. *Dev. Biol.* **24**, 209-228.

Huang, S., Shetty, P., Robertson, S. M. and Lin, R. (2007). Binary cell fate specification during C. elegans embryogenesis driven by reiterated reciprocal asymmetry of TCF POP-1 and its coactivator β-catenin SYS-1. *Development* **134**, 2685-2695.

Huelsken, J., Vogel, R., Brinkmann, V., Erdmann, B., Birchmeier, C. and Birchmeier, W. (2000). Requirement for beta-catenin in anterior-posterior axis formation in mice. *J. Cell Biol.* **148**, 567-578.

Kaletta, T., Schnabel, H. and Schnabel, R. (1997). Binary specification of the embryonic lineage in Caenorhabditis elegans. *Nature* **390**, 294-298.

Kawakami, Y., Rodriguez Esteban, C., Raya, M., Kawakami, H., Marti, M., Dubova, I. and Izpisua Belmonte, J. C. (2006). Wnt/beta-catenin signalling regulates vertebrate limb regeneration. *Genes Dev.* **20**, 3232-3237.

Kiecker, C. and Niehrs, C. (2001). A morphogen gradient of Wnt/beta-catenin signalling regulates anteroposterior neural patterning in Xenopus. *Development* **128**, 4189-4201

Korswagen, H. C., Herman, M. A. and Clevers, H. C. (2000). Distinct beta-catenins mediate adhesion and signalling functions in C. elegans. *Nature* **406**, 527-532.

Lee, P. N., Pang, K., Matus, D. Q. and Martindale, M. Q. (2006). A WNT of things to come: evolution of Wnt signalling and polarity in cnidarians. *Semin. Cell Dev. Biol.* **17**, 157-167.

Lin, R., Hill, R. J. and Priess, J. R. (1998). POP-1 and anterior-posterior fate decisions in *C. elegans* embryos. *Cell* **92**, 229-239.

Marikawa, Y. (2006). Wnt/beta-catenin signalling and body plan formation in mouse embryos. *Semin. Cell Dev. Biol.* **172**,175-184.

McMahon, A. P. and Moon, R. T. (1989). Ectopic expression of the proto-oncogene int-1 in *Xenopus* embryos leads to duplication of the embryonic axis. *Cell* **58**, 1075-1084.

Molina, M. D., Saló, E. and Cebrià, F. (2007). The BMP pathway is essential for re-specification and maintenance of the dorsoventral axis in regenerating and intact planarians. *Dev. Biol.* **311**, 79-94.

Morgan, T. H. (1898). Experimental studies of the regeneration of Planaria maculata. *Arch. Entw. Mech. Org.* **7**, 364-397.

Newmark, P. A., Reddien, P. W., Cebria, F. and Sanchez Alvarado, A. (2003). Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in planarians. *Proc. Natl. Acad. Sci. USA* **100 Suppl. 1**, 11861-11865.

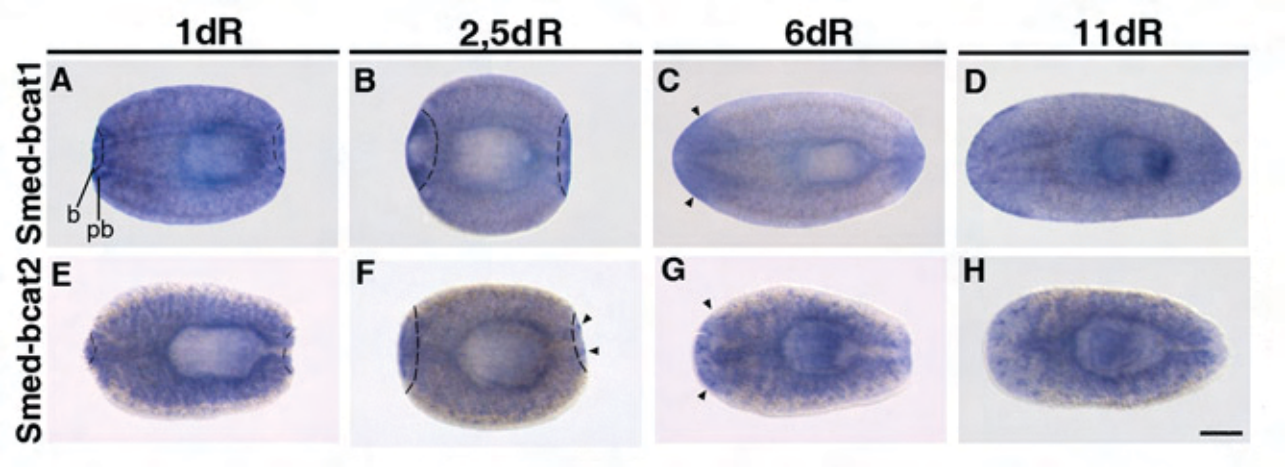
Niehrs, C. (1999). Head in the WNT: the molecular nature of Spemann's head organizer. *Trends Genet.* **15**, 314-319.

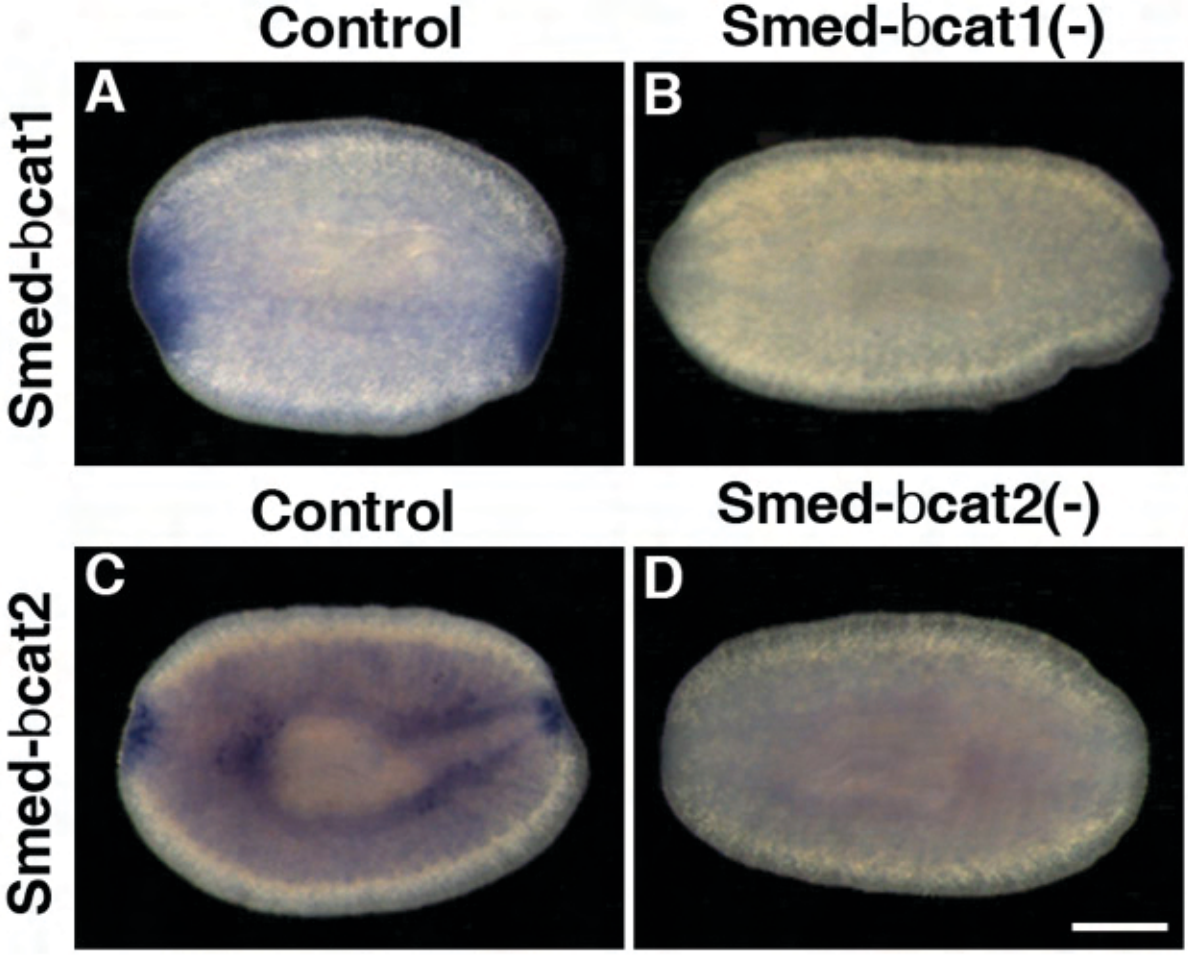
Niehrs, C. (2004). Regionally specific induction by the Spemann-Mangold organizer. *Nat. Rev. Genet.* **5**, 425-434.

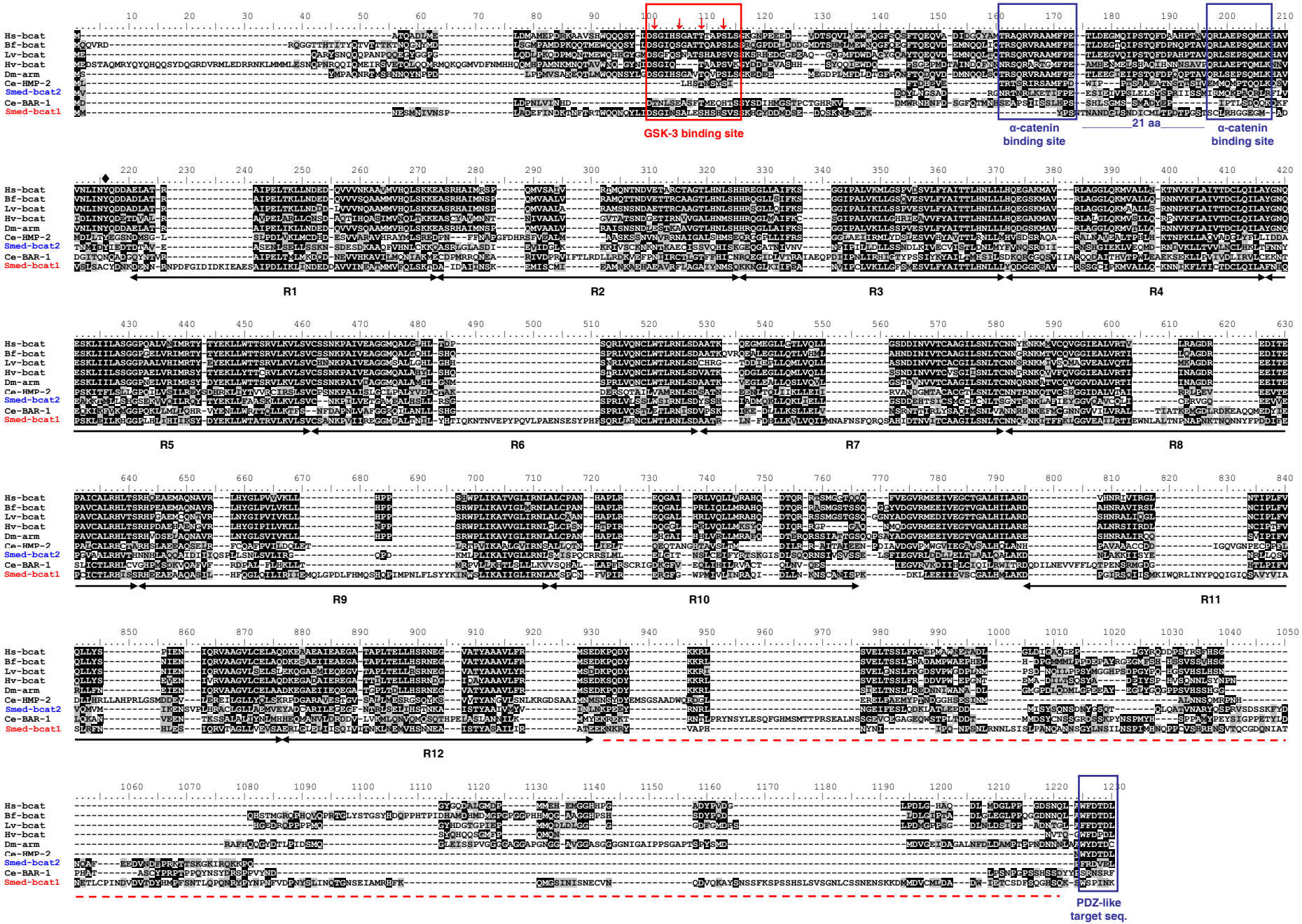
Nogi, N. and Levin, M. (2005). Characterization of innexin gene expression and functional roles of gap-junctional communication in planarian regeneration. *Dev. Biol.* **287**, 314-335.

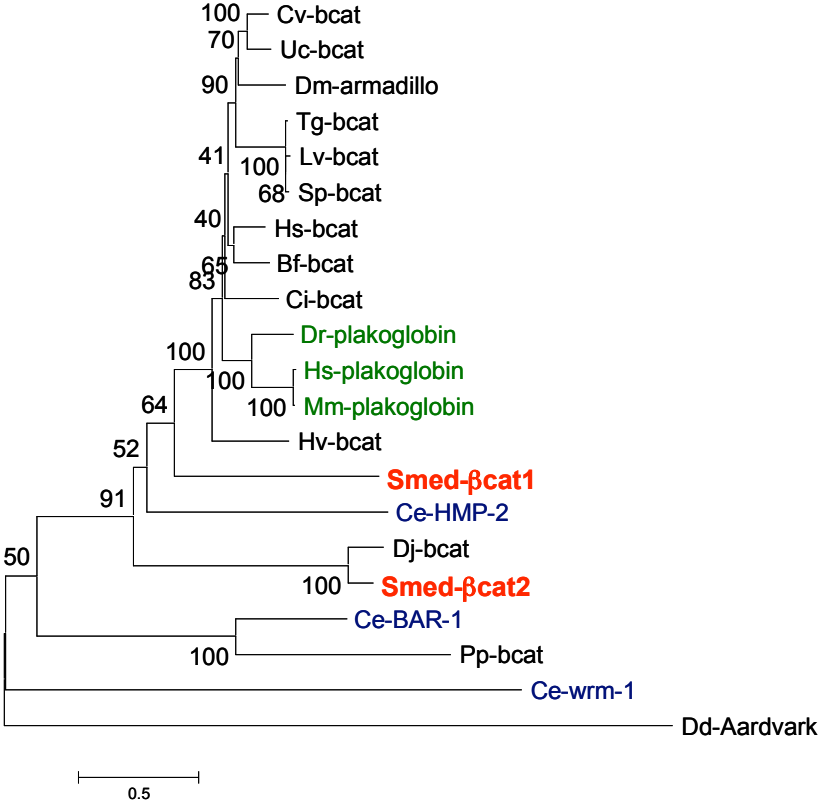
Nogi, T. and Watanabe, K. (2001). Position-specific and non-colinear expression of the planarian posterior (Abdominal-B-like) gene. *Dev. Growth Differ.* **43**, 177-184.
Nordstrom, U., Jessell, T. M. and Edlund, T. (2002). Progressive induction of caudal neural character by graded Wnt signalling. *Nat. Neurosci.* **5**, 525-532.
Okamoto, K., Takeuchi, K. and Agata, K. (2005). Neural projections in planarian brain revealed by fluorescent dye tracing. *Zoolog. Sci.* **22**, 535-546.
Orii, H., Kato, K., Umesono, Y., Sakurai, T., Agata, K. and Watanabe, K. (1999). The planarian HOM/HOX homeobox genes (Plox) expressed along the anteroposterior axis. *Dev. Biol.* **210**, 456-468.
Oviedo, N. J., Newmark, P. A. and Sanchez Alvarado, A. (2003). Allometric scaling and proportion regulation in the freshwater planarian *Schmidtea mediterranea*. *Dev. Dyn.* **226**, 326-333.
Petersen, C. P. and Reddien, P. W. (2008). Smed-betacatenin-1 is required for anteroposterior blastema polarity in planarian regeneration. *Science* **319**, 327-330.
Sakai, F., Agata, K., Orii, H. and Watanabe, K. (2000). Organization and regeneration ability of spontaneous supernumerary eyes in planarians – eye regeneration field and pathway selection by optic nerves. *Zool. Sci.* **17**, 375-381.
Saló, E. (2006). The power of regeneration and the stem-cell kingdom: freshwater planarians (Platyhelminthes). *BioEssays* **28**, 546-559.
Sánchez Alvarado, A. and Newmark, P. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl. Acad. Sci. USA* **96**, 5049-5054.
Sánchez Alvarado, A., Newmark, P. A., Robb, S. M. C. and Juste, R. (2002). The Schmidtea mediterranea database as a molecular resource for studying platyhelminthes, stem cells and regeneration. *Development* **129**, 5659-5665.
Schier, A. F. and Talbot, W. S. (2005). Molecular genetics of axis formation in zebrafish. *Annu. Rev. Genet.* **39**, 561-613.
Schneider, S. Q. and Bowerman, B. (2007). beta-Catenin asymmetries after all animal/vegetal-oriented cell divisions in Platynereis dumerilii embryos mediate binary cell-fate specification. *Dev. Cell* **13**, 73-86.
Schneider, S. Q., Finnerty, J. R. and Martindale, M. Q. (2003). Protein evolution: structure-function relationships of the oncogene beta-catenin in the evolution of multicellular animals. *J. Exp. Zoolog. B Mol. Dev. Evol.* **295**, 25-44.
Turner, D. L. and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. *Genes Dev.* **12**, 1434-1447.
Umesono, Y., Watanabe, K. and Agata, K. (1999). Distinct structural domains in the planarian brain defined by the expression of evolutionarily conserved homeobox genes. *Dev. Genes Evol.* **209**, 31-39.
Yokoyama, H., Ogino, H., Stoick-Cooper, C. L., Grainger, R. M. and Moon, R. T. (2007). Wnt/beta-catenin signalling has an essential role in the initiation of limb regeneration. *Dev. Biol.* **306**,170-178.
Zayas, R. M., Hernandez, A., Habermann, B., Wang, Y., Stary, J. M. and Newmark, P. A. (2005). The planarian Schmidtea mediterranea as a model for epigenetic germ cell specification: analysis of ESTs from the hermaphroditic strain. *Proc. Natl. Acad. Sci. USA* **102**, 18491-18496.


Smed-bcat1
Smed-bcat2
A
B
cg
vnc
agb
pgb
*
*


1dR
2,5dR
6dR
11dR
Smed-bcat1
Smed-bcat2
A
B
C
D
E
F
G
H
b
pb


Control
Smed-bcat1(-)
Smed-bcat1
A
B
Control
Smed-bcat2(-)
Smed-bcat2
C
D


Hs-bcat
Bf-bcat
Lv-bcat
Hv-bcat
Dm-arm
Ce-HMP-2
Smed-bcat2
Ce-BAR-1
Smed-bcat1
GSK-3 binding site
α-catenin binding site
21 aa
α-catenin binding site
R1
R2
R3
R4
R5
R6
R7
R8
R9
R10
R11
R12
PDZ-like target seq.


100
Cv-bcat
70
Uc-bcat
90
Dm-armadillo
Tg-bcat
41
100
Lv-bcat
68
Sp-bcat
40
Hs-bcat
65
Bf-bcat
83
Ci-bcat
Dr-plakoglobin
100
100
Hs-plakoglobin
64
100
Mm-plakoglobin
Hv-bcat
52
Smed-βcat1
91
Ce-HMP-2
50
Dj-bcat
100
Smed-βcat2
Ce-BAR-1
100
Pp-bcat
Ce-wrm-1
Dd-Aardvark
0.5