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BMP signaling regulates the dorsal planarian midline and is needed for asymmetric regeneration

Peter W. Reddien[1], Adam L. Bermange[2,*], Adrienne M. Kicza[1] and Alejandro Sánchez Alvarado[2,†]

Planarians can be cut into irregularly shaped fragments capable of regenerating new and complete organisms. Such regenerative capacities involve a robust ability to restore bilateral symmetry. We have identified three genes needed for bilaterally asymmetric fragments to regenerate missing body parts. These genes are candidate components of a signaling pathway that controls the dorsal-ventral patterning of many animal embryos: a BMP1/Tolloid-like gene (*smedolloid-1*), a SMAD4-like gene (*smedsmad4-1*), and a BMP2/4/DPP-like gene (*smedbmp4-1*). BMP signaling was involved in the formation of new tissues at the midline of regeneration, the dorsal-ventral patterning of new tissues, and the maintenance of the dorsal-ventral pattern of existing adult tissue in homeostasis. *smedbmp4-1* was normally expressed at the dorsal midline. Asymmetric fragments lacking a midline displayed new *smedbmp4-1* expression prior to formation of a regenerative outgrowth (blastema). Asymmetric fragments containing the midline displayed expanded *smedbmp4-1* expression towards the wound. We suggest injured animals that lack left-right symmetry reset their midline through modulation of BMP activity as an early and necessary event in regeneration.

KEY WORDS: Asymmetry, BMP, Planaria, Regeneration, Adult homeostasis

INTRODUCTION

Planarians are free-living flatworms and a classic model system for studies of regeneration (Reddien and Sánchez Alvarado, 2004). Planarians are members of the phylum Platyhelminthes and are currently viewed as part of the lophotrochozoan grouping of animal phyla (Adoutte et al., 2000). Planarians possess bilateral symmetry and are triploblastic (Hyman, 1951). Reproduction is accomplished either asexually through fission and regeneration or sexually as cross-fertilizing hermaphrodites. Among the anatomical features of planarians are a cephalized central nervous system (in addition to the cephalic ganglia, photoreceptors and other sensory cell types are present in the head), a musculature, a branched intestinal system for the delivery of nutrients, and a single, centrally located, body opening (the pharynx). The ability of planarians to regenerate entire animals from multiple types of body fragments has been known for almost two centuries, but there is a limited understanding of how this is achieved (Dalyell, 1814; Morgan, 1898; Randolph, 1897). Planarian regeneration involves formation of new tissues at the wound site (blastema formation) and re-modeling of pre-existing tissue to create an animal of proper proportions (morphallaxis) (Morgan, 1898). Blastema formation requires the action of a population of stem cells called neoblasts. Because almost any type of planarian fragment can regenerate, a blastema can form in cephalic, caudal, or lateral body regions and produce appropriate corresponding tissue. Irregularly shaped fragments must create new tissue and re-arrange pre-existing tissue in a coordinated manner to generate an animal with symmetry, a complete complement of organ systems, and the proper proportions and shape. How do wounded planarian tissues specify what to regenerate, and what rules govern the rearrangement of body coordinates? Recent technological developments (Newmark and Sánchez Alvarado, 2000; Newmark et al., 2003; Reddien et al., 2005a; Sánchez Alvarado and Newmark, 1999) make it now possible to begin to address these long-standing questions.

Two genes that are predicted to encode components of a BMP signal transduction pathway were found in a recent RNAi screen to be required for normal blastema morphology (Reddien et al., 2005a). Here we describe the roles of these genes, and of a *Schmidtea mediterranea* BMP-like gene (*smedbmp4-1*), in regeneration and homeostasis. BMPs are TGF-β signaling ligands that mediate a number of developmental events, such as embryonic dorsal-ventral patterning and *Drosophila* imaginal disc patterning (Diaz-Benjumea et al., 1994; Martindale, 2005; Padgett et al., 1987; Spencer et al., 1982). Little is known about the role this pathway may play in the maintenance of differentiated structures and their regeneration after amputation. Our data indicate that the regulated pattern of expression of a BMP-like gene in planarians has a conserved dorsal-ventral patterning function in regeneration and adult tissue maintenance, promotes midline patterning, and is needed for the initiation of regeneration in cases in which bilateral symmetry is disrupted by injury.

MATERIALS AND METHODS

Molecular biology

smedolloid-1 was derived from the cDNA H.14.4a and *smedsmad4-1* from the cDNA H.17.9e. The H.14.4a cDNA did not contain the complete *smedolloid-1* gene sequence. The central region of the *smedolloid-1* cDNA, 5′ of H.14.4a, was isolated with mixed oligonucleotide-primed amplification of cDNA (MOPAC). *S. mediterranea* head (H) cDNA library DNA (plasmid DNA pooled from all library clones) (Sánchez Alvarado et al., 2002) served as a template for the PCR. Gene-specific primers in nested PCR (5′-TGGACTGATTATGCGACC-3′) followed by the inner primer (5′-TGGATTTCCCCTCCGCAAAC-3′) were used. The degenerate primer pool (5′-CARGCNATGMGNCAYTGGG-3′) served as the 5′ primer in both rounds. The resulting 850 bp band was T/A cloned using the pGEM-T Easy Vector System II (Promega) and sequenced. The 5′-end of the *smedolloid-1* cDNA was isolated with 5′-RLM-RACE using the FirstChoice RLM-RACE Kit (Ambion). The outer primer (5′-CGACGTATGAACAA-

[1]MIT Biology, Whitehead Institute, 9 Cambridge Center, Cambridge, MA 02138, USA. [2]Howard Hughes Medical Institute, Department of Neurobiology and Anatomy, University of Utah School of Medicine, 401 MREB, 20N 1900E, Salt Lake City, UT 84132, USA.

*Present address: London Research Institute, Lincoln's Inn Fields Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX, UK
†Author for correspondence (e-mail: sanchez@neuro.utah.edu)

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CATCCG-3′) followed by the inner primer (5′-GGAATAAAGCTAAGG-CACG-3′) were used in the consecutive rounds of nested PCR. The resulting 650 bp band was T/A cloned into pGEM-T Easy (Promega) and sequenced. The *smedbmp4-1* gene was isolated by PCR amplifications from cDNA cloned into pBluescript (Sánchez Alvarado et al., 2002) with the primer 5′-GCACGGTTTTGGAATAGAAAGGTC-3′ and a primer that hybridizes to vector sequence. A second product used the primer 5′-ACTGTCCT-TACCCGTTATCAG-3′ and a vector primer to amplify the 3′ end of the *smedbmp4-1* gene. cDNAs were amplified by PCR and cloned into the vector pDONRdT7 for RNAi using the Gateway (Invitrogen) cloning procedure as previously described (Reddien et al., 2005a). RNA interference was performed by feeding bacteria expressing dsRNAs mixed with blended liver to planarians as previously described (Reddien et al., 2005a).

Antibody staining

Animals were fixed as previously described (Newmark et al., 2003; Sánchez Alvarado and Newmark, 1999). Following rehydration animals were blocked for 6 hours at room temperature (RT) in PBTxB (PBS+0.3% Triton X-100+0.25% BSA), then incubated overnight with 1:5000 VC-1 (kind gift of Dr K. Agata, RIKEN, Kobe, Japan), 1:133 anti-Synaptotagmin (kind gift of Dr K. Agata), 1:400 anti-acetylated tubulin (Robb and Sánchez Alvarado, 2002). Animals were rinsed with PBTxB, then washed six times in PBSTxB over 6 hours, and labeled overnight with 1:400 goat anti-mouse Alexa Fluor 568 (Molecular Probes). Animals were washed as before and mounted in Vectashield (Vector).

In situ hybridizations

Animals were fixed in Carnoy's fixative as described previously and stored in methanol (Sánchez Alvarado et al., 2002). Animals were rehydrated with a methanol:PBST (PBS, 0.1% Triton X-100) concentration series, pre-hybridized (50% formamide, 5× SSC pH 7.0, 100 μg/ml heparin, 0.05% Tween 20, 0.05% Triton X-100, 5 mM DTT, 1 mg/ml yeast RNA, 1× Denhardt's solution; wash Hybe) at 56°C for 2 hours, and hybridized with digoxigenin (DIG)-conjugated riboprobes for 16 hours (pre-hybridization solution plus 5% dextran sulfate). Animals were washed with 50% formamide, 5× SSC, 1× Denhardt's, then transferred through a wash Hybe dilution series (75%, 50%, 25% in 2× SSC+0.1% Triton X-100), then twice with 2× SSC+0.1% Triton X-100, then twice with 0.2× SSC+0.1% Triton X-100, and finally into MABT (100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20, pH7.5). Animals were blocked in MABT plus 1% BSA and 10% horse serum for 1 hour at RT, and then labeled with a sheep anti-DIG antibody (1:2000; Roche) in MABT+BSA+10% horse serum for 4 hours at RT. Animals were washed eight times with MABT and signal developed using nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3′-indolyl phosphate p-toluidine salt (BCIP). For some in situ hybridizations with *smedolloid-1* and *smedbmp4-1*, animals were killed in 10% *N*-acetyl cysteine for 5 minutes prior to fixation. Some in situ hybridizations were done with the assistance of a hybridization robot (Intavis, Köln, Germany).

RESULTS

A recent RNAi screen in *S. mediterranea* identified the first collection of genes required for normal regeneration in planarians (Reddien et al., 2005a). Phenotypic analyses were used to group screen isolates into categories that correspond to the sequential steps of regeneration – from wound healing and regeneration initiation to the restoration of normal behavior. Inhibition of two genes in this screen caused very similar abnormalities in blastema patterning. Specifically, *smedolloid-1(RNAi)* and *smedsmad4-1(RNAi)* animals regenerated new heads and tails that were indented at the midline. This result suggested the possibility that these two genes act together in planarians to regulate the midline patterning of cephalic and caudal blastemas of these bilaterally symmetric animals. *smedsmad4-1(RNAi)* animals that were challenged to go through two rounds of regeneration following gene inhibition completely failed to form blastemas (see below for more details). We report an analysis of the functions of these genes in controlling positional patterning in homeostasis, regeneration, and in the initiation of blastema formation in left-right asymmetric fragments. Fig. 1A defines relevant terminology for the planarian body regions and the surgical manipulations performed.

The *S. mediterranea* SMEDBMP4-1 pathway

The *smedolloid-1* and *smedsmad4-1* genes were identified from the cDNAs H.14.4a and H.17.9e, respectively. *smedbmp4-1* was identified by RT-PCR (see Materials and methods). *smedolloid-1* encodes a protein similar to the *Drosophila* Tolloid protein (see Fig. S1 in the supplementary material). Tolloid (BMP1-like) is a metalloprotease that activates Decapentaplegic (DPP; BMP2/4-like) signaling by inhibition of the BMP antagonist Short gastrulation (SOG; Chordin-like) (Ferguson and Anderson, 1992; Piccolo et al., 1997; Shimell et al., 1991; Wozney et al., 1988). *smedsmad4-1* encodes a protein similar to the SMAD4 protein (see Fig. S2 in the supplementary material). SMAD4 is a co-SMAD that mediates the signaling of multiple TGF-β pathways (Hahn et al., 1996; ten Dijke and Hill, 2004). A *bmp2/4*-like gene was previously identified in the planarian *Dugesia japonica* and found to be expressed in the dorsal midline (Orii et al., 1998). *smedbmp4-1* encodes a *S. mediterranea* protein similar to the *D. japonica* BMP, *Drosophila* DPP, and to other BMP proteins, including BMP2, 4, 5 and 7 proteins, and ADMP proteins (see Fig. S3 in the supplementary material). BMP2, 4 and DPP proteins are TGF-β signaling ligands that mediate the dorsal-ventral patterning of embryos (Holley and Ferguson, 1997; Martindale, 2005; Padgett et al., 1987; Spencer et al., 1982). By analogy to known functions for homologous proteins, the planarian pathway is predicted to act as follows: SMEDOLLOID-1 inhibits BMP antagonists, promoting the activity of SMEDBMP4-1; SMEDBMP4-1 acts as a signaling ligand, for which signal transduction is mediated by SMEDSMAD4-1.

smedolloid-1 is expressed in scattered dorsal cells and *smedbmp4-1* is expressed in dorsal midline cells (Fig. 1B,E). Significantly fewer cells on the ventral surface express *smedolloid-1* than on the dorsal surface (Fig. 1C). *smedbmp4-1* expression is broader in the head than in the body, expression weakens laterally, and an additional site of expression at the anterior end of the pharynx was observed (Fig. 1E). *smedbmp4-1*-expressing cells are dorsally localized, but subepidermal (Fig. 1F). RNAi of *smedbmp4-1* and of *smedolloid-1* eliminated detectable messages in in situ hybridizations (7/7 and 5/5, respectively, Fig. 1D,G). We could not detect an expression pattern for *smedsmad4-1* with our in situ hybridization methods, but we detected greater than 80% knockdown of *smedsmad4-1* by RNAi with quantitative RT-PCR (data not shown). Because of its dorsal and medial spatial distribution, the expression of *smedbmp4-1* suggested a candidate role in midline formation and dorsal-ventral patterning.

SMEDBMP4-1 signaling controls the maintenance of adult body form

During adult planarian life, differentiated cells age and are constantly replaced by the progeny of neoblasts (Reddien and Sánchez Alvarado, 2004). This process not only requires neoblasts, but presumably also the active control of body patterning even after development of adult structures has occurred. Given the prominent role of BMP signaling in the dorsal-ventral patterning of animal embryos (De Robertis and Kuroda, 2004) and the expression of BMP pathway genes in adult planarians, we sought to determine whether homeostatic cell turnover requires BMP signaling. The *C. elegans unc-22* gene was used as a negative RNAi control for these and other experiments presented throughout the text. This control

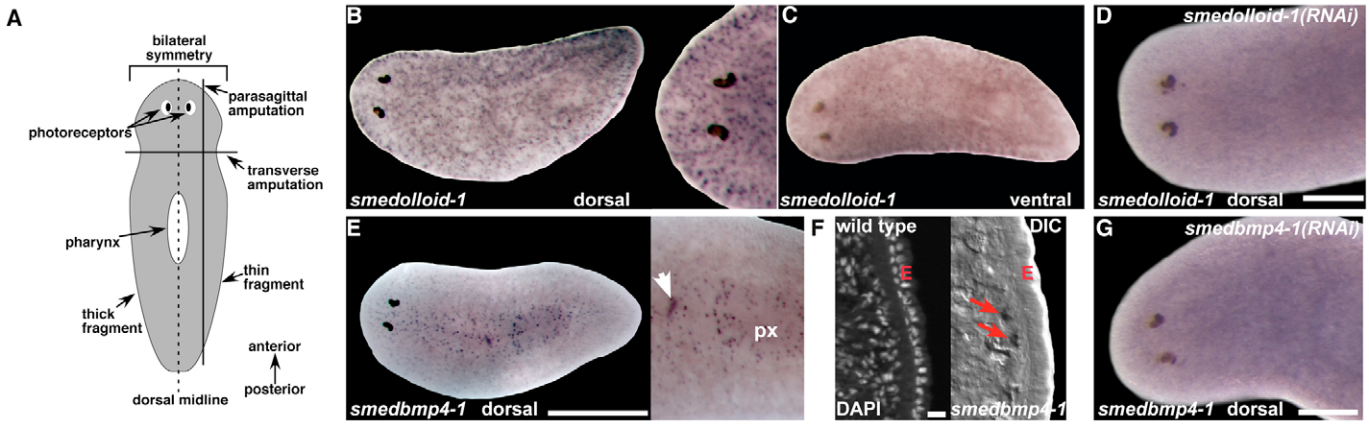

Fig. 1. *smedolloid-1* is expressed in small dorsally located cells and *smedbmp4-1* is expressed in cells along the dorsal midline. (**A**) Schematic of a planarian depicting body regions and terminology used throughout this manuscript. Anterior, up. Dotted line indicates the dorsal midline. Solid lines indicate surgical amputations. (**B**,**C**) Dorsal (B) and Ventral (C) views of wild-type animals labeled with the *smedolloid-1* riboprobe. An enlarged view of a head region is shown on the right in B. (**D**) A *smedolloid-1(RNAi)* animal showed no labeling in an in situ hybridization with a *smedolloid-1* riboprobe. Scale bar: 0.1 mm. (**E**) Wild-type animals were labeled with the *smedbmp4-1* riboprobe. px, pharynx. Arrowhead, *smedbmp4-1* signal at the anterior end of the pharynx. Scale bar: 0.5 mm. (**F**) Section of a wild-type animal showing expression of *smedbmp4-1* in sub-epidermal, dorsally localized cells. Following whole-mount in situ hybridization with a *smedbmp4-1* riboprobe, animals were sectioned as previously described (Reddien et al., 2005b). In both panels, dorsal is to the right. Left panel, DAPI staining; right panel, Nomarski optics. E, epidermis. Scale bar: 10 μm. (**G**) A *smedbmp4-1(RNAi)* animal showed no labeling in in situ hybridizations with a *smedbmp4-1* riboprobe. Anterior, left. Scale bar: 0.1 mm.

presents animals with dsRNA-containing bacteria, and because it lacks exact sequence identity to any known planarian sequence fails to cause perturbation of any aspect of planarian biology (Reddien et al., 2005a). We continuously inhibited *smedolloid-1*, *smedbmp4-1* and *smedsmad4-1* with RNAi and observed intact animals over an extended period of time (Fig. 2A). Neoblasts in these animals must have been capable of basic cell replacement because RNAi animals did not display any of the defects, such as regression, curling and lysis within approximately 20 days, that are always observed in irradiated animals that lack neoblasts (Reddien et al., 2005a). By contrast, intact *smedolloid-1(RNAi)*, *smedsmad4-1(RNAi)*, and *smedbmp4-1(RNAi)* animals did display body plan abnormalities that indicate a requirement for BMP signaling in the maintenance of the adult body plan (Fig. 2A-F).

smedolloid(RNAi) intact animals displayed homeostatic defects including a severely raised, wrinkled dorsal surface that appeared within 36 days of the initial dsRNA treatment (11/11) and that persisted throughout the remainder of the 65 day experiment (Fig. 2A). This phenotype differed from that observed in *smedsmad4-1* and *smedbmp4-1* RNAi animals. *smedsmad4-1(RNAi)* and *smedbmp4-1(RNAi)* animals developed an extra set of photoreceptors (Fig. 2A-C), abnormal body protrusions appeared on the dorsal surface (Fig. 2A), and there were signs of ventralization (Fig. 2D-F). Histological observations indicated that RNAi animals were slowly becoming ventralized in the process of tissue maintenance. For example, *smedsmad4-1(RNAi)* animals had two sets of nerve cords, one set ventral and the other dorsal, and lacked expression of *smedbmp4-1* on the dorsal surface (Fig. 2D,E). These observations are consistent with the recent observation of ectopic ventral nerve cord cells on the dorsal sides of *Djbmp(RNAi)* animals in the planarian species *D. japonica* (Orii and Watanabe, 2007). Furthermore, *smedsmad4-1(RNAi)* and *smedbmp4-1(RNAi)* animals lacked the normal pattern of dorsal cilia, instead possessing a ventral-like distribution of cilia on their dorsal surfaces (Fig. 2F). *smedsmad4-1(RNAi)* animals could eventually flip over and glide on their dorsal surface, indicating that the dorsal surface had in fact acquired functional ventral-like cilia and animals had become functionally ventralized. In other regeneration experiments, *smedbmp4-1(RNAi)* animals also displayed the ability to glide on their dorsal surface (data not shown). In short, animals largely consisted of two ventral sides. Unlike abnormal embryos in many model systems, in which gross patterning abnormalities can lead to embryonic lethality and preclude studies of function in later development or adult life, gene perturbation in otherwise normal adult planarians can result in live ventralized animals. Our RNAi experiments therefore allow the study of late functional roles for genes that also have essential embryonic patterning roles. Dorsal-ventral patterning throughout bilaterally symmetric animals appears to be controlled by BMP signaling (De Robertis and Sasai, 1996; Martindale, 2005). Our data indicate BMP signaling maintains the dorsal-ventral polarity of adult planarians, even after proper dorsal-ventral patterning has been previously set.

SMEDBMP4-1 signaling regulates midline tissue formation in symmetric regeneration

RNAi of *smedolloid-1*, *smedsmad4-1* or *smedbmp4-1* followed by decapitation and tail removal led to the regeneration of blastemas indented at the midline (e.g. *n*=62/62, 49/49, and 17/21, respectively; Fig. 3A). Control RNAi animals essentially never regenerated cephalic or caudal blastemas that were indented at the midline. Because blastemas still arose after RNAi, yet showed a midline defect, we surmised that BMP signaling is not needed for regenerative outgrowth. In order to examine the cellular defects underlying the observed morphological phenotypes, we examined the differentiation of distal blastema cells by assessing expression of the D.21.6 gene. The D.21.6 gene is expressed in cells that reside around the entire periphery of the animal (Kato et al., 1999). D.21.6 expression was perturbed at the midline of cephalic and caudal blastemas of RNAi animals (*smedolloid-1(RNAi)*: 9/10 cephalic and 9/10 caudal blastemas abnormal; *smedsmad4-1(RNAi)*: 8/8 cephalic and 7/8 caudal blastemas abnormal; *smedbmp4-1(RNAi)*: 3/10 cephalic and 10/10 caudal blastemas were abnormally dispersed in

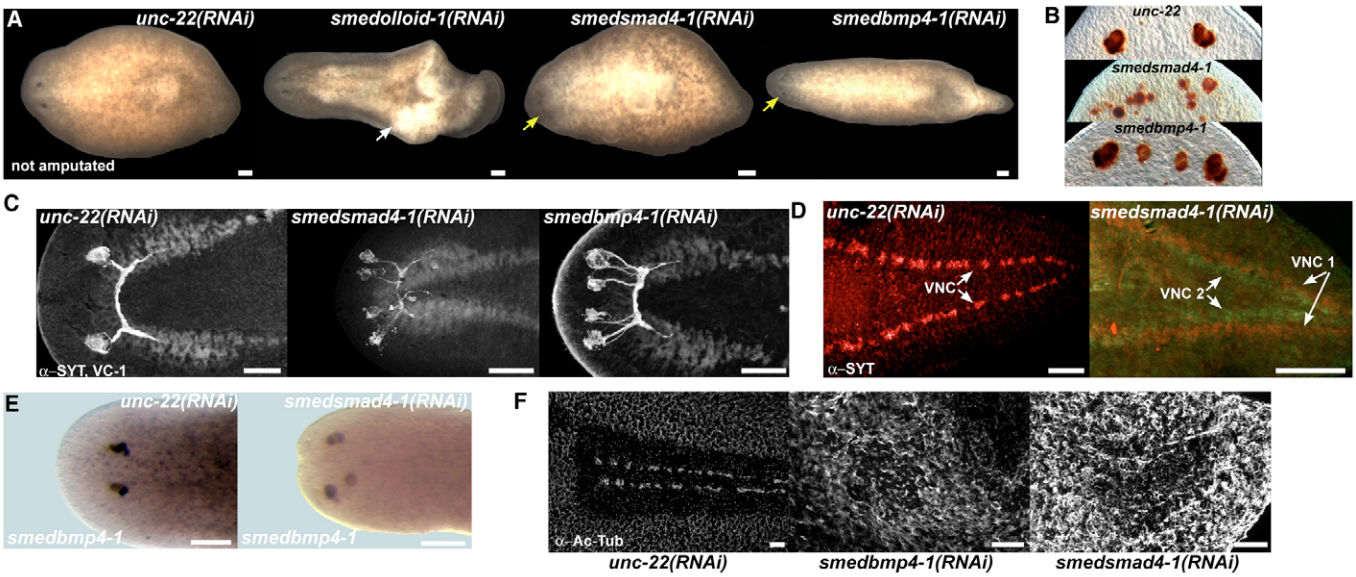

Fig. 2. A slow transformation of adult form occurs following prolonged perturbation of *smedbmp4-1* signaling. Control *unc-22(RNAi)* animals are shown on the left, or top, of each set of images. (**A**) *unc-22(RNAi)* and *smedsmad4-1(RNAi)* animals were pictured following ten dsRNA treatments and 83 total days of treatment. *smedbmp4-1(RNAi)* and *smedolloid-1(RNAi)* animals were pictured following seven dsRNA treatments and 65 days of total treatment. *smedolloid-1(RNAi)* animals display dorsal tissue ruffling (white arrow), and *smedsmad4-1* and *smedbmp4-1(RNAi)* animals display extra photoreceptors (yellow arrows). Anterior, left. Scale bars: 0.1 mm. (**B**) Magnification of fixed animals shows the extra photoreceptors in *smedsmad4-1(RNAi)* (126 days of RNAi) and *smedbmp4-1(RNAi)* (104 days of RNAi) animals. Anterior, up. (**C-E**) *smedsmad4-1(RNAi)* animals were fixed 126 days following initial dsRNA exposure and *smedbmp4-1(RNAi)* animals were fixed 104 days following initial dsRNA exposure. Anterior, left. Scale bars: 0.1 mm. (C,D) Animals were labeled with antibodies that recognize the photoreceptor neurons (VC-1, anti-Arrestin) and the cephalic ganglia (SYT, anti-Synaptotagmin). (D) Extra ventral nerve cords (green, vnc2) were present in a *smedsmad4-1(RNAi)* animal, located dorsal to the original nerve cords (red, vnc1). Animals were imaged with Zeiss Apotome-based optical sectioning, and the two sets of nerve cords false-colored. The regions shown are in the posterior of the animals. (E) In situ hybridizations with the *smedbmp4-1* riboprobe. *smedsmad4-1(RNAi)* animals had no detectable *smedbmp4-1* expression. (**F**) Animals were labeled with an antibody that recognizes cilia (anti-acetylated tubulin). The dorsal surface of *smedsmad4-1(RNAi)* animals display ventral-like cilia. The *smedbmp4-1(RNAi)* animals were fixed 117 days following initial exposure to dsRNA. Anterior, left. Scale bars: 0.05 mm.

dorsal-ventral directions with some gaps at the midline; Fig. 3B). These data indicate indented blastemas may arise because of absence of particular cell types.

SMAD4 proteins are co-SMADs that can mediate the signal transduction of multiple TGF-β ligands, including BMP2/4/DPP proteins (ten Dijke and Hill, 2004). Therefore, additional TGF-β signaling may be affected by inhibition of *smedsmad4-1*. We previously observed that cephalic blastema formation failed in *smedsmad4-1(RNAi)* animals during a second round of regeneration (Reddien et al., 2005a). We found that *smedsmad4-1(RNAi)* animals can fail to produce a blastema following a single amputation. Specifically, animals cut 17 days following the initial of four dsRNA feedings completely failed to regenerate blastemas (Fig. 3C). In some of these animals photoreceptors and outgrowths eventually developed in pre-existing tissues, indicating abnormal attempts at replacing missing tissues occurred in the absence of blastema formation (Fig. 3C). Minimal development of head structures appeared in the pre-existing tissue of *smedsmad4-1(RNAi)* animals that were cut 23 days following initial dsRNA exposure. Together, these results suggest that *smedsmad4-1* controls a process that is necessary for blastema formation. Similar RNAi protocols with the *smedbmp4-1* and *smedolloid-1* genes failed to block blastema formation. Because SMAD4 proteins can mediate the transduction of multiple TGF-β signals, *smedsmad4-1* may function with additional TGF-β proteins – separate from SMEDBMP4-1 – to mediate blastema formation.

SMEDBMP4-1 signaling affects patterning during symmetric regeneration

BMP signaling is known to regulate the dorsal-ventral patterning of embryos in many species (De Robertis and Sasai, 1996; Martindale, 2005) and, as described above, controls the dorsal-ventral patterning in adult planarian tissue homeostasis. To determine the patterning role of SMEDBMP4-1 signaling during symmetric regeneration, we assayed for the regeneration of dorsal midline cilia. In the cephalic blastemas of *smedbmp4-1(RNAi)* and *smedsmad4-1(RNAi)* animals, we detected abnormal cilia (Fig. 4A). Specifically, the normal stripe of dorsal cilia seen in control cephalic blastemas was absent in both *smedsmad4-1(RNAi)* and *smedbmp4-1(RNAi)* animals (10/10 and 2/4, respectively), accompanied by ventral-like and dispersed cilia (8/10 and 3/4, respectively). These observations suggest a dorsal-ventral patterning abnormality existed during regeneration in the animals. In the case of *smedolloid-1(RNAi)* animals, dorsal cilia were either not readily detected at all or displaced from the midline of blastemas after 7 days of regeneration (n=6; Fig. 4A).

We also determined patterns of photoreceptor neuron axons and the cephalic ganglia in RNAi animals. Normal photoreceptor neurons have projections that cross the midline and connect to the cephalic ganglia (Fig. 4B). In *smedolloid-1(RNAi)* animals, both photoreceptor neurons and the cephalic ganglia failed to reach and cross the midline (n=11/11). By contrast, the photoreceptor neuron axons and cephalic ganglia of *smedsmad4-1(RNAi)* and *smedbmp4-1(RNAi)* animals crossed the midline (11/11 and 10/10, respectively). However, the photoreceptor axons of *smedsmad4-1(RNAi)* and *smedbmp4-1(RNAi)*

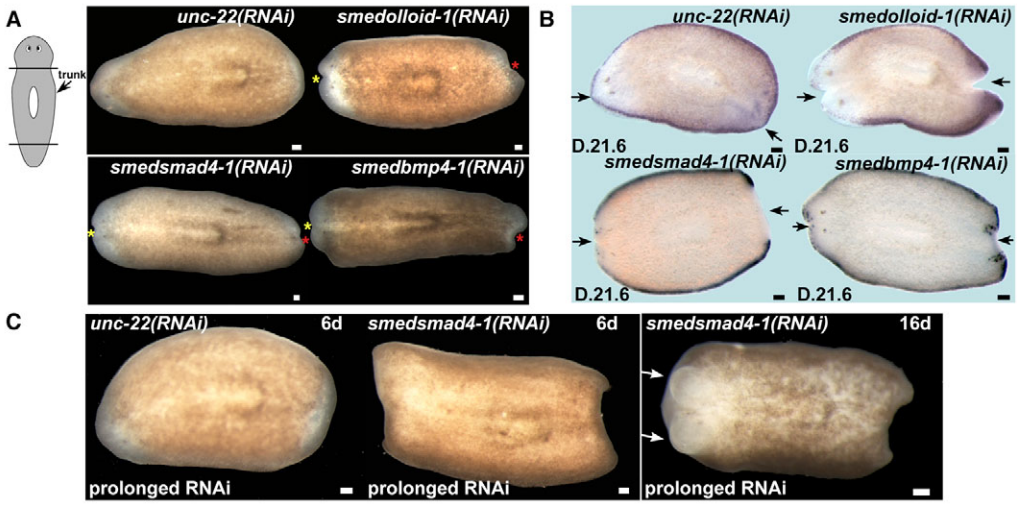

Fig. 3. The *smedbmp4-1* pathway is needed for regeneration of a normal midline. (**A**) The diagram on the left shows the sites of amputations (black lines); data for trunks regenerating new heads and tails are shown. Animals were at 7 (*unc-22* and *smedbmp4-1* RNAi animals), 8 (*smedolloid-1* and *smedsmad4-1* RNAi animals) and 6 days (*smedsmad4-1* prolonged RNAi) of regeneration. Yellow asterisks mark indented cephalic blastemas and red asterisks mark indented caudal blastemas. (**B**) Animals were labeled with the D.21.6 riboprobe that recognizes cells at the animal boundary (purple). Black arrows indicate cephalic and caudal blastema midline. The *unc-22(RNAi)* and *smedolloid-1(RNAi)* animals were at 11 days of regeneration, the *smedsmad4-1(RNAi)* animal was at 8 days of regeneration, and the *smedbmp4-1(RNAi)* animal was at 9 days of regeneration. (**C**) Prolonged exposure to *smedsmad4-1* dsRNA completely blocked blastema formation. Animals were fed dsRNA on days 1, 5, 13 and 17 and amputated the following day. Animals were decapitated and had tails removed. Blastema formation failed in the *smedsmad4-1(RNAi)* animals at 6 days following amputation (middle). Outgrowths (arrows) of tissue from pre-existing tissue in a *smedsmad4-1(RNAi)* animal showed head-like structures at 16 days of regeneration (right). Scale bars: 0.1 mm. Anterior, left.

animals projected in an atypical manner dorsally and laterally (11/11 and 9/10, respectively). Only one of 22 control RNAi animals displayed a similar dorsal projection. To determine whether the neuronal midline crossing abnormalities of *smedolloid-1(RNAi)* animals required SMEDBMP4-1 signaling, we inhibited both *smedbmp4-1* and *smedolloid-1* by feeding mixtures of bacteria expressing dsRNA for each of the two genes. Control animals were fed mixed sets of bacteria, in which one set expressed dsRNA for the *C. elegans unc-22* gene. Animals in which *smedbmp4-1* and *smedolloid-1* were both inhibited had more severely indented blastemas than did control single gene-inhibited animals (n=28; not shown). Whereas control *smedolloid-1(RNAi)* animals had midline crossing abnormalities (8/10), doubly inhibited animals had the *smedbmp4-1(RNAi)*-like appearance (Fig. 4B, 9/9). This observation suggests that some of the cellular abnormalities of *smedolloid-1(RNAi)* animals resulted from an aberrant pattern of SMEDBMP4-1 activity. Tolloid proteins generally function to enhance the signaling activity of BMP2/4/DPP-like proteins by cleaving a Chordin-like BMP antagonist (Piccolo et al., 1997). Why photoreceptor axon patterning differences between *smedolloid-1(RNAi)* and *smedbmp4-1(RNAi)* animals exist is unknown, but could reflect a role for SMEDOLLOID-1 in determining the normal pattern – as well as level – of SMEDBMP4-1 activity. It is also possible that there exists some independent roles for these genes. Together, our observations reveal midline and dorsal-ventral patterning abnormalities that occur following perturbation of BMP signaling in planarian regeneration.

SMEDBMP4-1 signaling is needed for lateral regeneration

Given the requirement for SMEDBMP4-1 signaling for normal formation of tissue at the distal midline in regeneration and the expression of *smedbmp4-1* at the midline, we asked whether this pathway is needed for regeneration of animal fragments challenged to re-establish their mediolateral axis. Individual inhibition of *smedolloid-1*, *smedsmad4-1* and *smedbmp4-1* perturbed the ability of left-right asymmetric animal fragments to regenerate (Fig. 5A-D). This observation indicates that blastema formation along sagittal or parasagittal cuts (both such amputations result in fragments that lack left/right symmetry about a midline, Fig. 1A) is different from blastema formation along transverse cuts (left/right symmetric fragments, Fig. 1A) in at least the requirement for normal BMP signaling. Parasagittal animal amputations were performed to generate thin and thick fragments (Fig. 1A and Fig. 5A-D); both such fragments normally regenerate, involving the formation of a blastema along the wound surface (Morgan, 1900). Blastemas were scored as unpigmented outgrowths from pigmented pre-existing tissue at the wound site. *smedolloid-1(RNAi)* thin fragments failed to regenerate along the body length (n=38/43), but all produced a head with both photoreceptors; thick fragments also failed to regenerate (n=45/49). *smedsmad4-1(RNAi)* thin (n=34) and thick (n=37) fragments failed to regenerate along the body length. RNAi of *smedbmp4-1* caused a less severe block in lateral regeneration than did RNAi of *smedolloid-1* or *smedsmad4-1* and was coupled with aberrant growths and tissue ruffling; however, animals fed dsRNA six times and amputated parasagittally 29 days following dsRNA exposure were robustly abnormal (n=18 thick fragments displayed minimal to no blastema formation and n=14 thin fragments displayed folding and ruffling of tissue along the wound site as well as minimal apparent blastema formation). In addition, perturbation of BMP pathway genes in animals cut sagittally – along the axis of left-right symmetry – produced animal halves that displayed failures in blastema formation (see Fig. S4 in the supplementary material).

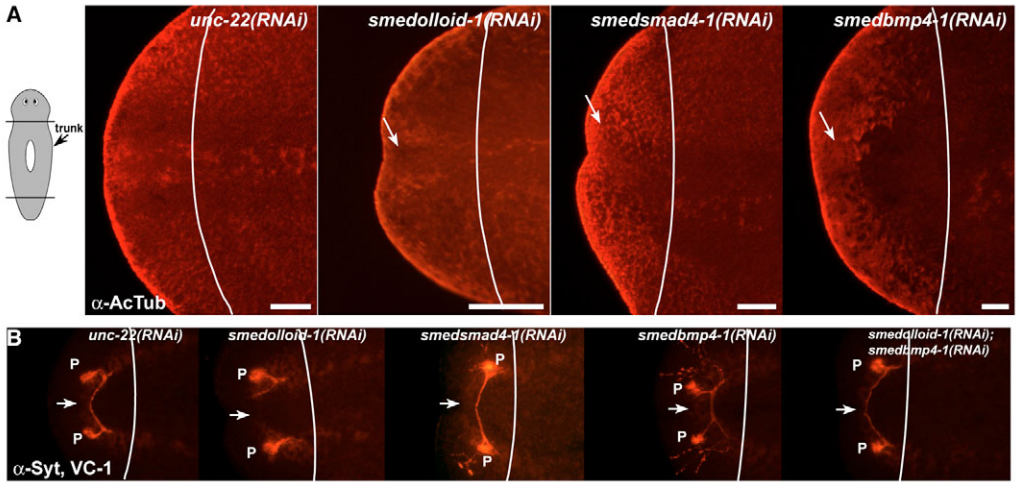

Fig. 4. *smedbmp4-1* signaling is required for blastema patterning. White lines indicate approximate boundary between pre-existing tissues and blastema. The diagram on the left shows the sites of amputations (black lines); data for trunks regenerating new heads are shown. Anterior, left. (**A**) Animals were labeled with an antibody that recognizes cilia (anti-acetylated tubulin). White arrows indicate aberrant cilia patterning at the midline of cephalic blastemas. The dorsal surface is shown. Animals had between 7 and 9 days of regeneration. Scale bars: 0.1mm. (**B**) Animals were labeled with antibodies that recognize the photoreceptor neurons (VC-1, anti-Arrestin) and the cephalic ganglia (SYT, anti-Synaptotagmin). Animals had between 7 and 8 days of regeneration. P, photoreceptors. Arrows indicate midline of cephalic ganglia and optic chiasmata.

The failure in lateral blastema formation of RNAi animals was also evident following labeling with the D.21.6 riboprobe (Fig. 5B,D). Thick and thin *smedolloid-1(RNAi)* fragments failed to regenerate D.21.6-expressing cells along the lateral wound (n=10/10 and 14/16, respectively). Similarly, thick and thin *smedsmad4-1(RNAi)* fragments failed to produce D.21.6-expressing cells along lateral, parasagittal wounds (n=8 and n=6, respectively). *smedbmp4-1(RNAi)* fragments were abnormal, with missing sections and abnormally dispersed sections of D.21.6 labeling (n=26/27). The *smedbmp4-1(RNAi)* phenotype was weaker than that of *smedolloid-1(RNAi)* or *smedsmad4-1(RNAi)* animals, indicating the possible existence of redundant BMP/DPP-like genes or independent roles for the genes used in this study. We used a second marker for tissues that are formed in lateral regeneration (H.1.3B, subepidermal marginal adhesive cells) and found that sagittally cut *smedolloid-1(RNAi)* animals failed to produce new

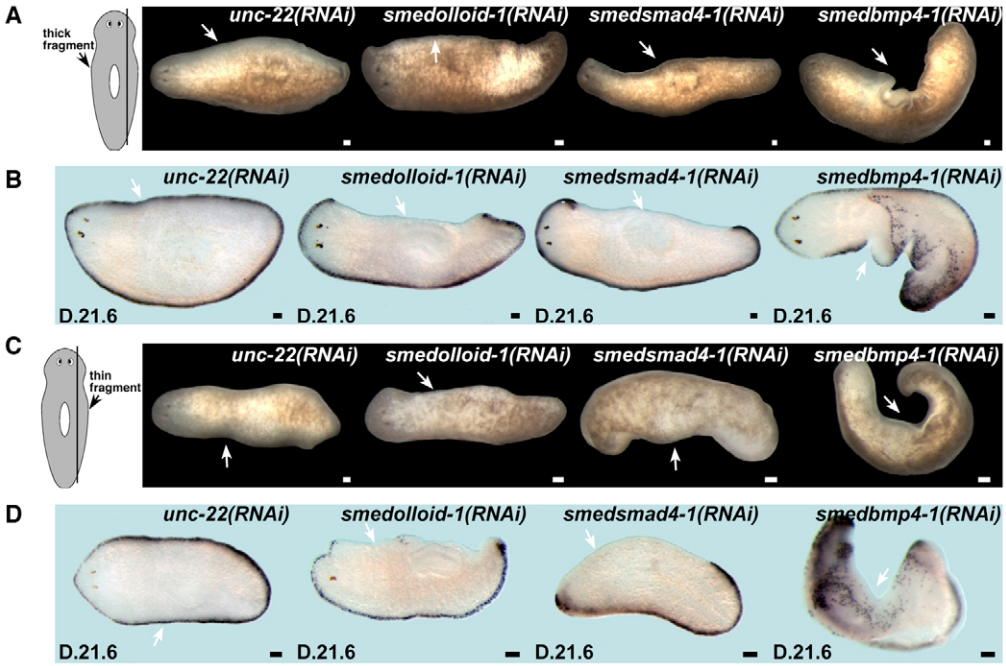

Fig. 5. Differentiation abnormalities of lateral blastemas in animals with perturbed *smedbmp4-1* signaling. (**A-D**) Diagrams in A and C show the sites of amputation (black lines) that generate thick or thin fragments that lack bilateral symmetry. Animals were examined at 14 days (*unc-22* and *smedsmad4-1* RNAi animals) and 22 days (*smedolloid-1* and *smedbmp4-1* RNAi animals) after amputation. White arrows indicate the wounded side. (A,B) Thick fragments. (C,D) Thin fragments. (B,D) Animals were labeled with the D.21.6 riboprobe that recognizes cells at the animal boundary (purple). Anterior, left. Scale bar: 0.1 mm.

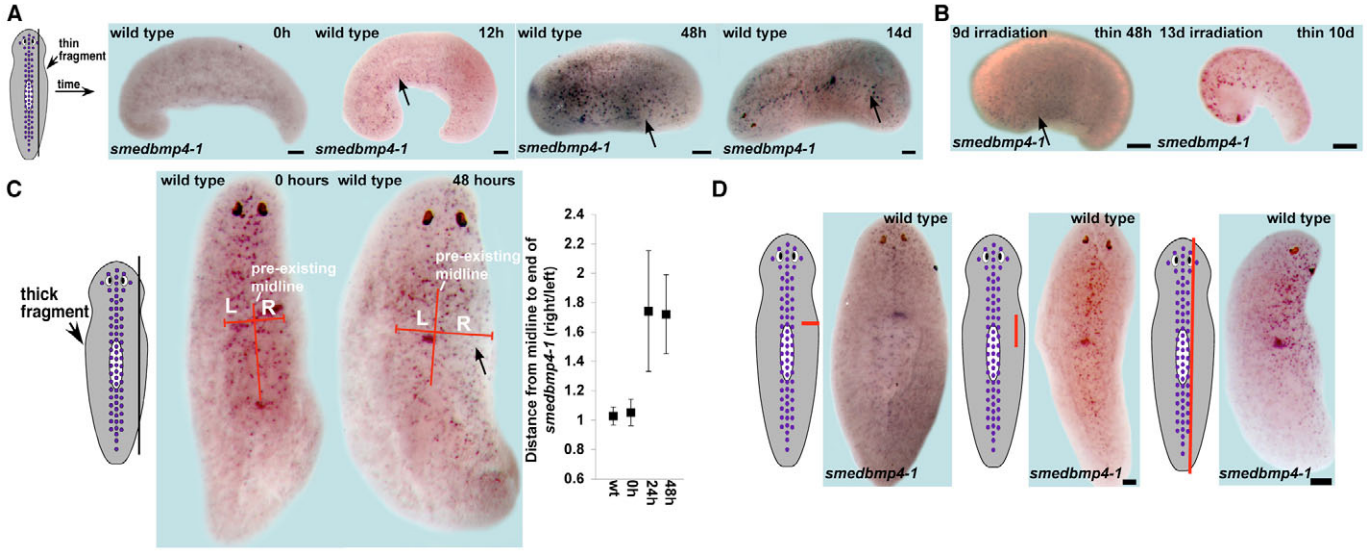

Fig. 6. *smedbmp4-1* is expressed in the pre-existing tissue of asymmetric fragments lacking a midline and expands towards wound sites in asymmetric fragments containing a midline. (**A-D**) Diagrams show a model of the *smedbmp4-1*-expressing midline, with black and red lines indicating the sites of amputation or injury. Arrows indicate new *smedbmp4-1* expression. In situ hybridizations were performed with the *smedbmp4-1* riboprobe. (A,B) Anterior, left. Thin, lateral wild-type fragments were generated that lacked a pre-existing midline. New *smedbmp4-1* expression was detected in these fragments by in situ hybridization. (B) Animals were irradiated with 6,000 Rad prior to amputation. New *smedbmp4-1* expression was detected 48 hours later (left), which failed to resolve to a midline pattern (right). (C) Representative animals (thick fragments) fixed and labeled using the *smedbmp4-1* riboprobe, 0 and 48 hours following amputation. '0' hours refers to all animals fixed shortly (between 0 and 3 hours) after surgery. The pre-existing midline of animals is defined as a line connecting the tip of the head through the photoreceptors and the middle of the pharynx, to the tip of the tail. The left (L) and right (R) regions of *smedbmp4-1* expression were determined by measuring the distance from the pre-existing midline to the end of the field of expression just above and below the pharynx. Means were taken per animal from these two measurements for both the left and right sides (red lines), and at least eight animals were scored for each time point. The graph indicates the mean ratios and standard deviations of measurements taken from wild-type animals and from animals fixed shortly after amputation (0 h), 24 hours after amputation, or 48 hours after amputation. Only animals missing their right sides were scored. The ratio of the right distance to the left distance indicated expansion towards the right, and was at each time point significantly different from the controls (P<0.0001, t-test). Furthermore, 66/73 fragments that were either in the 0 h or a 24 h or later group could be identified correctly in blind scoring. (D) Left, a wild-type animal was cut with a side nick perpendicular to the midline (red line in diagram). The nick was allowed to seal and animals were fixed 26 hours later; no obvious change in *smedbmp4-1* expression was detected (n=24). Middle, a wild-type animal was cut with an internal nick parallel to the midline (red line in diagram). The nick was allowed to seal and was fixed 48 hours later; no obvious change in *smedbmp4-1* expression was detected (n=15). Right, a wild-type animal was cut longitudinally (red line in diagram). 48 hours later no obvious change in *smedbmp4-1* expression was detected (n=11). Scale bars: 0.1 mm. h, hours; d, day.

H.1.3B-positive cells (see Fig. S4 in the supplementary material). Therefore, whereas the planarian BMP pathway appears to affect patterning of blastemas from symmetric blastemas (Reddien et al., 2005a), it appears to control a necessary step in blastema formation in bilaterally asymmetric fragments. Though RNAi may not result in complete gene knockdown, the difference between symmetric and asymmetric regeneration is highly penetrant and has been demonstrated with the same sets of RNAi animals. Therefore, we suggest that asymmetric and symmetric blastema formation have different genetic requirements.

New *smedbmp4-1* expression in pre-existing tissue of bilaterally asymmetric fragments

Our data suggest that the *smedbmp4-1* pathway is needed for asymmetric regeneration. Why is a pathway that is active at the dorsal midline needed in early events of blastema formation in fragments lacking old midline? We examined expression of *smedbmp4-1* and found that expression, like that of a BMP-like gene in *D. japonica* (Orii et al., 1998), appears anew in the pre-existing tissue of asymmetric fragments between 6 hours (0/8 expressed *smedbmp4-1*) and 12 hours following wounding (10/10 thin lateral fragments expressed *smedbmp4-1*; Fig. 6A). These new *smedbmp4-1*-expressing cells appeared initially along the wound site, resolved to a midline pattern within 8-14 days (Fig. 6A), and appeared similar in size and position to the *smedbmp4-1*-expressing cells of an intact animal.

To assess whether *smedbmp4-1* expression results from the production of new cells, requiring cell proliferation, we used irradiation. Irradiation has long been known to specifically eliminate the only known mitotically active cells in planarians, i.e. the neoblasts (Bardeen and Baetjer, 1904; Dubois, 1949; Reddien et al., 2005b). We amputated irradiated animals parasagittally and examined expression of *smedbmp4-1*. We found that *smedbmp4-1* expression can occur in irradiated thin fragments, indicating expression does not require cell proliferation. For example, we performed parasagittal amputations 7 days after irradiation and the resultant thin fragments, lacking original *smedbmp4-1* expression, newly expressed *smedbmp4-1* normally (n=15; Fig. 6B). The initial expression of *smedbmp4-1* in these irradiated fragments failed to resolve into a midline pattern before animal death, indicating resolution of the *smedbmp4-1* pattern requires regeneration (Fig. 6B). Regenerating planarian fragments not only produce new tissue at wound sites (blastema formation), but also dramatically re-arrange pre-existing tissues to result in a small animal with a complete complement of organ systems of appropriate proportions. Our data

suggest that new *smedbmp4-1*-expressing cells are produced from a change in function of other cells. We suggest that activity of *smedbmp4-1* at these new sites changes positional identities of existing tissues and plays an instructive role in the formation of lateral blastemas.

smedbmp4-1 expression expands towards missing sides

Why do thick fragments lacking a side, but containing a pre-existing midline, have blastema formation abnormalities when the *smedbmp4-1* pathway is perturbed by RNAi? We found that asymmetric thick fragments displayed changed expression of *smedbmp4-1* (Fig. 6C). Specifically, the field of cells expressing *smedbmp4-1* expanded towards the missing lateral side, such that cells expressed *smedbmp4-1* more strongly in more lateral regions than is normally observed in the wild type or in freshly amputated animals (Fig. 6C). By contrast, simple wounding was not sufficient to induce robust new expression of *smedbmp4-1*. This was demonstrated by generating side incisions, both perpendicular and parallel to the midline and which healed by resealing. We detected no obvious increase in expression of *smedbmp4-1* along these wound sites (Fig. 6D). These observations indicate that maintenance of *smedbmp4-1* signaling only at the midline requires an intact, lateral side. Furthermore, amputation near the midline did not obviously cause an expansion on the unwounded side, indicating expansion occurs towards missing sides only (Fig. 6D). Given *smedbmp4-1* pathway activity is needed for thick pieces lacking a side to regenerate, an expanded field of *smedbmp4-1* expression could have an instructive role in the regeneration of a missing side.

DISCUSSION

Regeneration requires not only the ability to produce new cells but also the ability to specify what to make. In planarians, regeneration is accomplished by a combination of new tissue growth at the wound site and change in pre-existing tissue. Both of these aspects of planarian regeneration require the tissue of the animal fragment to determine what is missing. How? Our data identify one key pattern-regulation mechanism needed for specific types of regeneration (the process of bilaterally asymmetric regeneration) that exists at the planarian dorsal midline. A BMP2/4/DPP-like cell signaling gene (*smedbmp4-1*) was expressed along the planarian dorsal midline and animal fragments generated by surgery to lack bilateral symmetry could not regenerate normally if *smedbmp4-1* signaling was perturbed. By contrast, wild-type planarian fragments can regenerate new organisms, even if they lack a pre-existing midline of bilateral symmetry.

The process of bilaterally asymmetric regeneration can be observed after parasagittal amputations that generate a thick fragment possessing the original midline – now off-center – and a thin fragment with no original midline tissue. How do these pieces re-acquire bilateral symmetry? Our data suggest that new *smedbmp4-1* expression in asymmetric fragments directs the changes to tissues that must occur in regeneration. In thin lateral pieces, for example, expression was induced within 12 hours of amputation on the side of the fragment near the old midline. This new *smedbmp4-1* expression did not require new cell production. Therefore, mechanisms exist that allow a rapid change in site of expression of a signaling protein regulating planarian body patterning within existing cells. We suggest change in lateral positional identity is a needed step in asymmetric blastema formation. Changes in the site of expression of *smedbmp4-1* are also involved in the regeneration of sides in thick asymmetric fragments that still possess the original midline. Specifically, lack of a side resulted in the spreading of the field of *smedbmp4-1*-expressing cells towards the wound. Because simple wounding did not induce *smedbmp4-1* expression, we suggest that the left and right flanks of the planarian body plan normally constrain *smedbmp4-1* expression. In this model, the absence of a side alleviates constraint and *smedbmp4-1* expression expands. Animals may detect that they are missing a side because SMEDBMP4-1 becomes expressed at or expands towards wound sites capable of generating a regeneration blastema. There are multiple known BMP antagonists; such an antagonist could act in planarians to normally restrict *smedbmp4-1* expression. Based upon data from other organisms, a Chordin-like molecule would be the best candidate, but no Chordin-like gene can, as yet, be identified in existing planarian gene and genome sequence. The ongoing planarian genome sequence project could allow exploration of such a hypothesis in the future.

BMP2/4/DPP functions in many animal embryos in dorsal-ventral patterning (Holley et al., 1995). Our data indicate that BMP signaling in planarians regulates dorsal-ventral patterning of new tissues in regeneration and during the maintenance of adult tissues. Given the current positioning of planarians in the understudied Lophotrochozoa (Adoutte et al., 2000), one of three main groupings of bilaterally symmetric animals, our data also support the view of a BMP system controlling dorsal ventral patterning throughout the Bilateria (De Robertis and Sasai, 1996; Martindale, 2005). Specifically, *smedbmp4-1* signaling regulated dorsal-ventral and midline patterning during adult tissue homeostasis and regeneration. Normal dorsal-ventral patterning is likely to be accomplished by regulated expression of *smedbmp4-1* to the dorsal midline. In the absence of planarian BMP signaling, we observed otherwise normal, fully formed adults slowly transform into animals with two ventral sides. This transformation of adult form did not preclude animal viability and is a striking demonstration of the role of signaling molecules in the maintenance of the pattern of adult tissues. Adult life requires the ability to functionally replace damaged and aged cells. These tasks are accomplished by stem cells and the descendants of stem cells; approximately 10 billion cells are produced per day in the human body during normal homeostasis (Heemels, 2000). How new cells are deployed to enter solid tissues and maintain adult form is an understudied arena of biology. Our data indicate that BMP signaling has an instructive role in the maintenance of dorsal character in tissues being replaced gradually by stem cells.

Animals lacking the normal function of a planarian Tolloid-like gene (*smedolloid-1*) or a BMP2/4/DPP-like gene (*smedbmp4-1*) were able to regenerate anterior and posterior blastemas, symmetrically around the midline, but these were abnormal (indented and lacking some differentiated tissues) at the midline. These data indicate BMP-like signaling regulates midline regeneration. Midline formation is a critical component of bilaterally symmetric body plans (Meinhardt, 2004). The conserved localization of BMP2/4/DPP activity at the dorsal midline suggests that universal BMP-mediated medial patterning strategies may be deployed to accomplish a multitude of developmental tasks across the metazoa.

Despite the similar aspects of the phenotypes associated with inhibition of the BMP pathway components studied in this report, some differences exist. For example, *smedolloid-1(RNAi)* animals display very severe midline patterning abnormalities (e.g. failures of axons to cross the midline), but do not display the ventralization defects observed in *smedbmp4-1* and *smedsmad4-1* RNAi animals. Furthermore, *smedbmp4-1(RNAi)* animals showed a generally weaker lateral regeneration defect than did animals in which *smedolloid-1* or *smedsmad4-1* were perturbed. The reasons for these differences are unknown, but could indicate independent functions for some of these

genes, or redundancy with undiscovered factors. Future investigation of these genes and other BMP pathway components should help illuminate the mechanisms by which the BMP pathway components act together, or separately, in regeneration.

Planarians constantly rebuild their adult bodies during normal homeostasis and are capable of dramatically altering pre-existing tissues during regeneration (Morgan, 1898; Morgan, 1900; Morgan, 1902; Reddien and Sánchez Alvarado, 2004). These properties of planarian regeneration have long puzzled biologists. Robust patterning and regulatory systems must exist that allow animal tissue to specify what to make and in what manner to re-pattern existing tissue. Understanding these patterning and regulatory systems should prove critical in understanding regeneration. The problems faced by a regenerating planarian bear some similarities to those faced by a surgically manipulated embryo. Specifically, some injured embryos and animals can restore the pattern of the entire animal and produce what would have existed in the removed tissue. Experimental embryology introduces these concepts as regulative development, in which some animal embryos replace missing cells via self-regulation. In extreme examples, one cell of the two-cell sea urchin embryo can develop into a smaller, but normally patterned larva, and the dorsal half of a *Xenopus* blastula embryo can produce an entire embryo (Driesch, 1893; Reversade and De Robertis, 2005; Spemann, 1924). Self-regulation of dorsal-ventral patterning, following surgical manipulation of *Xenopus* embryos, involves a BMP signaling system (Reversade and De Robertis, 2005). In planarians the ability to restore animal form can occur from a large array of wound types and involves the production of new tissue at wound sites acting in concert with changes in pre-existing tissue. These processes lack mechanistic explanation. Our data identify a key role for a BMP signaling system in lateral blastema formation. Our experiments with the *smedbmp4-1* pathway provide some of the first genetic insights into the topics of blastema specification and restoration of form in regeneration, and provide paradigms for future study of the genetic and cellular bases for the tissue patterning principles of planarian regeneration.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/22/4043/DC1

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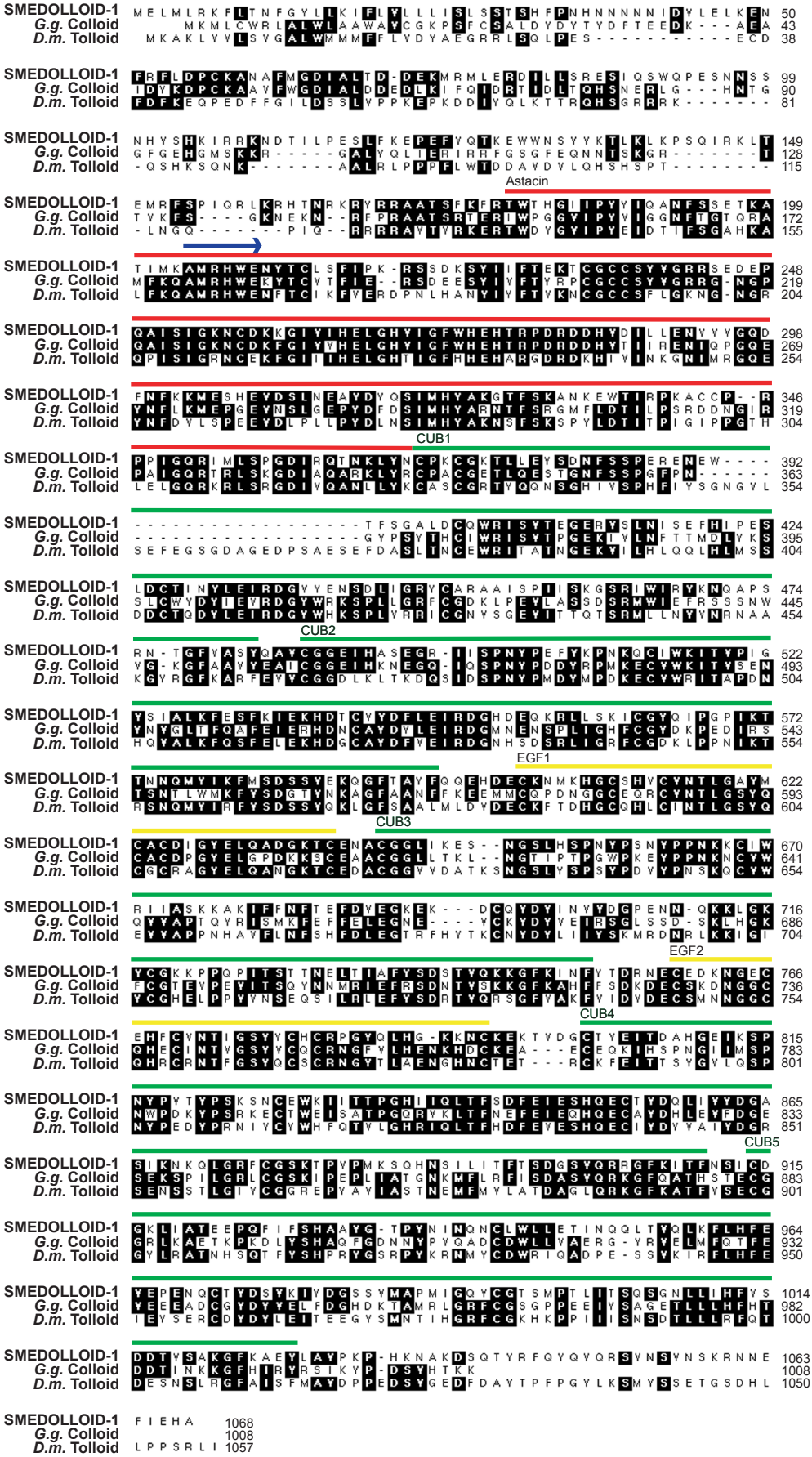
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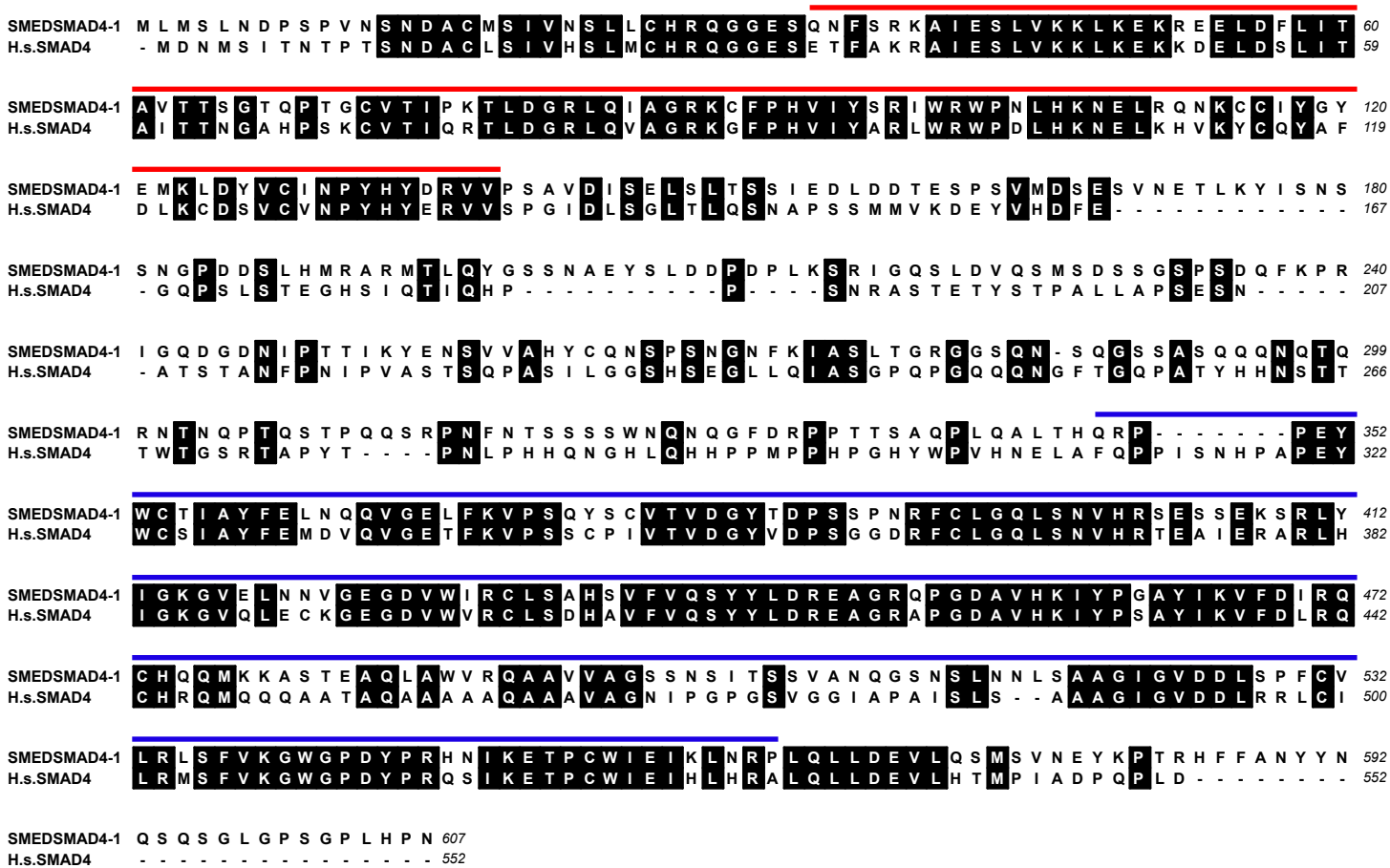
G.g. Colloid
D.m. Tolloid
Astacin
CUB1
CUB2
CUB3
EGF1
EGF2
CUB4
CUB5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SMEDSMAD4-1 S N G P D D S L H M R A R M T L Q Y G S S N A E Y S L D D P D P L K S R I G Q S L D V Q S M S D S S G S P S D Q F K P R 240
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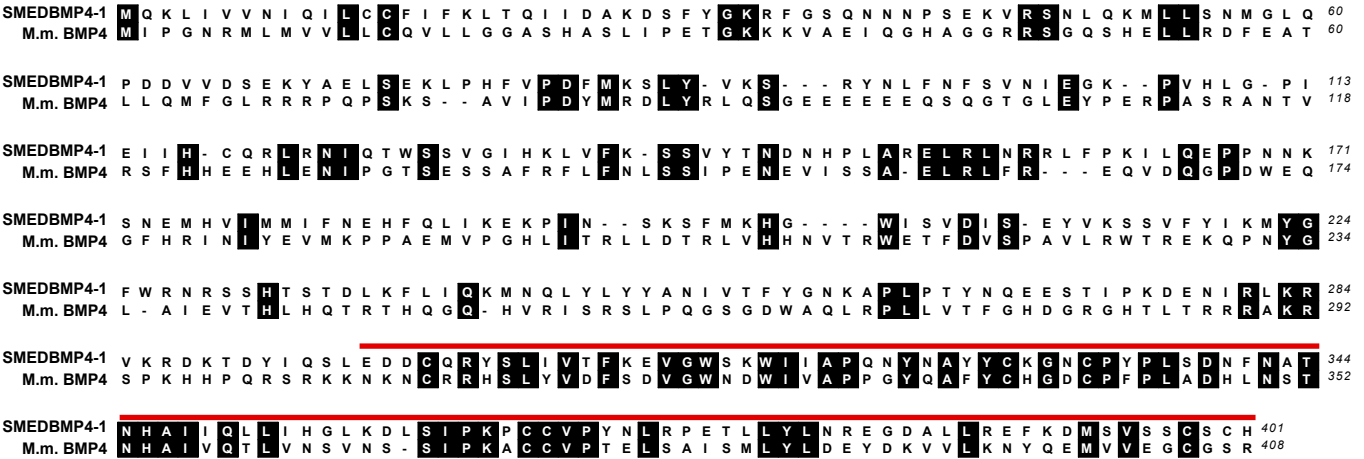
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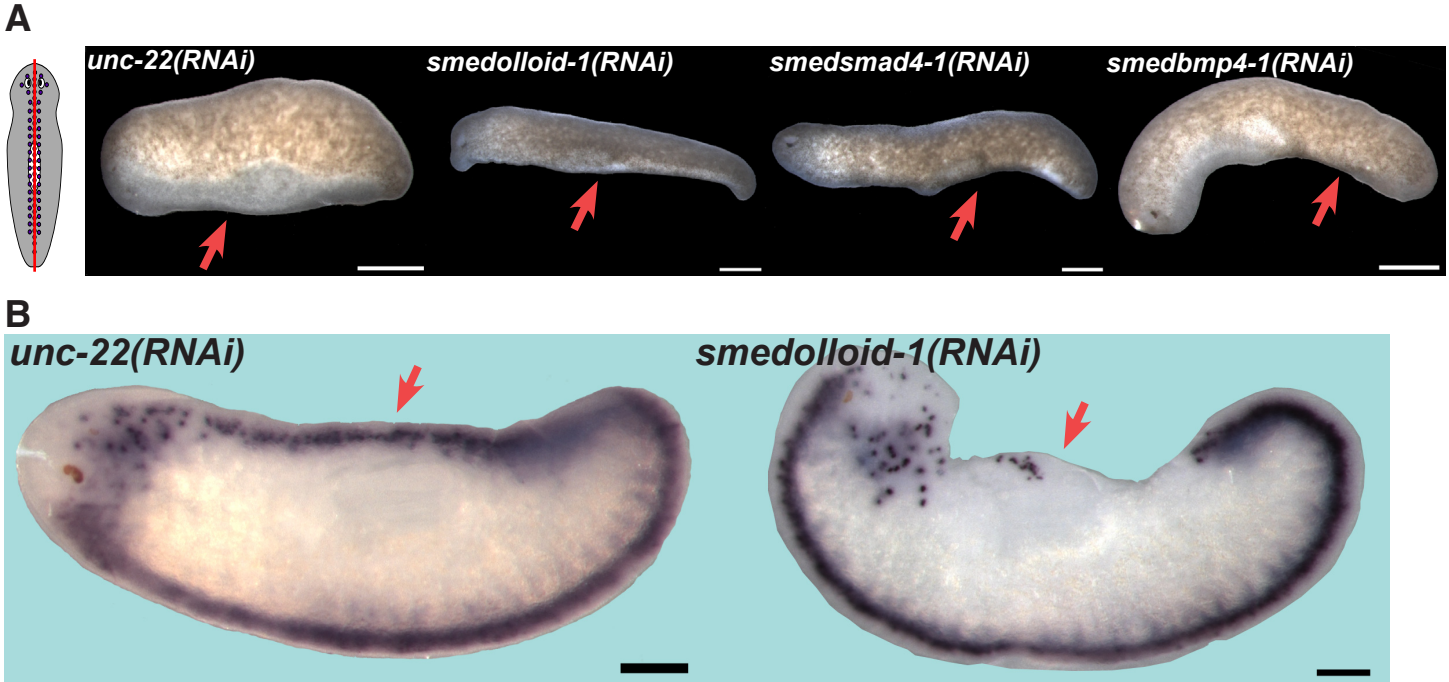
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