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Report

Noggin and Noggin-Like Genes Control Dorsoventral Axis Regeneration in Planarians

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Summary

Planarians regenerate a whole animal from a small body piece within a few days [1, 2]. Recent studies have shown that the bone morphogenetic protein (BMP) pathway is required to reestablish the dorsoventral (DV) axis [3–5]. In vertebrates, the specification of the DV axis depends on the coordinated action of a dual organizer defined by BMP and antidorsalizing morphogenetic protein (ADMP) under the control of several factors, including the inhibitors chordin and noggin [6]. Planarians have an expanded *noggin* family (up to ten members), which have been classified as canonical *noggin* (*nog*) and *noggin-like* (*nlg*) genes, the latter carrying an insertion within the *noggin* domain [7]. Here we show that a BMP/ADMP organizer governs DV axis reestablishment during planarian regeneration, highlighting a greater-than-thought conservation of the mechanisms that establish this axis in protostomes and deuterostomes. Also, we report that whereas *noggin* genes function as canonical BMP inhibitors, the silencing of planarian *nlg8* induces ectopic neurogenesis and enhances ventralizing *bmp (RNAi)* phenotypes. Finally, we show that *noggin-like* genes are conserved from cnidarian to vertebrates and that both planarian *nlg8* and *Xenopus nlg* ventralize *Xenopus* embryos when overexpressed. Remarkably, this ventralization is not associated with an increase in SMAD1/5/8 phosphorylation.

Results and Discussion

A BMP/ADMP Organizer Regulates Dorsoventral Patterning in Planarians

Bone morphogenetic protein (BMP) specifies dorsal fates in invertebrates and ventral fates in vertebrates, possibly because of an inversion of the dorsoventral (DV) axis within the chordate lineage [8]. In planarians, *bmp* is expressed along the dorsal midline [3, 5, 9], and silencing of *bmp* or *smad1* homologs results in ventralization, with duplications of the DV margin and central nervous system (CNS) and generation of conjoined twin-like planarians [3–5]. In *Xenopus*, the specification of the DV axis depends on the coordinated action of BMP and antidorsalizing morphogenetic protein (ADMP) that, being expressed at opposite sides of the embryo and under contrary transcriptional control, create a morphogenetic field that allows self-regulation in embryos that have been sectioned in half [10]. We have found one *admp* gene in *S. mediterranea*, named *Smed-admp-1*, that, complementarily to the dorsal midline expression of *bmp* [3, 5, 9], was expressed along the ventral midline, as well as around the body margin (see Figure S1A available online). Compared to control *gfp(RNAi)* treatment (Figure 1A), single *admp-1(RNAi)* did not alter normal planarian regeneration (Figure 1B); however, similarly to vertebrates, double *bmp(RNAi); admp-1(RNAi)* silencing enhanced ventralizing *bmp(RNAi)* phenotypes (compare Figures 1C and 1D). These *bmp(RNAi); admp-1(RNAi)* animals displayed stronger dorsal differentiation of both ectopic CNS and *Smed-eye53*-positive cells, duplication of the DV boundary, and a complete ventralization of their dorsal sides (Figure 1D and Table 1). These results imply that planarian ADMP-1 is able to partially compensate the loss of function of *bmp* and indicate for the first time that a dual BMP/ADMP organizer regulates DV polarity outside of deuterostomes.

Planarian *nog1* and *nog2* Genes Function as Canonical Inhibitors of BMP

The BMP signaling pathway is regulated by several well-known inhibitors such as the *noggin* genes [11]. Planarian *noggins* can be divided into two classes: canonical *noggin* (*nog*, two members) and *noggin-like* (*nlg*, eight members) genes that carry an insertion between the fifth and sixth cysteine residues of the *noggin* domain [7]. All of these genes show distinct expression patterns [7], with *Smed-nog1* expressed in the CNS and body margin [3, 7] and *Smed-nlg8* present all throughout the planarian dorsal side [7].

In order to characterize first the function of planarian *noggin* genes, we performed combinatorial RNA interference (RNAi) experiments in planarians. *Smed-nog1(RNAi)* and *Smed-nog2(RNAi)* single knockdowns did not show any significant phenotype (data not shown) compared to *gfp(RNAi)* controls (Figure 1E and Movie S1). However, after three rounds of *nog1(RNAi); nog2(RNAi)* double knockdown (Figure S1B), regenerating planarians showed a clear dorsalization along their ventral midline. Most animals were thinner, and around 13% bent their heads downward (Movie S2) and developed small anteroventral outgrowths where ectopic eyes occasionally differentiated (Figure 1F). In addition, ectopic ventral expression of the dorsal marker *Smed-septin* was detected in two to four cells, and an abnormal concentration of α-tubulin staining was evident around the ventral outgrowths (Figure 1F and Table 1). Finally, anteroventral expansion and broader posterior expression of the marginal marker *Smed-ifb* revealed DV boundary defects (Figure S1C). This ventralizing activity of NOG1 and NOG2 suggests that planarian NOGGINs have a canonical role in DV patterning through inactivation of BMP signaling. The low penetrance of the dorsalizing RNAi phenotypes is in agreement with results obtained in vertebrates in which *noggin*s need to be knocked out together with other BMP inhibitors in order to obtain a strong disruption of the DV axis [12].

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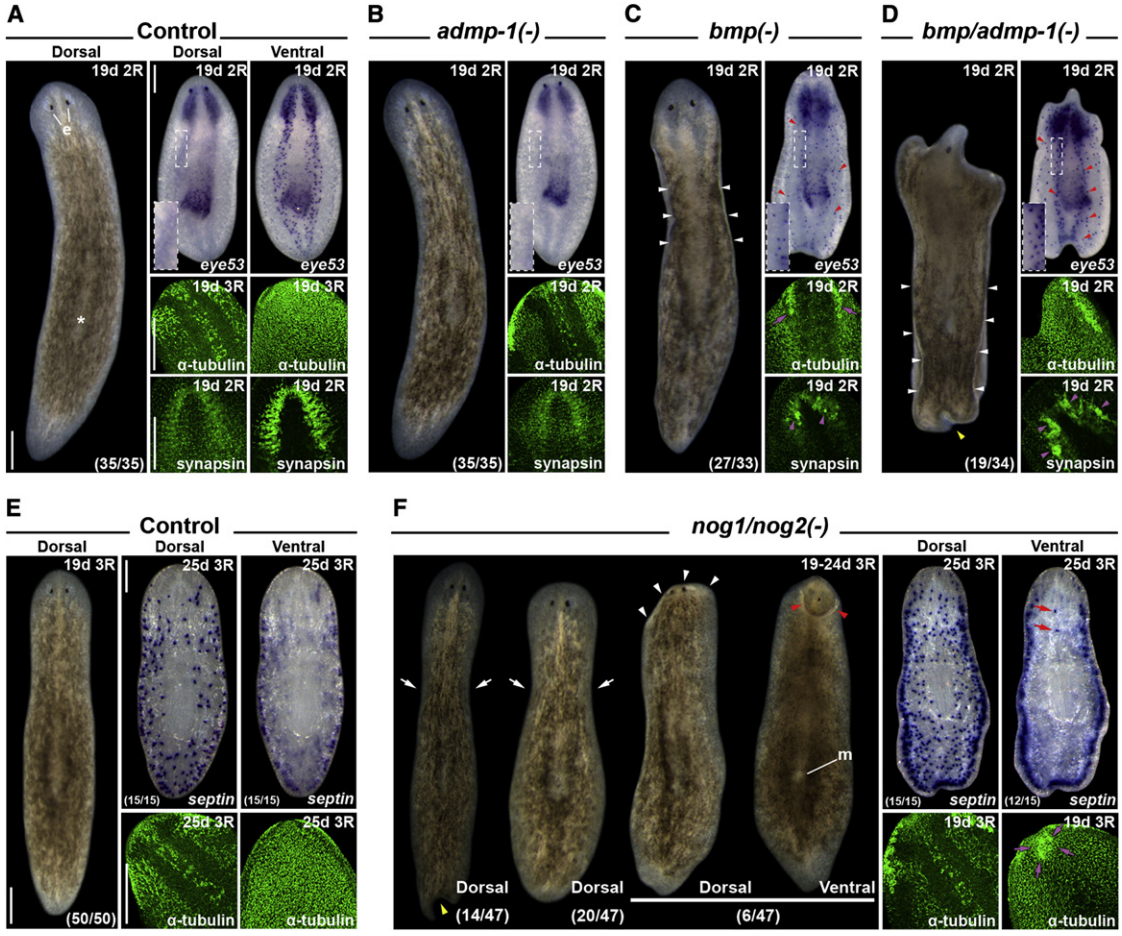


Figure 1. Planarian *admp-1* and *noggin* Homologs Play Canonical Functions in DV Patterning

(A–D) *admp-1(RNAi)* enhances *bmp* loss-of-function phenotypes.

(A) *gfp(RNAi)* control organisms. Left: live animal. Right panels: molecular markers. *Smed-eye53* is a marker associated with the planarian ventral CNS. Anti-tubulin immunostaining shows a homogeneous pattern of cilia throughout the ventral epidermis. However, dorsally, the cilia are mainly concentrated in a stripe along the midline and the lateral body regions. Anti-synapsin labels the dorsal submuscular plexus and the ventral CNS.

(B–D) *admp-1(RNAi)* and *bmp(RNAi)* single and combinatorial treatments. Images represent the strongest phenotypes obtained, and all are dorsal views. White arrowheads denote DV boundary duplication, yellow arrowhead denotes posterior indented blastema, and red arrowheads denote ectopic *eye53*-positive cells. Insets show higher magnifications of the boxed areas. Violet arrows denote expansion of the planarian dorsal midline, and violet arrowheads denote ectopic dorsal CNS differentiation.

(E and F) Silencing of *noggin* genes results in dorsalized planarians.

(E) *gfp(RNAi)* control organisms. *Smed-septin* is a marker of the planarian dorsal side.

(F) *nog1(RNAi);nog2(RNAi).* White arrows denote thinner prepharyngeal region, white arrowheads denote downward bending of the head, red arrowheads denote anteroventral outgrowth, yellow arrowhead denotes posterior indented blastema, red arrows denote ectopic *septin*-positive cells (n = 12 out of 15), and violet arrows denote ventral outgrowth. Anterior is to the top. The following abbreviations are used: e, eyes; m, mouth opening; *, pharynx. Scale bars represent 300 μm.

nlg8(RNAi) Yields Similar Phenotypes to *bmp* Loss of Function

Whereas *Smed-nlg1(RNAi)* to *nlg7(RNAi)* did not give any evident phenotypes, *Smed-nlg8(RNAi)* treatment resulted in clear alterations. In addition to transient defects in blastema formation (asymmetry, reduction, or even absence; Figure 2B and Figures S2A and S2B), and in contrast to the dorsalization observed after simultaneous *nog1* and *nog2* silencing (Figure 1F), *nlg8(RNAi)* treatment revealed an unexpected *bmp*-like RNAi phenotype (Table 1). Ectopic *Smed-eye53*-positive cells appeared all along the dorsal side of *nlg8(RNAi)*-treated organisms, and a duplicated CNS differentiated dorsally (compare Figures 2A and 2B). Also similar to *bmp(RNAi)* [3–5], spreading of *cintillo*-positive cells, dorsal expansion of the cephalic ganglia, and aberrant projections of the planarian visual axons were evident (Figure S2C). However, in contrast to *bmp(RNAi)* phenotypes, in which an ectopic dorsal CNS differentiates from posterior toward anterior regions [3], after *nlg8(RNAi)* the ectopic CNS first appeared anteriorly and gradually differentiated at different levels along the anteroposterior (AP) axis. Also, despite some expansion of submarginal markers, no thickening, dorsal bulges, DV border duplication (Figure S2D), or ventralization of the stereotypical dorsal cilia pattern (Figure 2B) typical of *bmp(RNAi)* phenotypes [3–5] were observed after several consecutive rounds of *nlg8 (RNAi)*. Thus, *nlg8(RNAi)* seems to recapitulate the neural phenotypes obtained after *bmp(RNAi)* [3–5], but without the further disturbance of the DV axis observed with *bmp*

Table 1. Summary of the Phenotypes of the RNAi Experiments Described

RNAi	Dorsal Side: Ventral-Like Cilia (α-Tubulin)	Dorsal Side: Ectopic CNS (*eye53* + Synapsin)	Ventral Side: Dorsalization (*septin* + α-Tubulin)
nog1/nog2	np	np	*
nlg8	np	***	np
bmp	***	**	np
admp-1	np	np	np
nlg8/bmp	****	*****	np
bmp/admp-1	****	*****	np
nog1/nog2/nlg8	np	****	****
nog1/nog2/gfp	np	np	np
nog1/nog2/nlg8/gfp	np	***	***
nog1/nog2/nlg8/bmp	**	***	np
nog1/nog2/nlg8/admp-1	np	**	**

The number of asterisks indicates the severity of the phenotypes. np denotes no phenotype.

silencing. Overall, *nlg8* silencing clearly differs from the canonical noggin phenotype of *nog1(RNAi); nog2(RNAi)* and partially resembles that of *bmp(RNAi)*.

Simultaneous silencing of *nlg8*, *nog1*, and *nog2* did not abolish the differentiation of an ectopic CNS compared to single *nlg8(RNAi)* (Figure 3A), suggesting that NLG8 does not function as a dominant-negative protein of NOG1 and/or NOG2. In fact, ectopic neural differentiation was significantly enhanced (Figure 3A and Table 1). Furthermore, compared to *nog1/nog2* inhibition, triple RNAi knockdown planarians also showed stronger dorsalization of their ventral sides (Figure 3B and Table 1) and head-like ventral outgrowths (Figures 3C and 3D, Figure S3A, and Movie S3). The fact that the simultaneous silencing of *nog1*, *nog2*, and *nlg8* did not rescue but rather enhanced their individual phenotypes indicates that, similarly to vertebrates, some regulatory elements might function as ventralizing or dorsalizing factors, depending on the genetic context in which they act [13, 14].

Next, in order to investigate the relation between *bmp* and *nlg8*, we silenced both genes simultaneously (Figure S3B). Similarly to simultaneous *bmp* and *admp-1* loss of function, double *nlg8(RNAi); bmp(RNAi)* silencing significantly enhanced single *bmp(RNAi)* phenotypes (compare Figures 3E and 3F), suggesting that NLG8 is able to partially compensate for the ventralization observed after *bmp(RNAi)*. Therefore, BMP and NLG8 might configure a regulatory circuit that patterns the planarian DV axis; however, SMED-BMP would be the main factor responsible for establishing dorsal identity (Table 1). Moreover, the facts that *admp-1(RNAi)* did not phenocopy *nlg8* silencing and that double *bmp(RNAi); nlg8(RNAi)* enhanced the effects of *bmp* silencing would suggest that the dorsal ectopic CNS differentiation after *nlg8(RNAi)* is somehow independent of BMP and ADMP-1, indicating that additional molecules might mediate NLG8 activity.

Both *bmp(RNAi)* and *admp-1(RNAi)* Rescue the Dorsalization Observed after *nog1/nog2* and *nog1/nog2/nlg8* Loss of Function

Because *bmp* is expressed along the planarian dorsal midline [3–5], one might expect that the dorsalization observed after either *nog1/nog2* or *nog1/nog2/nlg8* silencing would occur through a dorsal-to-ventral expansion of the BMP-mediated dorsalizing activity. However, ectopic *bmp*-expressing cells appeared to be restricted at the ventral midline of both *nog1/nog2* (data not shown) and *nog1/nog2/nlg8* (Figure S3C) silenced planarians, in the same region where a head-like outgrowth will develop later. Indeed, these ectopic *bmp*-expressing cells would act as a dorsalizing center in the ventral side, because cosilencing of *nog1*, *nog2*, *nlg8*, and *bmp* blocked the ectopic differentiation of dorsal markers and head-like outgrowths on the ventral side of treated planarians (Figure 3G, Table 1, and Figures S3A and S3D).

Similarly, regardless of the early disappearance of *admp-1* expression along the planarian ventral midline after either *nog1/nog2* (data not shown) or *nog1/nog2/nlg8* loss of function (Figure S3C), cosilencing of *nog1*, *nog2*, *nlg8*, and *admp-1* resulted in a reduction of nearly 30% of dorsalized animals (Figure 3G and Figure S3D); moreover, those dorsalized organisms displayed weaker phenotypes compared to *nog1/nog2/nlg8* loss of function (Figure 3H and Table 1). Altogether, these experiments suggest that *nog1(RNAi);nog2(RNAi);nlg8(RNAi)* dorsalizing phenotypes depend on ADMP-1 and BMP. We propose that triple *nog1/nog2/nlg8* silencing might allow ADMP-1 activation. Thus, and similar to vertebrates, a positive ADMP-1 regulation on *bmp* expression at the planarian ventral midline might induce the formation of a new BMP dorsalizing center, which in turn would inhibit *admp-1* expression. Therefore, the same transcriptional regulation of the BMP/ADMP organizer described in *Xenopus* might be conserved in planarians. No interaction between NOGGINs and ADMP has been found yet in other organisms [15]; however, chordin, a BMP signaling inhibitory molecule, inhibits ADMP in *Xenopus* [10]. The fact that no *chordin* homolog has been found yet in the *S. mediterranea* genome opens the door to the possibility that *noggin* and *noggin-like* genes might have this inhibitory action on ADMP.

Noggin-Like Genes Are Evolutionary Conserved, and Their Overexpression Ventralizes *Xenopus* Embryos

We next investigated the gain-of-function phenotype of planarian *nog* and *nlg* in *Xenopus laevis* embryos (Figure S4A). In agreement with the knockdown experiments in planarians, *nog1* and *nog2* showed conserved dorsalizing effects when overexpressed in *Xenopus* (Figures 4A and 4B; Figure S4B), whereas *nlg8* overexpression caused ventralizing *bmp*-like phenotypes (Figures 4A and 4B; Figure S4B).

Xenopus has two canonical *nog* genes, *Xlnog1* and *Xlnog2* [16, 17], plus a third gene that, similar to planarian *nlg*s, carries an insertion between the fifth and sixth cysteine residues (*Xlnog4* [17], hereafter *Xlnlg*) and has not been functionally characterized. Strikingly, overexpression of *Xlnlg* in *Xenopus* embryos caused ventralization (Figures 4A and 4B; Figure S4B), suggesting that not only *noggin* but also *noggin-like* gene functions are conserved. Moreover, genomic searches in silico and phylogenetic analyses revealed the presence of *nog* and *nlg* in phyla as varied as cnidarians, annelids, and hemichordates (Figure 4C and Figures S4C and S4D). This suggests that *nog* and *nlg* define two distinct evolutionarily conserved families that diverged before the origin of bilaterians. *nlg*s are present in a single copy in most animal groups; in contrast, in those Platyhelminthes with available genomic data, there are at least two *noggin-like* genes (Figure S4C). This is particularly striking in the case of *S. mediterranea*, with up to eight members [7]. Similarly, several other gene families have been specifically expanded in the *S. mediterranea* genome [18, 19], suggesting that duplication may be a general phenomenon in this lineage. However, until more genomic data is available, it will not be possible to discern whether these gene expansions have been originated

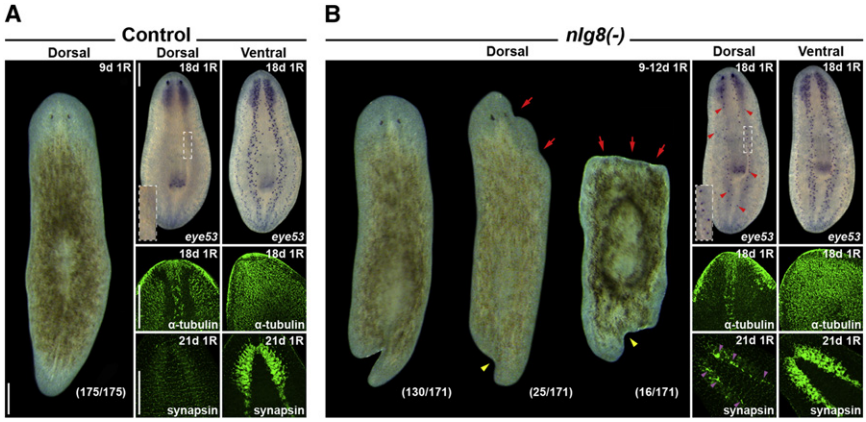


Figure 2. *nlg8* Silencing Produces *bmp*-like Phenotypes

(A) *gfp(RNAi)* control organisms.

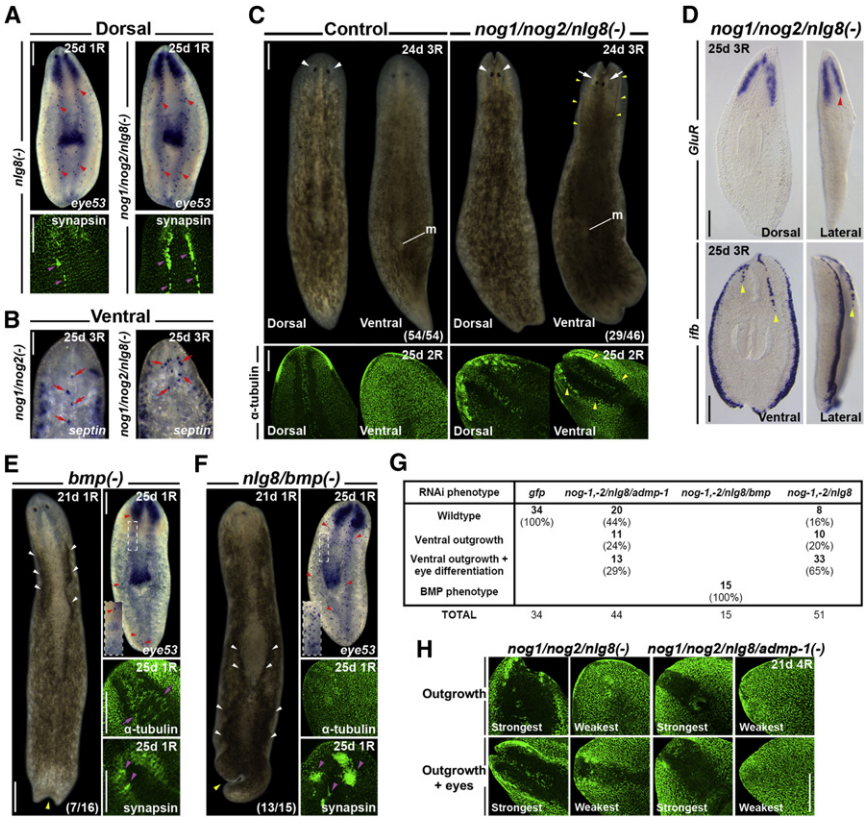
(B) *nlg8(RNAi)*. Red arrows denote asymmetric and absent anterior blastemas, yellow arrowheads denote indented posterior blastemas, and red arrowheads denote ectopic *Smed-eye53*-positive cells (n = 16 out of 16). Insets show higher magnifications of the boxed areas. Violet arrowheads denote ectopic dorsal CNS differentiation. Anterior is to the top. Scale bars represent 300 μm.

from internal duplications within specific families or are the consequences of large-scale episodes such as whole-genome duplications.

In order to analyze whether the opposite function of *nog* and *nlg* depended on the insertion within the *noggin* domain, we swapped the region located between the fifth and sixth cysteine residues of XInog1 and XInlg (Figure 4A). Remarkably, *XInlg_CnogC* mRNA injection dorsalized rather than ventralized *Xenopus* embryos. On the other hand, even though injections of *XInog_CnlgC* mRNA did not result in ventralized animals, the percentage of embryos with clear secondary axes was significantly reduced compared to injections of *XInog1* (Figure 4A). These results suggest that although the insertion in *noggin-like* genes may be important, additional residues in *noggin* and *noggin-like* genes would be determinant for their function.

Finally, to assess whether the ventralizing activity of XInlg depended on BMP and ADMP signaling, we checked the level of phosphorylation of SMAD1/5/8 after a set of combinatorial mRNA injections in *Xenopus* embryos. Remarkably, *XInlg* overexpression did not significantly modify the levels of P-SMAD1/5/8, either alone or when injected together with *Xlbmp4* and *Xladmp* (Figure 4D and Figure S4E). These results are in agreement with the BMP- and ADMP-independent role of *nlg8* loss-of-function phenotypes found in planarians and suggest that XInlg ventralizing activity might function through mechanisms others than SMAD1/5/8 C-terminal phosphorylation.

In summary, we have shown that the establishment and patterning of the planarian DV axis might depend on the



RNAi phenotype	*gfp*	*nog-1,-2/nlg8/admp-1*	*nog-1,-2/nlg8/bmp*	*nog-1,-2/nlg8*
Wildtype	34 (100%)	20 (44%)		8 (16%)
Ventral outgrowth		11 (24%)		10 (20%)
Ventral outgrowth + eye differentiation		13 (29%)		33 (65%)
BMP phenotype			15 (100%)	
TOTAL	34	44	15	51

Figure 3. Combinatorial Silencing of *nog1*, *nog2*, *nlg8*, *bmp*, and *admp-1* in Planarians

(A–F) Anterior is to the top.

(A–D) Triple *nog1/nog2/nlg8* silencing enhances both *nog1/nog2* and *nlg8* loss-of-function phenotypes.

(A) Red arrowheads denote dorsal expression of *Smed-eye53*. Violet arrowheads denote ectopic dorsal CNS.

(B) Red arrows denote ventral expression of *Smed-septin*. Note the higher number of ectopic *septin*-expressing cells after *nog1/nog2/nlg8* silencing.

(C and D) Triple-silenced organisms develop ventral outgrowths that differentiate ectopic eyes (white arrows) and brain (red arrowhead) and display a typical dorsal pattern of cilia and duplication of the DV margin (yellow arrowheads). White arrowheads denote dorsal eyes. m denotes mouth opening.

(D) In the lateral view, dorsal is to the left.

(E and F) Combinatorial silencing of *bmp* and *nlg8*. Images represent the strongest phenotypes obtained, and all are dorsal views. White arrowheads denote DV boundary duplication, yellow arrowheads denote posterior indented blastemas, and red arrowheads denote ectopic *Smed-eye53*-positive cells. Insets show higher magnifications of the boxed areas. Violet arrows denote expansion of the planarian dorsal midline, and violet arrowheads denote ectopic dorsal CNS differentiation.

(G) Table summarizing the effect of *admp-1* or *bmp* loss of function on the dorsalization phenotype seen in the planarian ventral midline after *nog1/nog2/nlg8* inhibition.

(H) Strongest and weakest phenotypes obtained after *nog1/nog2/nlg8/gfp* and *nog1/nog2/nlg8/admp-1* silencing experiments. All images are ventral views of anti-tubulin immunostaining. Anterior is to the left, except the two top left panels, where anterior is to the top. Scale bars represent 300 μm.

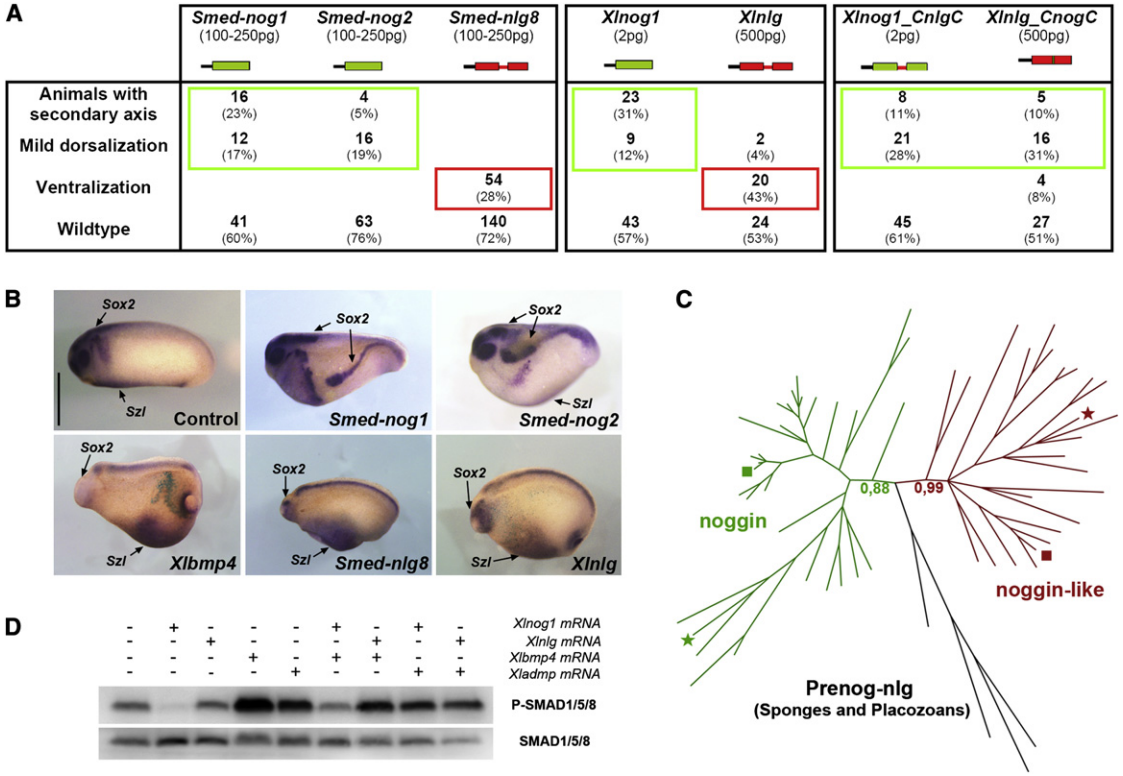


Figure 4. *nog* and *nlg* mRNA Injection in *Xenopus* Embryos

(A) Table summarizing the effects of mRNA overexpression in *Xenopus*. Overexpression of planarian *nog1*, *nog2*, and *Xenopus nog1* dorsalize the embryos; *nlg8* and *Xenopus nlg* ventralize them. Overexpression of *Xlnog1_CnlgC* (*Xlnog1* mRNA with the W215-Q227 peptide domain of *Xlnlg* inserted between C197 and C205) produces mild dorsalization. Overexpression of *Xlnlg_CnogC* (*Xlnlg* mRNA with the Q198-K204 peptide domain of *Xlnog1* inserted between C214 and C228) produces dorsalization.

(B) Lateral views of *Xenopus laevis* embryos at stage 25 of development double stained for the dorsal and ventral markers *Sox2* and *Szl*, respectively (arrows). Embryos injected with planarian *nog1* and *nog2* show an extra axis clearly visible by the ectopic *Sox2* expression. Injection of *Xenopus Bmp4*, planarian *nlg8*, or *Xenopus nlg* cause ventralization of the embryos, as determined by the reduced *Sox2* expression in the dorsal structures, including the truncated head, and the dorsal expansion of the ventral marker *Szl*. Anterior is to the left. Scale bar represents 1 mm.

(C) Phylogenetic analysis showing that *nog* and *nlg* genes are two different gene families in eumetazoans. ★ denotes planarian *nog* (green) and *nlg* (red) genes. ■ denotes *Xenopus nog* (green) and *nlg* (red) genes.

(D) Western blot against phospho-SMAD1/5/8 (P-SMAD1/5/8) and total SMAD1/5/8 (SMAD1/5/8) after indicated mRNA injections in *Xenopus* embryos. *Xlnog1* mRNA overexpression was used as a positive control of P-SMAD1/5/8 downregulation. *Xlbmp4* and *Xladmp* mRNA overexpression were used as positive controls of P-SMAD1/5/8 upregulation. Remarkably, *Xlnlg* did not significantly modify the levels of P-SMAD1/5/8 either alone or when injected together with *Xlbmp4* or *Xladmp*. In contrast, *Xlnog1* rescued the upregulation promoted by *Xlbmp4* overexpression, whereas it did not alter the effect of *Xladmp* mRNA injection (see also Figure S4E). Values are relative to uninjected organisms at the same point of development.

activity of NOGGINs, NLG8, BMP, and ADMP-1. *Noggin-like* genes are novel evolutionary-conserved regulatory elements with a function that clearly differs from the BMP antagonistic role of *noggin* genes. Our results suggest that NOGGIN-LIKE does not inhibit NOGGINs but acts synergistically with BMP through SMAD1/5/8 C-terminal phosphorylation independent mechanisms. Future biochemical analyses should elucidate the mechanism of action of this new family of regulators. The presence of a BMP/ADMP organizer in planarians together with the fact that *noggin-like* genes are functionally conserved between planarians and *Xenopus* raises the possibility that these novel proteins could also control DV axis formation in other organisms.

Experimental Procedures

Planarian Experiments

The *Schmidtea mediterranea* clonal line BCN-10 was used for all experiments and maintained as described elsewhere [3]. In silico gene searches and cloning, immunostaining, and RNAi were performed as previously described [7, 20]. The efficiency of the RNAi treatment was checked by quantitative real-time PCR. In situ hybridizations were done following standard protocols [3] adapted into an Intavis InsituPro hybridization robot. In all images, d refers to days after amputation and R to rounds of injection and amputation. For a detailed protocol, see the Supplemental Experimental Procedures.

Identification of *noggin* and *noggin-like* Genes across Metazoans and Phylogenetic Analysis

An extensive search and annotation of *noggin* and *noggin-like* orthologs in sponges, cnidarians, placozoans, ecdysozoans, lophotrocozoans, and deuterostomes was carried out using the available genomic and expressed sequence tags (ESTs) databases. Amino acid sequences of the identified orthologs were aligned with MUSCLE [21], and the resulting alignment was manually curated. A phylogenetic tree was generated by the Bayesian method with MrBayes 3.1.2 [22, 23]. For a detailed protocol, see the Supplemental Experimental Procedures.

Xenopus Microinjection of mRNA, In Situ Hybridization, and Western Blotting

The entire coding regions of *Smed-noggins*, *Smed-noggin-like* genes, *Xlbmp4* [24, 25], *Xlnog1* [16], *Xlnog4* [17] (renamed *Xlnlg* after this work), *Xlnog1_CnlgC*, and *Xlnlg_CnogC* were inserted into the multicloning site of

pCS2+ [26]. mRNAs prepared as previously described [27] were injected in *Xenopus* embryos at the two-cell stage in a single blastomere. X-Gal staining was performed as described elsewhere [28]. The *Xenopus* (*XInIg* or *XInog4*) clone was obtained from the National Institute for Basic Biology/National Institute of Genetics (NIBB/NIG) *Xenopus laevis* EST project (clone XL003k11). Antisense RNA probes were prepared from *Sox2* and *Szl* cDNAs using digoxigenin (Roche). *Xenopus* specimens were hybridized as described [29]. Total protein was extracted from a pool of treated embryos at stage 10, and western blot was performed following standard protocols. For a detailed protocol, see the Supplemental Experimental Procedures.

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.2011.01.016.

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