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A Bmp/Admp Regulatory Circuit Controls Maintenance and Regeneration of Dorsal-Ventral Polarity in Planarians

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Summary

Animal embryos have diverse anatomy and vary greatly in size. It is therefore remarkable that a common signaling pathway, BMP signaling, controls development of the dorso-ventral (DV) axis throughout the Bilateria [1–8]. In vertebrates, spatially opposed expression of the BMP family proteins Bmp4 and Admp (antidorsalizing morphogenetic protein) can promote restoration of DV pattern following tissue removal [9–11]. *bmp4* orthologs have been identified in all three groups of the Bilateria (deuterostomes, ecdysozoans, and lophotrochozoans) [12]. By contrast, the absence of *admp* orthologs in ecdysozoans such as *Drosophila* and *C. elegans* has suggested that a regulatory circuit of oppositely expressed *bmp4* and *admp* genes represents a deuterostome-specific innovation. Here we describe the existence of spatially opposed *bmp* and *admp* expression in a protostome. An *admp* ortholog (*Smed-admp*) is expressed ventrally and laterally in adult *Schmidtea mediterranea* planarians, opposing the dorsal-pole expression of *Smed-bmp4*. *Smed-admp* is required for regeneration following parasagittal amputation. Furthermore, *Smed-admp* promotes *Smed-bmp4* expression and *Smed-bmp4* inhibits *Smed-admp* expression, generating a regulatory circuit that buffers against perturbations of Bmp signaling. These results suggest that a Bmp/Admp regulatory circuit is a central feature of the Bilateria, used broadly for the establishment, maintenance, and regeneration of the DV axis.

Results and Discussion

Spatially Opposed Expression of Bmp and Admp Genes in Adult Planarians

Planarians are flatworms famous for their regenerative capacities. The ability of planarians to regenerate entire adult animals from small tissue fragments makes them well suited for the study of body axis polarization and patterning [13]. Furthermore, their phylogenetic position as a member of the protostome superphylum Lophotrochozoa makes them ideal for identifying features that are conserved across the Bilateria. Planarians utilize a dorsally expressed *bmp4* ortholog, *Smed-bmp4* (in short, *bmp4*), to maintain and regenerate the dorso-ventral (DV) axis [2–4]. We isolated a putative *admp* ortholog in the planarian *Schmidtea mediterranea* that is, to our knowledge, the first characterized in a protostome [14–16] (see Figure S1 available online and functional data below). We cloned two highly similar *admp* sequences (*Smed-admp-1a* and *Smed-admp-1b*; see Figure S1 and Supplemental Experimental Procedures for details); it is unknown whether these

sequences reflect the existence of distinct *admp* alleles or of highly similar *admp* paralogs. We refer to a single gene in this text as *Smed-admp* (in short, *admp*).

admp expression was detected in subepidermal cells on the ventral animal midline and around lateral animal edges at the dorsal/ventral boundary (Figures 1A and 1B). These ventral and lateral domains spatially oppose the *bmp4* expression domain on the dorsal midline. Double labeling with *admp* and *bmp4* RNA probes revealed that expression of these genes does not detectably overlap (Figure 1C). *admp* expression opposing *bmp4* expression in planarians is noteworthy, because it provides the first example of spatially opposed *bmp* and *admp* expression outside of the deuterostome lineage. Following head and tail amputation, lateral *admp* expression first appeared at wound sites by 48 hr, whereas ventral *admp* expression decreased at 24 hr, 48 hr, and 60 hr before increasing again at 72 hr (Figure S2A). These data indicate that ventral *admp* expression is regulated following transverse amputation, possibly by wound-induced factors. *admp* expression was not detected dorsally at any point during regeneration (Figure S2A). Together, *admp* and *bmp4* form complementary expression domains that identify the dorsal and ventral midlines, as well as the lateral, dorsal-ventral boundary, of animals.

Smed-admp Is Required for Lateral Planarian Regeneration

To investigate the role of *admp* in regeneration, we inhibited *admp* expression with RNA interference (RNAi) and amputated animal heads and tails. Planarian regeneration involves new tissue outgrowth at wound sites called a blastema [13]. *admp(RNAi)* fragments displayed regeneration defects including indented head and tail blastemas, a hallmark phenotype of planarian Bmp pathway dysfunction [2, 3], as well as uncoordinated movement (Figure 2A, Movie S1, and Movie S2). In situ hybridization with a marker for lateral-edge cells identified defects in regeneration of lateral DV boundary tissue (Figure 2B). We conclude that *admp* is required for the regeneration of tissues at lateral animal edges, at the midpoint between dorsal and ventral poles, following transverse amputation.

Bmp signaling is crucial for lateral planarian regeneration following sagittal amputations [2, 3]. Parasagittal amputation produces two left-right asymmetric fragments: a thin fragment that must regenerate an appropriately sized *bmp4* expression domain de novo, and a thick fragment that must reposition and rescale its *bmp4* expression domain to accommodate new animal dimensions. Parasagittal amputations therefore present a stringent test of establishment and scaling of DV, as well as medial-lateral (ML), pattern. *admp(RNAi)* thin fragments were able to regenerate some structures along the anteroposterior (AP) axis within preexisting tissue (photoreceptors and pharynx); however, they failed to regenerate a new side and corresponding lateral marker expression (Figure 2C). *admp(RNAi)* thick fragments also failed to regenerate a side (Figures 2C and 2D; Figure S2D). Furthermore, nonamputated *admp(RNAi)* animals displayed aberrant body dimensions and ML marker expression following several months of *admp* inhibition (Figure 2E, Figure S3, and Movie S3),

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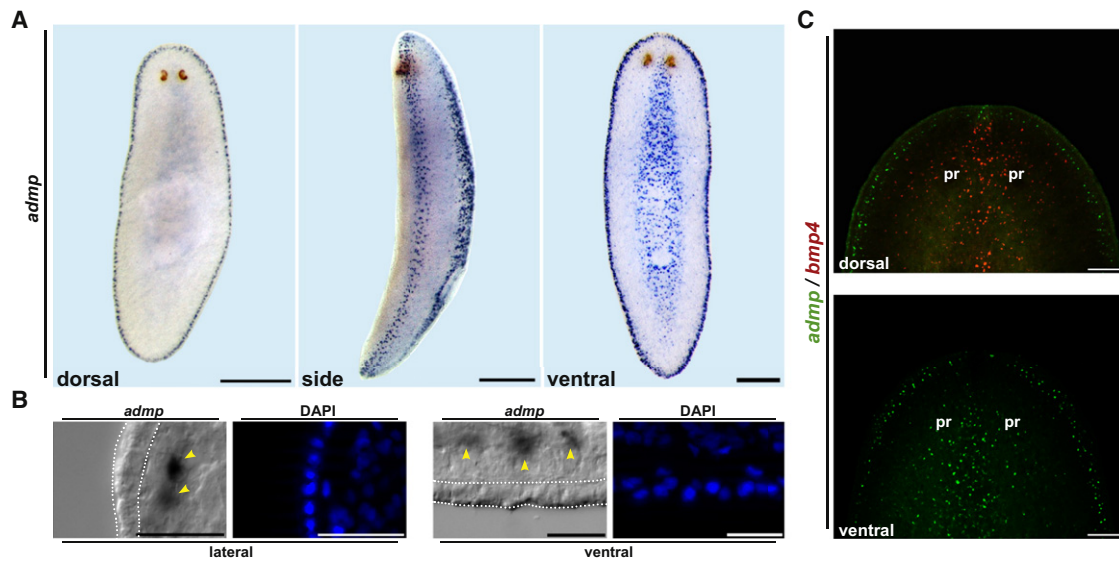


Figure 1. *Smed-admp* Is Expressed Ventrally and Laterally

(A) In situ hybridization with *admp* RNA probe displayed ventral and lateral expression.

(B) Transverse sections (20 μ m), differential interference contrast (DIC) images: *admp*-expressing cells were subepidermal (yellow arrowheads). White lines represent epidermis.

(C) Wild-type animals double labeled with *admp* (green) and *bmp4* (red) RNA probes. pr denotes photoreceptors.

Scale bars represent 200 μ m for (A) and (C) and 20 μ m for (B). Anterior is up.

suggesting that *admp* is crucial for maintaining body form and proper ML pattern during animal homeostasis. We conclude that *admp* is required for lateral planarian regeneration and ML pattern maintenance.

***Smed-admp* Promotes *Smed-bmp4* Expression**

The indented head and tail blastemas and the failed lateral regeneration in *admp(RNAi)* animals are consistent with a defect in Bmp signaling [2, 3]. *bmp4* promotes dorsal tissue maintenance and regeneration; we therefore investigated the role of *admp* in DV patterning. Whereas animals inhibited for *admp* expression alone did not show dorsal expansion of ventral markers, *admp(RNAi)* animals exposed to a low dose of *bmp4* double-stranded RNA (dsRNA) became more ventralized near wound sites than did control animals exposed to the same *bmp4* dsRNA dose (Figure 3A). These results indicate that animals depleted of *admp* activity become hypersensitive to small decreases in Bmp signaling level during DV axis regeneration.

We next assessed whether *admp* influences *bmp4* expression. Thin fragments produced by parasagittal amputation must reexpress *bmp4* during regeneration. *admp(RNAi)* thin fragments displayed reduced *bmp4* expression, and this expression domain did not reposition to reflect a new dorsal midline (Figure 3B). These results indicate that *admp* promotes *bmp4* expression and controls the positioning of *bmp4* expression during regeneration of left-right asymmetric fragments. Whether Admp signaling regulates *bmp4* expression by direct action or through some other mechanism is unknown.

In addition to regenerating, planarians undergo extensive tissue turnover and growth as adults—processes that also require patterning genes for instructing new cell identities [17–19]. Consequently, if *admp* promotes *bmp4* expression, nonamputated *admp(RNAi)* animals should display reduced *bmp4* expression. Quantitative RT-PCR confirmed that *bmp4*

expression was reduced in intact *admp(RNAi)* animals (Figure 3C). This decrease in *bmp4* expression was particularly apparent in cells more distal from the dorsal midline (Figure 3C). Together, these data indicate that Admp signaling is required to maintain the appropriate level and broad spatial distribution of *bmp4* expression during adult tissue maintenance and growth.

***Smed-bmp4* Inhibits *Smed-admp* Expression**

To determine whether *admp* is regulated by Bmp4 signaling, we examined the effect of Bmp pathway inhibition on *admp* expression. In both transversely and parasagittally amputated *bmp4(RNAi)* animals, *admp* expression was increased and expanded dorsally (Figures 4A and 4B). To conversely test whether an increase in Bmp signaling leads to a reduction in *admp* expression, we inhibited a ventrally expressed planarian *noggin* homolog (*Smed-nog1*, or *nog1* in short) [3]. Noggins are well-characterized inhibitors of Bmp signaling [20]. Parasagittally amputated *nog1(RNAi)* animals indeed displayed a marked decrease in ventral *admp* expression (Figure 4B). Together these results indicate that *admp* expression is negatively regulated by Bmp signaling.

We next investigated whether the change in *admp* expression observed in *bmp4(RNAi)* animals reflected failure of specific regulation of *admp* or whether it was the simple consequence of ventralization. Following Bmp pathway inhibition, we compared expression of *admp* to genes with similar ventral or lateral expression domains. Whereas regeneration occurs quickly (within days), intact nonamputated animals inhibited for *bmp4* or the Bmp effectors *smad1* or *smad4* gradually become ventralized over a period of weeks [2, 3]. This slow transformation allows for greater temporal resolution in assessing the changes in gene expression that occur following Bmp signaling loss. After 3 weeks of RNAi, intact *bmp4(RNAi)*, *smad1(RNAi)*, and *smad4(RNAi)* animals all displayed dorsal expression of *admp*, despite little to no expansion in the

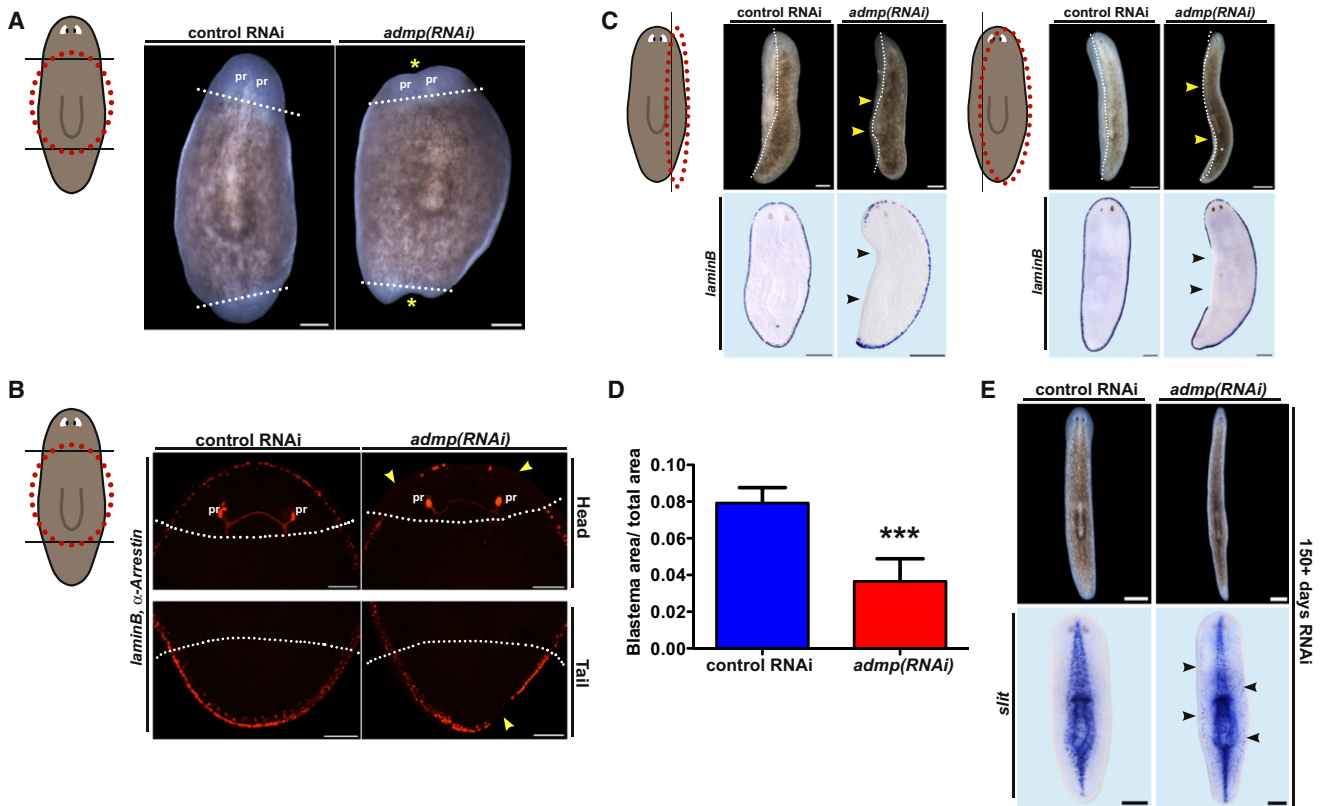


Figure 2. *Smed-admp* Is Required for Planarian Regeneration

(A) Transversely amputated *admp(RNAi)* animals displayed aberrant midline regeneration (yellow asterisks, $n = 14$ of 20). pr denotes photoreceptors. Scale bars represent 200 μm .

(B) Transversely amputated *admp(RNAi)* animals failed to regenerate cells at the DV boundary, as assayed by detection of *lamininB*⁺ cells (yellow arrowheads, $n = 7$ of 7). Scale bars represent 200 μm .

(C) *admp(RNAi)* animals failed to regenerate a missing side (yellow arrowheads, $n = 22$ of 26 thin and 19 of 19 thick fragments) or lateral *lamininB*⁺ tissue (black arrowhead, $n = 13$ of 16 thin and 19 of 19 thick fragments) following parasagittal amputation. Scale bars represent 500 μm (top) and 200 μm (bottom).

(D) Blastema size: unpigmented tissue at amputated sides (thick fragments), from anterior pharynx tip to tail, divided by worm area (difference with control was significant, $p = 0.0006$, unpaired t test).

(E) Nonamputated *admp(RNAi)* animals became thinner than control animals following 155 days of RNAi and aberrantly expressed the ventral midline marker *slit* [31] at lateral positions following 163 days of RNAi ($n = 7$ of 7, black arrowheads). RNAi of *admp* was shown to be effective and specific (see Figures S2B and S2C). White lines in (A)–(C) denote approximate blastema boundary. Dorsal view in (A)–(C) and (E), top; ventral view in (E), bottom. Anterior is up in all.

expression of other tested genes (Figure 4C). Strikingly, *smad4* inhibition resulted in broad dorsal expansion of *admp* expression after 3 weeks of RNAi and in ubiquitous DV expression of *admp* after 82 days of RNAi (Figure 4D). In both of these cases, inhibition of *smad4* resulted in more extensive dorsal expression of *admp* than did inhibition of either *smad1* or *bmp4* (Figure 4C and Figure S4C). Because Smad4 proteins are required for all forms of TGF β superfamily signaling [21], these results suggest that *admp* expression may also be regulated by non-Bmp TGF β signaling. In contrast to Bmp pathway RNAi, 3 weeks of *nog1* RNAi in intact animals caused a potent reduction in the ventral *admp* expression domain without affecting other ventral markers (Figure 4D and Figure S4A). These data indicate that *admp* expression is negatively regulated by Bmp signaling during adult homeostasis and growth. Together with the observation that *admp* promotes *bmp4* expression, we propose that inhibition of *admp* expression by Bmp4 produces a feedback circuit that buffers against fluctuations in Bmp signaling levels, conferring robustness in DV and ML patterning. This model is supported by the observation that *admp* depletion results in animals that are hypersensitive to *bmp4* inhibition. Although planarians lack identified orthologs

of the Bmp modulators *chordin* [22] and *sizzled* [23], the presence of a homolog of the Bmp pseudoreceptor *bambi* [24] (Figure S4D), as well as a greatly expanded family of *noggin* genes [25] and the ability of Admp to regulate *nog1* expression (Figures S2D and S3D), suggests that additional regulatory mechanisms likely function to fine-tune the activity of this central Bmp/Admp circuit.

Admp Orthologs Are Widespread among Protostomes

Because *Smed-admp* represents the first *admp* ortholog characterized in a protostome, we searched the genomes of other lophotrochozoans to determine whether *admp* orthologs are widespread in protostomes. Indeed, predicted *admp* orthologs were identified in the genomes of the snail *Lottia gigantea*, the leech *Helobdella robusta*, and the polychaete annelid *Capitella teleta* (Figure S1C). The presence of putative *admp* orthologs in these species, coupled with the expression pattern and functional properties of *Smed-admp*, suggest that a Bmp/Admp regulatory circuit is an ancestral and central feature of the DV axis (Figure 4E). This model predicts that an *admp* gene was present in the ancestor of *C. elegans* and *Drosophila* but was subsequently lost in the evolution of these

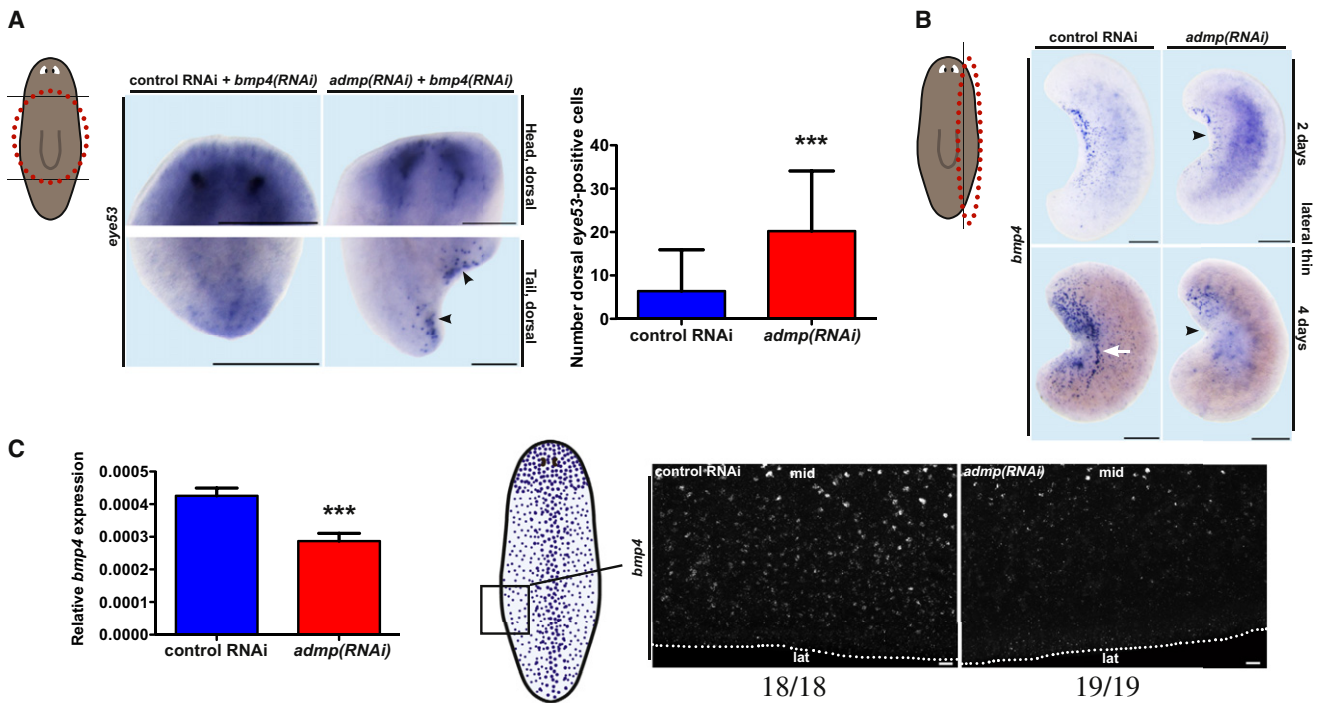


Figure 3. *Smed-admp* Inhibits Ventral Fates and Is Required for Normal *Smed-bmp4* Expression

(A) *bmp4* dsRNA addition in *admp(RNAi)* animals caused ectopic dorsal *eye53* expression (black arrowheads). Out-of-focus signal in control animals is from ventral cells. Difference in dorsal *eye53*-expressing cell numbers was significant, $p < 0.0001$, unpaired t test. RNAi of *bmp4* was shown to be effective (see Figure S4B).

(B) *admp(RNAi)* thin fragments had reduced *bmp4* expression (black arrowheads, $n = 5$ of 6 and $n = 9$ of 15 at 2 and 4 days, respectively) and failed to reposition *bmp4* expression as in control animals (white arrow).

(C) Left: *bmp4* expression was reduced in intact *admp(RNAi)* animals (by quantitative RT-PCR). Difference was significant, $p < 0.0001$, paired t test. Right: *bmp4* expression depiction. Eight micron optical sections images were acquired, with identical exposure times, of the postpharyngeal left side of intact *admp(RNAi)* and control RNAi animals. *admp(RNAi)* animals had reduced *bmp4* expression (37 of 37 animals blindly scored correctly, three independent experiments). mid denotes medial dorsal, lat denotes lateral dorsal. White lines denote approximate lateral animal edge.

Scale bars represent 200 μm for (A) and (B) and 20 μm for (C). Dorsal view, anterior is up.

species; consequently, the potential widespread significance of *admp* genes for the DV axis of Bilaterians was previously unknown.

Conclusions

Development proceeds in a remarkably reliable fashion despite the myriad forms that embryos assume and the widely varying conditions they encounter [26]. Robust patterning of the DV axis is a crucial component of this process. The DV axis can scale during growth and, in some cases, is capable of restoration following surgical manipulation [9, 27–29]. How Bmp signaling is able to generate consistent DV pattern in diverse species and respond appropriately to perturbation has been a central mystery in developmental biology. Our data demonstrate that a molecular circuit of spatially opposed *bmp4* and *admp* expression is crucial for the regeneration and maintenance of both DV and ML pattern in planarians. This circuit may function to buffer against changes in Bmp level that naturally arise from differences in patterned tissue size, the genotype of individuals, or environmental influences encountered. The requirement of a Bmp/Admp circuit for both planarian regeneration and deuterostome self-regulation [10, 11, 16] suggests that restoration of the DV axis in embryonic regulation and adult axial regeneration share mechanistic features. The presence of spatially opposed expression of *bmp* and *admp* in planarians (lophotrochozoans) and in

several deuterostomes [15, 16, 30] suggests that a Bmp/Admp circuit is a widespread feature of the DV axis that emerged concurrent with the first bilaterally symmetric animals.

Supplemental Information

Supplemental Information includes four figures, Supplemental Experimental Procedures, and three movies and can be found with this article online at doi:10.1016/j.cub.2011.01.017.

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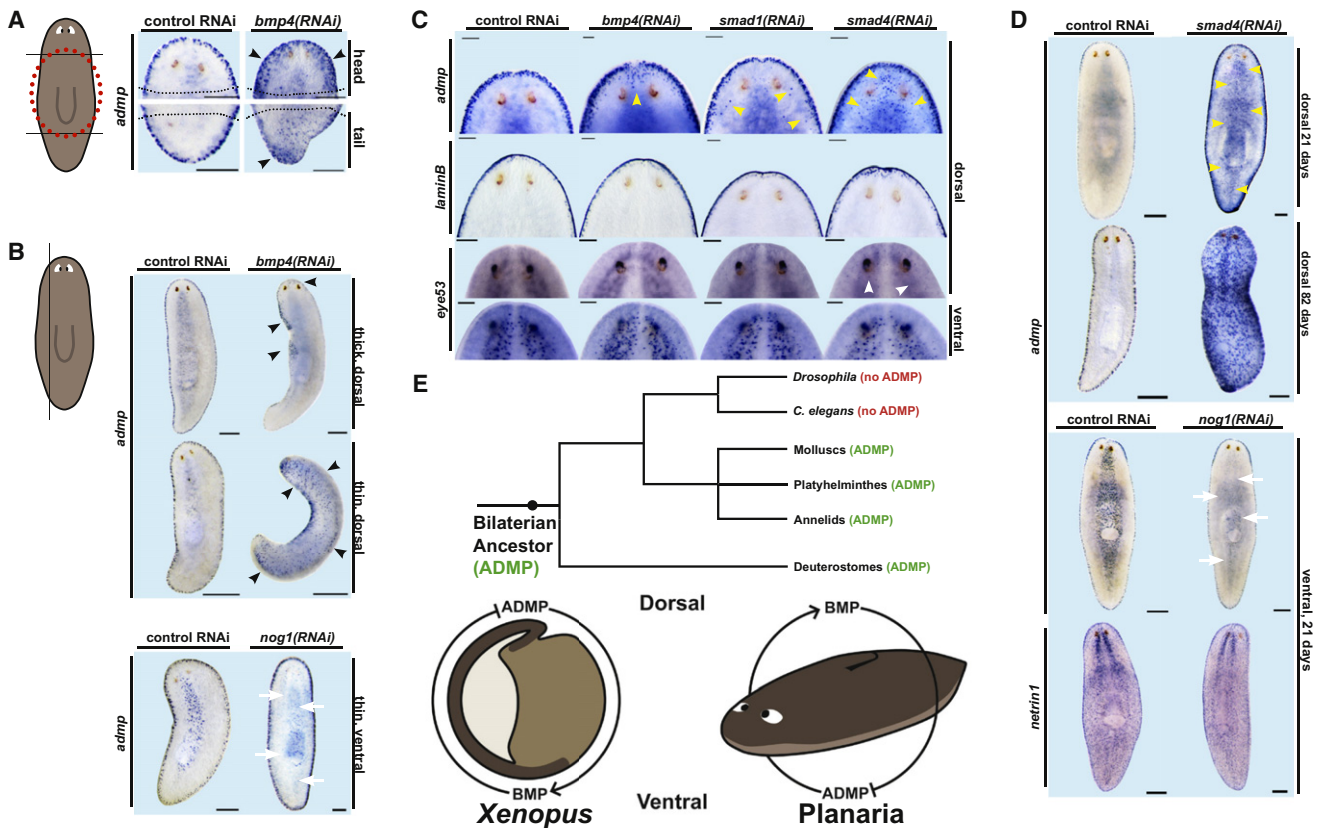


Figure 4. *Smed-admp* Expression Is Negatively Regulated by Bmp Signaling

(A) Transversely amputated *bmp4(RNAi)* animals had ectopic, dorsal *admp* expression (black arrowheads, $n = 14$ of 14) in preexisting tissue. Nineteen days of regeneration, dorsal view. Dotted lines denote blastema boundary.

(B) Parasagittally amputated *bmp4(RNAi)* animals had ectopic dorsal *admp* expression (black arrowheads, $n = 5$ of 5) in preexisting tissue; parasagittally amputated *nog1(RNAi)* animals had reduced ventral *admp* expression (white arrows, $n = 20$ of 20). Fourteen and 19 days of regeneration for *bmp4(RNAi)* and *nog1(RNAi)* animals, respectively.

(C) Nonamputated animals inhibited for Bmp pathway components displayed ectopic dorsal *admp* expression after 21 days of RNAi (yellow arrowheads, $n > 9$ of 9 for each condition). Weak dorsal expression of the ventral marker *eye53* was detected in *smad4(RNAi)* animals (white arrowheads, $n = 5$ of 11). Ventral and lateral marker expression was otherwise unaffected.

(D) Nonamputated *smad4(RNAi)* animals displayed broad dorsal *admp* expression after 21 days of RNAi (yellow arrowheads, $n = 23$ of 23) and ubiquitous *admp* expression after 82 days of RNAi ($n = 5$ of 5). Nonamputated *nog1(RNAi)* animals displayed reduced ventral *admp* expression (white arrows, $n = 8$ of 8) but normal *netrin1* expression.

(E) Top: phylogenetic diagram of bilaterians annotated with the existence of putative *admp* orthologs. Bottom: schematic of proposed conserved Bmp-Admp circuit in *Xenopus* embryos (left) and planarians (right). RNAi of *smad1*, *smad4*, and *nog1* was shown to be effective (see Figure S4B). Scale bars represent $100 \mu\text{m}$ for (A) and (C) and $200 \mu\text{m}$ for (B) and (D). Anterior is up.

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