SNW1 Is a Critical Regulator of Spatial BMP Activity, Neural Plate Border Formation, and Neural Crest Specification in Vertebrate Embryos

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Abstract
Bone morphogenetic protein (BMP) gradients provide positional information to direct cell fate specification, such as patterning of the vertebrate ectoderm into neural, neural crest, and epidermal tissues, with precise borders segregating these domains. However, little is known about how BMP activity is regulated spatially and temporally during vertebrate development to contribute to embryonic patterning, and more specifically to neural crest formation. Through a large-scale in vivo functional screen in Xenopus for neural crest fate, we identified an essential regulator of BMP activity, SNW1. SNW1 is a nuclear protein known to regulate gene expression. Using antisense morpholinos to deplete SNW1 protein in both Xenopus and zebrafish embryos, we demonstrate that dorsally expressed SNW1 is required for neural crest specification, and this is independent of mesoderm formation and gastrulation morphogenetic movements. By exploiting a combination of immunostaining for phosphorylated Smad1 in Xenopus embryos and a BMP-dependent reporter transgenic zebrafish line, we show that SNW1 regulates a specific domain of BMP activity in the dorsal ectoderm at the neural plate border at post-gastrula stages. We use double in situ hybridizations and immunofluorescence to show how this domain of BMP activity is spatially positioned relative to the neural crest domain and that of SNW1 expression. Further in vivo and in vitro assays using cell culture and tissue explants allow us to conclude that SNW1 acts upstream of the BMP receptors. Finally, we show that the requirement of SNW1 for neural crest specification is through its ability to regulate BMP activity, as we demonstrate that targeted overexpression of BMP to the neural plate border is sufficient to restore neural crest formation in Xenopus SNW1 morphants. We conclude that through its ability to regulate a specific domain of BMP activity in the vertebrate embryo, SNW1 is a critical regulator of neural plate border formation and thus neural crest specification.

Introduction
The development of all metazoans involves the sequential specification of restricted cell fates from pluripotent progenitors. Understanding the spatial and temporal dynamics of cell differentiation is of fundamental importance to developmental biology research, in particular, how sharp borders and domains are delineated in a field of progenitor cells. For example, the neural crest, a vertebrate-specific embryonic cell type that gives rise to many different cell types (e.g., cartilage and bones of the face, melanocytes, neurons and glia of the peripheral nervous system, and smooth muscle of the heart), is induced at a very specific domain in the developing ectoderm between the neural plate and the epidermis [1] (Figure 1A).

Patterning of the vertebrate ectoderm across its dorsal-ventral axis is known to be dependent on a gradient of bone morphogenetic protein (BMP) activity. BMP ligands are capable of functioning as morphogens, providing positional information to a field of cells depending on the strength and duration of signaling activity [2]. In Xenopus and zebrafish embryos, a high ventral to low dorsal gradient of BMP activity is thought to contribute to embryonic patterning. The classical model proposes that in the ectoderm, high BMP activity is required for epidermal fate, a moderate level of activity is necessary for neural plate border fates, including competency to become neural crest, and BMP inhibition is a prerequisite for neural fate [3] (Figure 1A). Analogous to the observation that gastrulating cells become competent to respond to BMP signals in a temporal fashion along the anterior-posterior axis [4], neural and neural crest fates require BMP signaling at temporally distinct periods [5,6], in addition to the requirement for different levels of BMP activity. However, whilst these studies have demonstrated a particular requirement in time and space for...
**Author Summary**

A subset of cells in the ectoderm of vertebrate embryos becomes the neural crest, which contributes to the bones and cartilage of the adult face. The neural crest arises in a location between the epidermis, which becomes the future skin, and the neural plate, which becomes the future central nervous system. Through our studies in both frog and fish embryos, we have discovered that the protein SNW-domain containing protein 1 (SNW1) is absolutely essential for defining the edge of the neural plate, where neural crest forms. SNW-domain containing proteins have been implicated in a variety of nuclear activities ranging from transcriptional regulation and elongation to RNA splicing. We show that SNW1 functions upstream of bone morphogenetic protein (BMP) receptors to regulate BMP activity, and is necessary for the activity of the BMP signaling pathway at the neural plate border where the neural crest is specified. In the absence of SNW1, BMP activity is reduced in this region and neural crest cells are lost. Given that SNW1 family proteins are highly conserved from nematodes to humans, SNW1's BMP regulatory function is likely conserved in other animals.

BMP activity to specify certain fates, they have provided little insight into how the BMP activity may change spatially and temporally to provide the means for these developmental events to occur, or how this is regulated. Indeed, while the BMP activity gradient has been visualized in gastrulating *Xenopus* and zebrafish embryos [4,7], no comprehensive visualization of BMP activity domains at post-gastrula stages has been reported in *Xenopus* or zebrafish. The ability to directly observe how BMP activity is remodeled at different stages will help determine how this contributes to the specification of particular cell fates and the formation of sharp tissue boundaries, as well as clarifying which BMP ligands are likely to contribute to BMP activity in specific tissues.

Here, we report the identification of a novel regulator of BMP activity in the vertebrate embryo, SNW1, in a large-scale functional screen in *Xenopus* embryos for neural crest fate. The expression of *SNW1* is enriched in dorsal tissues during neurula stages in both *Xenopus* and zebrafish embryos. Depletion of SNW1 in both organisms leads to a loss of neural crest, concomitant with an expansion of the neural plate and loss of the sharp neural plate border. We have established that dorsally expressed SNW1 is critical for neural crest formation and that the effects of SNW1 occur independently of mesoderm formation. In addition to its effect on neural crest specification, we have shown that SNW1 is necessary for BMP activity during neurula stages. More specifically, in the absence of SNW1, a specific domain of BMP activity is lost in the ectoderm, adjacent to the neural plate. Experiments in mammalian cell culture and *Xenopus* embryo explants indicate that SNW1 does not directly affect the core BMP signaling pathway, suggesting that SNW1 must function upstream of the BMP receptors. Finally the proximity/overlap of the SNW1 expression domain, neural crest cells, and the BMP activity domain prompted us to test whether SNW1 affects neural crest formation via its ability to regulate BMP activity. Indeed, targeted overexpression of BMP2b was able to rescue the neural crest phenotype of *Xenopus* Δ*SNW1* morphants. Thus, SNW1 is a critical factor that is required for a specific BMP activity domain in the ectoderm in order to specify neural crest fate during neurulation.

**Results**

Identification of *SNW1* in a Functional Overexpression Screen for Neural Crest Regulators

To discover how a new cell population can be specified at a defined location in a developing vertebrate embryo we performed a large-scale in vivo functional screen in *Xenopus laevis* for neural crest fate, which occurs at the neural plate border (Figure 1A and 1B). A *Xenopus tropicalis* full-length cDNA library was used as a template for transcribing mRNA in vitro. The mRNAs were then pooled and injected into *Xenopus* embryos for overexpression. Embryos were fixed at the early neurula stage 14 and subjected to whole-mount in situ hybridization (WISH) for the neural crest marker Slug. Positive pools were then deconvoluted by injection of subpools and again assessed by WISH for Slug until single clones were isolated. We found a total of 14 proteins whose overexpression altered the levels of the neural crest marker Slug, and the screen was validated by the fact that some of the positive hits were known modulators of signaling pathways required for neural crest fate, or factors previously identified as neural crest regulators. Examples were c-Myc, whose overexpression resulted in increased Slug staining [8], and the Wnt antagonist *Dickkopf1* (*Dkk-1*) and the BMP antagonist Noggin2 [1,9,10], both of which repressed neural crest fate (Figure 1C). One unexpected hit was *SNW1*, which substantially inhibited Slug staining when overexpressed (Figure 1C). *SNW1*, which is a nuclear protein, has been variously implicated in transcription regulation in response to several different signals [11–13], and in transcriptional elongation through its ability to interact with pTEFb [14], and in yeast it is thought to be involved in pre-mRNA splicing [15]. It is extremely well conserved at the amino acid sequence level throughout its entire sequence from *Caenorhabditis elegans* to humans (Figure S1).

*SNW1* is Ubiquitously Expressed during Gastrulation, but Is Enriched in the Dorsal Ectoderm and Mesoderm during Neurulation

Semi-quantitative reverse transcription PCR (RT-PCR) indicated that *SNW1* is expressed at all stages of *Xenopus* development examined (Figure S2A), and the presence of *SNW1* mRNA in unfertilized eggs suggests that it is maternally deposited during oogenesis. Quantitative PCR (qPCR) on dorsal and ventral halves of *Xenopus* embryos confirms that *SNW1* transcripts are present in both halves of the embryo (Figure S2B), but WISH for *SNW1* mRNA at two neurula stages (stage 14 and 17) reveals that *SNW1* expression is specifically enriched on the dorsal side, in the neural plate region (Figure 1D). Western blotting demonstrated that SNW1 protein levels were relatively low up to the end of gastrulation (stage 12), at which point the levels increased and continued to do so through the rest of development to stage 32 (Figure 1E). The 61-kDa SNW1 band disappears when SNW1 is knocked down by injection of a translation-blocking antisense morpholino (MO), MoSNW1a (Figures 1E and S3A). Interestingly, this MO efficiently reduced SNW1 protein levels from stage 12 onwards (Figure 1E), suggesting that the protein detected prior to this stage is maternally deposited and thus resistant to the MO, whereas the protein detectable after stage 12 is zygotically produced.

To investigate whether *SNW1* expression was conserved in another vertebrate species, we cloned zebrafish *SNW1* and examined its expression pattern. WISH on the naturally translucent zebrafish embryos provided higher resolution in terms of identifying the tissues where *SNW1* mRNA is enriched. During gastrula stages, *SNW1* is expressed ubiquitously. By 11 h post-fertilization (hpf), *SNW1* is detected ubiquitously in the embryo,
SNW1 Spatially Regulates BMP Activity

A

V

A

D

P

Neural/dorsal tissue: low BMP activity
Neural crest/neural plate border tissue: intermediate BMP activity
Epidermal/ventral tissue: high BMP activity

B

Pools of mRNA transcribed in vitro from X. tropicalis full-length cDNA library are injected into X. laevis embryos
Screen by whole-mount in situ hybridization for Slug, a neural crest marker, at stage 14
Identification of hit clone by injection of row and column subpools of library

C

X. laevis

 Slug

 10/10

Control

 10/10

c-Myc

 10/10

Dkk-1

 10/10

Noggin2

 10/10

SNW1

D

X. laevis

 SNW1

St 10

St 14

St 17

St 32

E

X. laevis

SNW1

Tubulin

Stage:

clayage blastula gastrula neurula tailbud clayage blastula gastrula neurula tailbud

WT

MoSNW1a

F

zebrafish

SNW1

lateral

shield

75% epiboly

6.5–8 hpf

lateral

11 hpf

12 hpf

13 hpf

G

zebrafish

blastula gastrula segmentation

hpf: E 2 4 6 8 9 10 12 14 16

SNW1

MCM6

WT
SNW1 Spatially Regulates BMP Activity

Figure 1. SNW1 was identified in a functional screen for neural crest fate in X. laevis. (A) A diagram of a stage 14 embryo depicting the classical model, which proposes that different levels of BMP signaling are required for ectodermal patterning and neural crest formation. (B) An overview of the functional screen. (C) The screen identified proteins whose overexpression modulated levels of Slug expression. 200 pg of mRNA expressing the X. tropicalis c-Myc, Dkk-1, Noggin2, or SNW1 was injected at the one-cell stage, with 250 pg of GFP mRNA as tracer. Control embryos received the GFP mRNA only. Embryos were fixed at stage 14 and analyzed for Slug expression by WISH. The number of embryos out of the total analyzed that showed the presented staining pattern is given. (D) X. laevis embryos were analyzed for SNW1 expression by WISH at the indicated stages. (E) Whole X. laevis embryo extracts were prepared from either wild-type (WT) embryos or embryos injected with 20 ng of a translation-blocking MO, MoSNW1a, at the one-cell stage, and analyzed by Western blotting using antibodies against SNW1 and Tubulin, the latter as a loading control. The developmental stages are indicated. (F) Zebrafish embryos were analyzed for SNW1 expression at the indicated times. Two gastrulation stages (the shield stage and 75% epiboly) and three segmentation stages (11, 12, and 13 hpf) are shown. Lateral views are shown, and for the later stages, also a dorsal-anterior view. (G) Whole zebrafish embryo extracts were prepared from wild-type embryos at the indicated times post-fertilization and analyzed by Western blotting using antibodies against SNW1 and MCM6, the latter as a loading control. The developmental stages are indicated. E, unfertilized egg.

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except in the yolk-associated tissue. It appears to be enriched anteriorly on the dorsal side and is expressed in both the ectoderm and mesoderm (Figure 1F). The same expression pattern persists until 13 hpf. Consistent with what we observed in Xenopus embryos, the SNW1 protein is deposited maternally in zebrafish embryos and increases in levels from 6 hpf (Figure 1G).

SNW1 Is Absolutely Required for Neural Crest Induction in Xenopus

SNW1 was isolated as a neural crest repressor in the overexpression screen, so we expected that depletion of SNW1 in embryos would induce more neural crest. Surprisingly, injection of MoSNW1a into one cell of a two-cell Xenopus embryo resulted in a dramatic loss of the neural crest markers Snail, Slug, and Sox9 [16,17] on the injected side (Figure 2A). To check that the loss of Snail, Slug, and Sox9 staining reflected an inhibition of neural crest fate and not merely a delay, we investigated the effect of SNW1 knockdown later in development, when the neural crest cells have migrated and invaded the branchial arches [1], using Snail and Twist as markers [18]. At stage 28 the amount of Twist-positive neural crest cells in morphant embryos was severely reduced on the injected side compared with the uninjected side and both sides of control embryos (Figure 2B). Similarly, a dramatic loss of Snail-stained neural crest cells was observed in MoSNW1a-injected embryos, and the neural crest cells that were induced did not migrate normally (Figure S3D). This was also evident when a different MO was used (MoSNW1b) (Figure S3A, S3C, and S3D).

We also examined neural crest derivatives at later stages of development. Cranial neural crest cells differentiate into cartilage and bones of the face, among other cell types [19]. Examination of branchial cartilage formation using alcian blue staining of MoSNW1a-injected embryos at the tadpole stages indicated that where SNW1 was depleted in half the embryo, the cartilage of the face did not form correctly (Figure 2C). Closer examination of the tadpoles demonstrated that non-neural-crest derivatives of the face formed correctly, such as the eyes (Figure S3E) and the blood vessels (data not shown).

Given that both overexpression and depletion of SNW1 had the same inhibitory effect on neural crest induction in Xenopus, it was important to determine whether X. tropicalis SNW1 mRNA could rescue X. laevis SNW1 depletion. The five mismatches between the MoSNW1a sequence and the X. tropicalis SNW1 sequence (Figure S3A) are sufficient to protect the X. tropicalis mRNA from silencing by the MO (Figure S3B). Xenopus embryos injected with the MO alone or with X. tropicalis SNW1 mRNA alone showed reduced expression of Slug and Sox9. However, injection of the MO with the X. tropicalis SNW1 mRNA restored both Slug and Sox9 expression (Figure S4A), confirming the specificity of both the overexpression and knockdown phenotypes. Interestingly, opposite effects of knockdown and overexpression on tailbud stage morphology were observed (Figure S4A, bottom row). Depletion of SNW1 resulted in dorsalized embryos that curled downwards because of more tissue on the dorsal side, whereas overexpression of SNW1 resulted in slightly ventralized embryos with smaller heads and anterior structures. This suggests that distinct mechanisms account for the repression of neural crest when SNW1 is overexpressed versus when it is depleted, which is discussed in more detail below.

The Role of SNW1 in Neural Crest Fate Is Evolutionarily Conserved

To investigate whether the requirement of SNW1 for neural crest fate was conserved in zebrafish we designed an antisense MO, MoZFSNW1, to block splicing (Figure S3A). Despite being injected within the first hour post-fertilization, this MO is effective only after 8 hpf, suggesting it inhibits only zygotic transcripts, analogous to the situation with the Xenopus SNW1 MOs. Knockdown of SNW1 in zebrafish using MoZFSNW1 inhibited neural crest fate as demonstrated by Sox10 and FoxD3 staining (Figure 2D). A dorsalized-like phenotype was also observed when SNW1 was depleted in zebrafish embryos with MoZFSNW1 (Figure S4B). This phenotype, which was characterized by loss of ventral tail fin tissues, and loss of posterior structures including the most posterior somites, was partially rescued by overexpression of X. laevis SNW1 (Figure S4C). Thus, the requirement for SNW1 for neural crest fate is conserved in zebrafish.

SNW1 Is Required in the Dorsal Ectoderm for Neural Crest Fate

As demonstrated by qPCR on dorsal and ventral halves of Xenopus embryos, SNW1 is expressed in both of these domains (Figure S2B). However, in situ staining in Xenopus and zebrafish embryos revealed that SNW1 mRNA is enriched in the dorsal ectoderm and mesoderm (Figure 1D and 1F). Specifically, in both organisms the enriched domain of expression in the ectoderm overlaps the neural plate as well as the neural crest (Figure 3A). To determine whether it was the dorsally expressed SNW1 that was required for neural crest specification we targeted the injection of MoSNW1a to either a dorsal or ventral quadrant of Xenopus embryos at the four-cell stage. Only depletion of SNW1 on the dorsal side, where its expression is enriched, affected neural crest fate (Figure 3B).

Since SNW1 is also enriched in dorsal mesoderm, it was important to establish that the effects of SNW1 depletion on neural crest fate were direct and not the result of perturbing earlier mesoderm induction or gastrulation movements, both of which could influence neural crest specification [1]. Depletion of SNW1 had no effect on the mesoderm marker Xbra, nor on the dorsal and ventral mesoderm markers Goosecoid (Gsc) and Sizzled (Szl), respectively, as examined by WISH at stage 12 (Figure S5A).
Figure 2. SNW1 knockdown inhibits neural crest induction. (A) Injection of MoSNW1a (Figure S3) results in the loss of neural crest markers Snail, Slug, and Sox9 on the injected side at stage 14. MoSNW1a (20 ng) was co-injected with Fdx as a tracer in one cell of a two-cell Xenopus embryo. The tracer was detected using an anti-fluorescein antibody. Control embryos were uninjected. (B) Two-cell Xenopus embryos were injected in one cell with MoSNW1a. (C) X. laevis Stage 42. (D) zebrafish.
Knockdown of SNW1 also had no effect on mesoderm induction or convergence-extension movements in an animal cap assay [20] (Figure S5B). Moreover, in zebrafish, depletion of SNW1 had no effect on the mesoderm markers No tail a, Even-skipped-like 1, Cdx4 (Figure S5C), or MyoD (Figure 2D).

To demonstrate that the loss of neural crest fate upon depletion of SNW1 is a direct effect on ectodermal patterning, we inhibited mesoderm induction by overexpressing Cerberus short (CerS) in Xenopus embryos. Full-length Cerberus inhibits Nodal, Wnt, and BMP signaling, whereas the N-terminally truncated form, named

Figure 3. Dorsally expressed SNW1 is required for neural crest specification, and this is independent of mesoderm. (A) Left panels. Wild-type (WT) zebrafish embryos at the indicated times post-fertilization were doubly stained for the neural crest marker Sox10 (dark blue) and SNW1 (grey-purple), which is localized in the neural plate (see Figure 1F). Right panels. Wild-type stage 14 Xenopus embryos were stained for either SNW1, the neural crest marker Slug or the neural plate marker Sox3. The orange lines mark the edges of the neural crest staining to enable direct comparison of expression domains. (B) One dorsal or one ventral blastomere of four-cell Xenopus embryos was injected with Fdx with or without 10 ng of MoSNW1a and fixed at 13 hpf for WISH using a probe against the neural crest marker Sox10, or at 12 hpf for WISH with probes against FoxD3 and the adaxial and paraxial mesoderm marker MyoD. A group of embryos is shown, as well as a representative single embryo. The neural crest cells are marked by grey arrowheads, and the MyoD-positive cells by an asterisk (righthand panels). For the MyoD staining, only the adaxial mesoderm is visible, and this is not affected by SNW1 depletion. In all cases the number of embryos out of the total analyzed that showed the presented staining pattern or phenotype is given.

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with either 20 ng of control MO (MoControl) or 20 ng of MoSNW1a. WISH was performed at stage 28 using a probe against Twist. (C) Xenopus embryos were injected with control MO or MoSNW1a as in (B). At stage 42 the facial cartilage was stained with alcian blue and then dissected. The injected side is indicated with an arrow. (D) Zebrafish embryos were injected with 15 ng of either a control MO or MoZFSNW1 and fixed at 13 hpf for WISH with probes against FoxD3 and the adaxial and paraxial mesoderm marker MyoD. A group of embryos is shown, as well as a representative single embryo. The neural crest cells are marked by grey arrowheads, and the MyoD-positive cells by an asterisk (righthand panels). For the MyoD staining, only the adaxial mesoderm is visible, and this is not affected by SNW1 depletion. In all cases the number of embryos out of the total analyzed that showed the presented staining pattern or phenotype is given.

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SNW1 Spatially Regulates BMP Activity
CerS, inhibits only Nodal signaling and hence mesoderm formation [21]. The expression of the mesoderm markers Xbra and Foxa was completely repressed by CerS overexpression, and the embryos develop without gastrulation movements, as evidenced by the lack of a blastopore (Figure 3C). Despite a lack of mesoderm formation, the neural plate and neural crest cells are induced, as seen by robust staining for Sox3 and Slug, respectively, in embryos overexpressing CerS (Figure 3C, lower two rows). Importantly, injection of MoSNW1a expands the expression domain of Sox3, consistent with the dorsalized morphology observed previously (Figure 5A), and represses the expression of Slug in both wild-type and CerS-overexpressing embryos (Figure 3C). These results indicate first that, contrary to what was previously thought [19], neural crest fate appears to be induced in *Xenopus* embryos in the absence of mesoderm, and second that the effect of SNW1 depletion on neural crest fate occurs entirely by perturbing ectoderm patterning.

**SNW1 Is Required for Formation and Sharpening of the Neural Plate Border**

Our observation that SNW1 depletion leads to an expansion in the expression domain of Sox3, as well as loss of neural crest, prompted us to assess how overall dorsal/ventral patterning was affected when SNW1 was knocked down. At the neurula stages, dorsal midline/notochord markers such as Goosecoid, Chordin, Xnot, and Noggin were strongly expressed in morphant embryos, although the notochord was visibly shortened and wider (Figure 4A), consistent with a shortening of the anterior-posterior axis seen at later stages (Figure 5A). This could be an effect on convergence-extension movements, but we do not believe that this contributes to the effect on the neural crest, since in the absence of morphogenetic movements when CerS is overexpressed, we still observed the specific effect of depleting SNW1 on the neural crest marker Slug (Figure 3C). Expression of Xnot and Noggin at the anterior neural plate border was also absent in the morphant embryos (Figure 4A, arrows). The paraxial mesodermal expression of Wnt8 was lost, although the ventral mesodermal expression near the blastopore was maintained (Figure 4A, arrows), and the somitic mesodermal marker MyoD was not affected (Figure 4A), similar to what was seen in the fish (Figure 2D).

Next, we examined a panel of ectodermal markers, starting with the neural plate marker Sox3. As previously shown (Figure 3C), the domain of Sox3 expression was expanded in SNW1 morphants, and the border of the neural plate was very diffuse (Figure 4A and 4B). Consistent with this, the domain of Epidermal keratin (EpiKer) staining was reduced in SNW1-depleted embryos, and the sharp border between neural and non-neural tissue was lost. Examination of the anterior neural marker Otx2 revealed little difference, except a slight expansion laterally, consistent with the observation that the neural plate is wider and that the embryos exhibit a dorsalized morphology later in development. The same was seen for Otx2 staining in fish embryos (Figure 5C). Interestingly, in *Xenopus* the neural expression of Zic3 parallels what was observed for Otx2, whereas the expression normally found at the neural plate border was fainter and more diffuse. The same was true for BMP4, which is normally expressed at the neural plate border but is more diffuse in SNW1 morphants (Figure 4A).

Most importantly, neural plate border markers (Mx3,1), including neural crest markers (Sox9, Snail, Slug, and FoxD3), were completely absent in morphant embryos (Figure 4A). Thus, neural plate border specification is completely lost when SNW1 is depleted. Careful examination of Sox3-stained *Xenopus* morphant embryos, especially from a lateral view, reveals that the neural plate border is consistently diffuse and expanded towards the ventral side (Figure 4B). There is a clear loss of the sharp border of the neural plate. We see a similar expansion of the neural plate in zebrafish morphants and loss of neural crest as seen by Vimentin and Sox10 double in situ hybridizations (Figure 4C). Furthermore, in *Xenopus* embryos the neural plate and epidermal ectoderm appear to merge together without a distinct border between the two tissue types (Figure 4D). Without neural plate border specification and the expression of Msx3, neural crest cells cannot be induced [22,23].

**SNW1 Is Required for Post-Gastrulation BMP Activity in Whole Embryos**

Among factors known to be important for neural plate border specification are the transforming growth factor β (TGF-β) superfamily ligands of the BMP family [1]. The loss of neural plate border markers in the SNW1 morphant embryos and the clear disruption of the sharp neural/non-neural border seen in embryos stained for Sox2/3 and Epidermal keratin (Figure 4B and 4D) raised the exciting possibility that SNW1 might directly affect BMP activity in vivo that is required for dorsal/ventral patterning of the ectoderm and neural crest specification.

BMP signaling leads to phosphorylation of the intracellular signal transducer Smad1 [2] and thus phosphorylated Smad1 (p-Smad1) can be used as a readout of BMP activity. BMP activity is first detected in developing *Xenopus* embryos at stage 9, and its activity increases through gastrulation and early neurulation as indicated by the level of p-Smad1 detected in whole embryo lysates (Figure 5A). At stage 13 (early neurula), SNW1-depleted embryos exhibited lower p-Smad1 compared to control embryos, and SNW1 overexpression resulted in higher levels of p-Smad1 compared to control embryos (Figure 5A). This would account for the opposite phenotypes seen when SNW1 is knocked down versus overexpressed (Figure 5A).

The significant stage-specific decrease in p-Smad1 levels in SNW1-morphant *Xenopus* embryos prompted us to examine how BMP activity is affected spatially. When stage 14 *Xenopus* embryos were bisected into dorsal and ventral halves, the majority of p-Smad1 detected in whole embryo lysates was derived from the ventral halves of embryos (Figure 5B). This ventral BMP signaling was substantially decreased in the morphant embryos, but slightly increased when SNW1 was overexpressed (Figure 5B). Similarly, knockdown of SNW1 in zebrafish embryos led to a dose-dependent decrease in p-Smad1 levels from 12 hpf (4–5 somites) (Figure 5C).

To visualize the spatial differences in BMP activity detected by Western blotting (Figure 5B) and determine how they are affected by SNW1 depletion, we initially used immunostaining for p-Smad1 in fixed embryos at stage 9, and its staining was reduced in SNW1-depleted embryos, but slightly increased when SNW1 was overexpressed (Figure 5B). Similarly, knockdown of SNW1 in zebrafish embryos led to a dose-dependent decrease in p-Smad1 levels from 12 hpf (4–5 somites) (Figure 5C).

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Figure 4. SNW1 is essential for specification of the border between neural and non-neural ectoderm. (A) *Xenopus* embryos were either uninjected (control) or injected with 20 ng of MoSNW1a at the one-cell stage. WISH was carried out for the dorsal mesoderm marker Goosecoid (Gsc), the dorsal midline markers Chordin (Chd), Xnot, and Noggin (Nog), the paraxial mesoderm markers Wnt8 and MyoD, the neural plate marker Sox3, the
BMP2b in zebrafish, which is functionally equivalent to plate and tailbud, and zebrafish in the endoderm (Figure S7). Importantly, zebrafish BMP4 [26], is specifically expressed at this region, whereas Xenopus activity found at the lateral edges of the neural plate. Furthermore, SNW1 is specifically required for the localized BMP activity investigated in vivo, we generated a zebrafish reporter transgenic line (Figures 5E and S7B). An initial characterization of this BRE-mRFP line is shown in Figure S6. WISH for mRFP in control embryos at 11–13 hpf reveals known domains of BMP activity, such as the tailbud (Figure 5E; see dorsal-posterior views), where high p-Smad1 staining has been reported [24,25]. In the anterior we detect a novel horseshoe-shaped domain of BMP activity at the border region between neural and non-neural ectoderm. This domain progressively sharpens from 11 hpf to 13 hpf (see red arrows, Figure 5E). In SNW1 morphant embryos, mRFP levels in this domain are significantly reduced. Very interestingly, mRFP is most strongly reduced at the lateral edges of the horseshoe-shaped domain in SNW1 morphants, whereas the tailbud and the most anterior neural plate border activities are not significantly affected (Figures 5E and S7B).

We examined which BMP ligands may be responsible for this localized activity at the neural plate border by carrying out in situ staining for BMP2b, BMP4, and BMP7 in zebrafish embryos. BMP2b in zebrafish, which is functionally equivalent to BMP4 in Xenopus [26], is specifically expressed at this region, whereas zebrafish BMP4 is mainly expressed in the anterior prechordal plate and tailbud, and zebrafish BMP7 is predominantly expressed in the endoderm (Figure S7). Importantly, BMP2b transcripts were unaltered in SNW1 morphants, indicating that SNW1 must act downstream of BMP2b transcription (Figure S7B). The BRE-mRFP zebrafish line provided us with increased resolution when observing BMP activity in vivo. This prompted us to use immunostaining of p-Smad1 on transversely bisected Xenopus embryos to see whether, indeed, BMP activity at the neural plate border is conserved and whether this localized BMP activity requires SNW1. Fluorescein dextran (Fdx) with or without MoSNW1a was injected into one cell at the two-cell stage (Figure S8). In embryos injected with Fdx alone, p-Smad1 could be detected at the region of the embryo that corresponds to the neural plate border on both the injected and un.injected sides. In embryos injected with Fdx and MoSNW1a, p-Smad1 could be detected only on the un injected side of the embryo (see enlarged images, Figure S8). Thus, the requirement for SNW1 for BMP activity at the end of gastrulation is conserved in the two species examined. Furthermore, SNW1 is specifically required for the localized BMP activity found at the lateral edges of the neural plate.

Neural Crest Cells Form in a Distinct Region at the Intersection of the Dorsally Enriched SNW1 Expression Domain and the BMP Activity Domain

We have demonstrated that SNW1 is required for neural crest fate, and have characterized the essential role of SNW1 in regulating BMP activity post-gastrulation. To find the link between these two activities of SNW1, we further exploited the BRE-mRFP fish line to visualize how SNW1 expression, neural crest cells, and the neural plate border BMP activity relate to each other spatially over time. Double in situ hybridization was carried out on BRE-mRFP embryos at 11 and 13 hpf. At 11 hpf, Sox10-positive neural crest cells and the enriched SNW1 expression domain in the neural plate overlap (Figure 6A and 6B). Neural crest cells also overlap with the broad horseshoe domain of BMP activity, as detected by in situ staining for mRFP (Figure 6A and 6B). Double in situ staining for SNW1 and mRFP demonstrates that at 11 hpf, there is no detectable separation between these domains (Figure 6A and 6B). Thus, neural crest cells appear to form where SNW1 expression and BMP activity meet. At 13 hpf, when the broad domain of BMP activity has sharpened, the embryos have undergone elongation, and the neural tube has started forming, there is clear separation between the SNW1 expression domain, including neural crest cells, and the domain of BMP activity (Figure 6A and 6B).

We carried out a similar analysis in Xenopus embryos by transversely bisecting embryos and carrying out WISH for Slug on one half, and immunostaining for p-Smad1 on the other half. Overlay of the p-Smad1 staining on the Slug staining reveals that the domain of BMP activity at the neural plate border overlaps with the neural crest cells (Figure 6C).

SNW1 Is Not a Core Component of the Intracellular BMP Signal Transduction Pathway and Must Function at the Level of, or Upstream of, the BMP Receptors

Since two studies have previously suggested a role for SNW1 in BMP- and TGF-β-dependent transcription [11,27], and mammalian SNW1 was originally discovered in a two-hybrid screen for proteins that interact with the transcriptional repressor of TGF-β superfamily signaling pathways, Ski [28,29], we investigated whether SNW1 might elicit its effects on neural crest fate by functioning intracellularly in TGF-β superfamily signaling pathways. We decided to use MDA-MB-231 cells for these assays as they express the necessary receptors and were previously shown to have a robust response to both TGF-β and BMPs at the levels of Smad phosphorylation and transcriptional output [30]. Small interfering RNA (siRNA) depletion of SNW1 in MDA-MB-231 cells, however, had no effect on the levels of phosphorylated Smad1/5 or Smad2 in response to TGF-β signaling pathways at the level of Smad activation in these cells (Figure 7A). Furthermore, SNW1 depletion using either the siRNA pool or individual siRNAs had no effect on the induction of a BMP-dependent reporter, BRE-Luciferase (Figure 7B), or a TGF-β-dependent reporter, CAGA-Luciferase (Figure S9A), although depletion of Smad4 reduced the activity of both of these reporters as expected [31] (Figures 7B and S9A). Moreover, despite reports of SNW1 interacting with Smad2, Smad3, and Ski when the proteins were overexpressed [11,32], we found no evidence for such interactions for the endogenous proteins (Figure S9B), although we could confirm the previously demonstrated interaction between Ski and Smad proteins [28] in.
Figure 5. SNW1 regulates BMP activity in *Xenopus* and zebrafish embryos and is essential for BMP activity at the border between neural and non-neural tissue. (A) One-cell *Xenopus* embryos were either uninjected (control) or injected with 20 ng of MoSNW1a or 500 pg of *X. tropicalis* MoSNW1. Embryos were harvested at the stages indicated, and whole embryo extracts were analyzed by Western blotting using antibodies against p-Smad1 and Tubulin, the latter as a loading control. Quantification of p-Smad1 levels relative to Tubulin is shown below the blots. (B) *Xenopus* embryos were either uninjected (control) or injected with 20 ng of MoSNW1a or 500 pg of *X. tropicalis* SNW1 mRNA at the one-cell stage, and bisected at stage 14 into dorsal (D) and ventral (V) halves. Whole cell extracts were analyzed by Western blotting using antibodies against SNW1, p-Smad1, Smad1, and Tubulin, the last as a loading control. WE, whole embryo. (C) Zebrafish embryos were either uninjected (U) or injected with increasing amounts (5, 10, 15, or 20 ng) of the splice-blocking MO MoZFSNW1. Embryos were harvested at 8, 10, or 12 hpf. Whole embryo extracts were analyzed by Western blotting using antibodies against SNW1, p-Smad1, and MCM6, the last as a loading control. (D) Knockdown of SNW1 strongly reduces ventral p-Smad1 levels at stage 13, but has no effect on the p-Smad1 ventral/dorsal gradient at stage 10. Embryos that were either uninjected (control) or injected with 20 ng of MoSNW1a at the one-cell stage were fixed at either stage 10 or stage 13, sagittally bisected along the midline, and immunostained with an antibody against p-Smad1. For these panels and subsequent p-Smad1 immunostaining, the specific p-Smad1 staining is nuclear and punctate. A, anterior; D, dorsal; P, posterior; V, ventral. (E) Transgenic BRE-mRFP embryos were injected with 15 ng of control MO or MoZFSNW1. They were fixed at 11, 12, and 13 hpf, when WISH was performed for mRFP, which indicates domains of BMP activity. In each case three different views of the same embryo are shown. In control embryos, mRFP is expressed in the tailbud (see dorsal-posterior view) and in a horseshoe-shaped domain at the dorsal anterior at 11 hpf (red arrowheads), which becomes increasingly sharpened at 12 and 13 hpf. In SNW1 morphants, the mRFP in this dorsal-anterior domain is reduced or absent. However, mRFP expression, and hence BMP activity, in the posterior is only slightly reduced or unchanged. In (D) and (E) the number of embryos out of the total analyzed that showed the presented staining pattern is given. In (E), the MO starts to knockdown SNW1 only from 8 hpf, and thus its effects become apparent only after 10 hpf. This explains why only a small percentage of embryos at 11 hpf shows the presented phenotype. By 13 hpf, when the MO effectively knocks down SNW1, 66% of the embryos show the phenotype. doi:10.1371/journal.pbio.1000593.g005

the same experiment (Figure S9B). These results argue against SNW1 functioning in either the core BMP or TGF-β pathways.

The experiments described above were performed in tissue culture cells using purified ligand. To determine whether the results hold true in *Xenopus*, we compared the effect of SNW1 knockdown on p-Smad1 levels in whole embryos at stage 14 with that in animal caps isolated at stage 8.5 then cultured to stage 14. In both of these cases the inducing ligands are endogenously produced. In whole embryos, SNW1 depletion resulted in a reduction of p-Smad1 levels equivalent to that caused by overexpression of the BMP antagonist Noggin [33] (Figure 7C). In contrast, in animal caps, depletion of SNW1 had no effect on p-Smad1 levels, whereas Noggin overexpression substantially reduced p-Smad1 (Figure 7C). SNW1 therefore affects BMP signaling only in the context of the whole embryo, and not in an isolated tissue or in cell culture, further indicating that SNW1 is not a core component of the intracellular BMP pathway.

Taking these results together with the results in the previous two sections, we conclude that SNW1 is required for BMP activity in vivo, that it plays an important role in demarcating the boundary of BMP activity at the neural plate border, and that it must act downstream of ligand transcription, but at the level of, or upstream of, the BMP receptors.

**Disruption of BMP Activity by Localized Expression of Noggin Mimics the Effect of SNW1 Depletion on Neural Crest Fate**

Having shown that the major effect of SNW1 depletion or overexpression is a change in BMP activity, we asked whether this was sufficient to explain the inhibitory effects of SNW1 depletion on neural crest fate and neural plate border formation. We therefore devised an alternative method of specifically lowering BMP activity after the mid-blastula transition to determine whether this would mimic the effect of SNW1 knockdown. We targeted a plasmid expressing the secreted diffusible BMP antagonist Noggin to the dorsal ectoderm, where BMP signaling is normally very low. Noggin produced in this region from mid-blastula transition onwards diffuses into neighboring tissue, thus inhibiting BMP activity. This was sufficient to lower BMP activity in the embryo, equivalent to the effect seen when SNW1 is depleted (Figures 5, 8A, and 8B). Most importantly, we observed a loss of Slug staining, disruption of the neural plate border, expansion of the neural plate as judged by Sox2 staining, and a dorsalized phenotype similar to what we observed with SNW1 depletion (Figure 8C). This strongly supports the idea that BMP activity between stages 12 and 14 is required for neural border sharpening/formation.

**Zebrafish BMP2b Can Rescue the Effect of SNW1 Depletion on Neural Crest Fate in *Xenopus* Embryos**

Since we know that zebrafish BMP2b/*Xenopus* BMP4 is responsible for the BMP activity at the neural plate border, we carried out an epistasis experiment to determine whether targeted expression of zebrafish BMP2b post-zygotically on the dorsal side of *Xenopus* embryos could rescue the neural crest phenotype in *XW1* morphant embryos. We therefore injected a plasmid expressing zebrafish BMP2b into dorsal blastomeres that would give rise to cells in or at the neural plate border. As expected, neural crest cells are very sensitive to the level of BMP activity, and the higher dose attempted (16 pg) resulted in the loss of Slug staining on the injected side of control embryos (Figure 8D), suggesting that this dose was too high. Similarly, this dose was unable to rescue neural crest fate in *XW1* morphant embryos. However, when we decreased the dose by half, we were able to maintain or slightly increase Slug staining in control embryos. Very importantly, at this dose, we managed to rescue Slug expression on the injected side of a significant number of *XW1* morphant embryos (Figure 8D). This experiment unequivocally links the effects of SNW1 depletion on BMP activity and the requirement of SNW1 for neural crest fate. Our work has therefore demonstrated that the effect of SNW1 on BMP activity post-gastrulation is critical for neural plate border formation and neural crest fate.

**Discussion**

**SNW1 Is a Critical Regulator of BMP Activity in Vertebrate Embryos**

We identified SNW1 in a large-scale in vivo functional screen in *Xenopus* for neural crest fate. Our characterization of SNW1 has revealed that it is a key novel regulator of in vivo BMP activity in vertebrate embryos. Specifically, SNW1 is absolutely essential for the regulation of BMP activity at the end of gastrulation and affects critical domains of BMP activity. Using MOs to study the loss-of-function effects of SNW1 in *Xenopus* and zebrafish embryos, we depleted zygotic SNW1, thereby targeting our analysis to post-gastrulation stages of development, and showed that this reduced
Figure 6. In post-gastrula zebrafish and *Xenopus* embryos the neural crest domain overlaps with the dorsal-anterior domain of BMP activity. (A) At early segmentation stages of zebrafish development the domains of expression of SNW1 and Sox10 partially overlap with the anterior “horseshoe” domain of BMP activity. Transgenic BRE-mRFP zebrafish embryos were stained for SNW1 (grey-purple) and Sox10 (dark blue), Sox10 (dark blue) and mRFP (grey-purple), mRFP (dark blue) and SNW1 (turquoise), mRFP (dark blue) and Hgg1 (maroon), or SNW1 (dark blue) and Hgg1 (maroon).
all readouts of particular domains of BMP activity at these stages of development. Since BMP activity is crucial for dorsal/ventral patterning during gastrulation, it is possible that maternal SNW1 may play important roles in regulating BMP activity and tissue patterning during these earlier developmental stages.

In wild-type embryos, immunostaining for p-Smad1 and analysis of the BRE-mRFP fish revealed that BMP activity is spatially and temporally dynamic during the stages of development examined. We have identified a novel horseshoe-shaped domain of BMP activity in 11- to 13-hpf zebrafish embryos at the border between the neural and non-neural ectoderm, and we have evidence that an analogous domain exists in *Xenopus*. Detailed spatial analysis further demonstrated that depletion of SNW1 results in a specific loss of BMP activity in this domain, at the lateral neural plate border (Figure 9), and also on the ventral side of the embryo, but morphant embryos maintain most of the BMP activity found at the tailbud and at the most anterior neural plate.

**SNW1 and BMP Activity on the Dorsal Side of Embryos Are Required for Neural Crest Fate**

**SNW1** is enriched dorsally during post-gastrulation stages, and we further isolated the effects of SNW1 depletion on neural crest fate to the dorsal side of the embryo. These results suggest that the BMP activity at the neural plate border, which overlaps with neural crest cells and the enriched domain of **SNW1** expression during the early neurula stage, is responsible for the loss of neural crest fate (Figure 9). Indeed, zygotic expression of the zebrafish BMP2b near or at the neural plate border is sufficient to rescue the loss of neural crest fate in **SNW1** morphant embryos. We did observe additional effects of SNW1 depletion, such as the loss of ventral/posterior tissues in zebrafish (Figure S4B and S4C), and the loss of some ventral BMP activity when SNW1 is depleted in whole embryos may be responsible for this phenotype.

In summary, we have found that disruption of the BMP activity at this particular stage of development (the end of gastrulation) and at a particular location in the embryo (the neural plate border) through the depletion of SNW1 results in the disruption of the sharp neural plate border and virtually complete loss of neural crest fate. This is an ectoderm-specific effect since SNW1 depletion has exactly the same effect when embryos fail to induce mesoderm or undergo gastrulation, as occurs when CerS is overexpressed. We have thus characterized how the BMP activity changes during the neural stages of development, found a factor that is required for particular domains of BMP activity in the embryo during this time, and demonstrated that this factor plays an essential role in ectodermal patterning.

**SNW1 Is Essential for Neural Plate Border Formation**

Consistent with previous reports that the ectoderm responds to signals before or during gastrulation to specify neural tissue [5,6], we found that Sox2 and Sox3 expression is induced in the expected region of the embryo when SNW1 is depleted. However, the expression of these markers is expanded more ventrally, consistent with the dorsalized phenotype of the morphant embryos seen at the tailbud stages. Unlike previous studies where expansion of the neural plate merely shifts the neural plate border, with the neural crest and other border fates still induced [16], we observed a complete loss of border fates. In the markers examined, Noggin and Wnt8B are induced in post-gastrulation embryos at the neural plate border region, and this specific domain of expression is lost in **SNW1** morphant embryos (Figure 4A). Since both Noggin and Wnt8B are secreted molecules involved in signaling required for neural crest fate [1,10], it is possible that this loss of expression contributes to the robust loss of neural crest cells in morphant embryos. However, we think that the loss of their expression at the neural plate border is more likely a consequence of the fact that the border does not form in **SNW1** morphants, because of the loss of BMP signaling in this region, rather than that they are direct targets of SNW1, since expression of Noggin and Wnt8 in the notochord and posterior mesoderm, respectively, is not affected by SNW1 knockdown (Figure 4A).

The two tissues that normally form on either side of the neural crest, neuroectoderm and epidermis, are both specified in **SNW1** morphant embryos, although there is no distinct border between them, as judged by the expression of Sox2 and Epker, respectively (Figure 4D). We have demonstrated that SNW1 is a fundamental regulator of BMP activity. To confirm it is **SNW1**’s effects on BMP activity that account for its effects on neural plate border formation at the end of gastrulation, we mimicked the effect of the **SNW1** MO by targeting the zygotic overexpression of a diffusible BMP antagonist, Noggin, to the dorsal ectoderm using DNA injection. Injection of mRNA for overexpression or the non-targeted overexpression of Noggin results in a gross dorsalization of the embryo, with a majority of the ectoderm being respecified as neural (data not shown) [16,34]. In contrast, localized expression of Noggin after the mid-blastula transition has a milder phenotype, phenocopying the **SNW1** morphant, demonstrating that late alteration of the BMP activity in whole embryos affects just border formation and not neural induction per se.

We also performed the reverse experiment, expression of a BMP ligand via injected exogenous cDNA at the correct stage of development, at the correct locale, and at a correct dose. We showed that this is sufficient to rescue neural crest fate in **SNW1** morphant embryos. If BMP activity however is increased earlier in development through injection of BMP mRNA for expression, the embryos develop with a severely ventralized phenotype, with complete loss of neural and head structures (data not shown). Thus, **SNW1** is a key regulator of BMP activity at the end of gastrulation, and, at this stage, BMP activity at the neural plate border is absolutely required for the formation of a sharp neural plate boundary and the specification of border fates.

During embryonic development, the same pathways are redeployed at different times and in different contexts to elicit distinct responses. We have demonstrated, to our knowledge for the first time, that neural induction, which is dependent on regulation of BMP activity during gastrulation, is uncoupled temporally from neural border sharpening and border fate specification, which require BMP activity, albeit at a later stage.

**SNW1 Functions Upstream of the BMP Receptors to Regulate BMP Activity**

In overexpression studies, SNW1 was previously reported to function with receptor-activated Smad proteins to regulate the
Figure 7. SNW1 does not function downstream of the receptors in the BMP pathway in tissue culture cells or in Xenopus animal cap explants. (A) Knockdown of SNW1 had no effect on the levels of p-Smad1/5 or p-Smad2 in response to ligand stimulation. SNW1 was depleted in MDA-MB-231 cells using an siRNA pool containing four individual duplexes that specifically target the human SNW1 mRNA. 72 h after siRNA transfection, cells were either left uninduced or induced for 1 h with 20 ng/ml BMP4 (R&D Systems) or 2 ng/ml TGF-β (PeproTech). Whole cell extracts...
were analyzed by Western blotting using antibodies against SNW1, p-Smad1/5, p-Smad2, and Smad2/3, the last serving as a loading control. In this gel system p-Smad1 and p-Smad5 are resolved into two bands. RiscFree (RF) siRNA is a non-targeting duplex used as a control. (B) siRNA-mediated knockdown of SNW1 in mammalian cells does not affect a BMP-responsive reporter activity. An MDA-MB-231 cell line stably expressing TK-Renilla and BRE-Luciferase was transfected with a non-targeting siRNA (NT), an siRNA pool against SNW1, the four individual siRNAs that make up the pool, or an siRNA against Smad4. 72 h after transfection, cells were induced with 200 ng/ml BMP7 (PeproTech). Samples were taken for Western blotting after 1 h and for Luciferase/Renilla assays after 8 h. The efficiency of knockdown is demonstrated in the Western blot shown. (C) SNW1 depletion affects BMP activity only in whole embryos and not in animal caps. Embryos were either un.injected or injected at the one-cell stage with 20 ng of MoSNW1a or 10 pg of a plasmid expressing Noggin. Animal caps were cut at stage 8.5 and cultured until sibling embryos had reached stage 14. Whole cell extracts from whole embryos or the animal caps were analyzed by Western blotting using antibodies against p-Smad1, Smad4, and Tubulin, the last serving as a loading control. doi:10.1371/journal.pbio.1000593.g007

Materials and Methods

Xenopus and Zebrafish Embryos, Microinjection, and Morpholinos

In vitro fertilization of X. laevis embryos and their culture, staging, manipulation, injection, and dissection was carried out as previously described [36], with the exception that animal caps were cultured in Danilchik’s blastocoeal buffer [37]. Zebrafish were maintained and staged as previously described [38,39]. Synthetic mRNA for injection was prepared as previously described [36]. X. tropicalis SNW1 mRNA for injection was synthesized using cDNA template from clone TEGg900909 from the X. tropicalis full-length cDNA library (Source BioScience; [40]), and X. laevis SNW1 mRNA was synthesized from l.M.A.G.E. Consortium clone ID 5083691. Noggin DNA injections were carried out using pcS2-Noggin [34], and BMP2b DNA injections used pcS2-zBMP2b. pcS2-zBMP2b was made by amplifying the coding region of BMP2b from zebrafish total gastrula cDNA and cloning the appropriately digested PCR product into pcS2+. The insert was checked by sequencing. Green fluorescent protein (GFP) mRNA was synthesized from pcS2-GFP (a gift from P. Blader), and CerS mRNA from pcS2-CerS [21]. Antisense MOs were designed and obtained from GeneTools and are listed below. In Xenopus, MOs were injected at the one- to 32-cell stages as indicated, whilst in zebrafish they were injected at the one- to eight-cell stage.

Morpholinos

The following MOs were used: MoControl (against human β-globin), CCTCTTACCTCAGTTACAATTTATA; MoSNW1a (against X. laevis SNW1), CTAGGGCATTTTCTCTGTGCATC; MoSNW1a (against X. laevis SNW1), GGCATATGGAGGAAGTGTCAGT-GACCTAAGAG; and MoZFSN1 (against zebrafish SNW1), AGACGTTTCTGCTGCATTCTTGT.

The Overexpression Screen

The X. tropicalis full-length expressed sequence tag library was obtained from Source BioScience (http://www.lifesciences. sourcebioscience.com/clone-products/cdna-and-orf-resources/- xenopus-tropicalis-est-clones.aspx). At the time of ordering, the collection comprised just over 6,900 unique clones. Pools of 48 individual clones (~7.5 μg of DNA) were linearized using Ascl and used as templates for in vitro transcription reactions with SP6 RNA polymerase to generate 5’ capped mRNA for injection into one-cell X. laevis embryos. Embryos were fixed at stage 14 and stained for Slug expression. Pools showing a strong effect on Slug staining were then deconvoluted. Individual clones isolated after deconvolution include the following: Dkk-1 (TGas131c10), c-Myc (TEgg042131), Noggin2 (TNeu122a14), and SNW1 (TEgg09009).

Cloning of Zebrafish SNW1

Zebrafish SNW1 cDNA including 5’ UTR was amplified from total cDNA (somitogenesis stage) using the following primers: 5’-TCCAA-

transcription of TGF-β superfamily target genes [11,27], placing it downstream of the receptors. However, we found that SNW1 in a model tissue culture system is not required for either TGF-β- or BMP-dependent transcription, and endogenous SNW1 did not co-immunoprecipitate with any endogenous Smad proteins or Ski. Together, these results argue against SNW1 being a component of the core BMP signal transduction pathway.

Similar to our observations in cell culture experiments, we found that depleting SNW1 in animal caps has absolutely no effect on p-Smad1 levels, whereas intact morphant embryos have substantially lower levels of p-Smad1 compared to control embryos. The animal cap is an isolated, mostly homogeneous tissue, cultured ex vivo. The fact that depletion of SNW1 does not affect p-Smad1 levels in this context further confirms that SNW1 is not a direct component of the BMP pathway downstream of the receptors. Furthermore, we can rule out any effect of SNW1 on p-Smad1 levels, whereas intact morphant embryos have substantially lower levels of p-Smad1 compared to control.

Since SNW1 is a nuclear factor that has been implicated in transcriptional regulation [11–13], we performed a microarray analysis comparing the expression profiles of control versus morphant embryos in an attempt to identify direct targets of SNW1. However, we did not detect any significant changes in the mRNA levels of known modulators, ligands, or components of the BMP signaling pathway (unpublished data). SNW1 has also been reported to be involved in transcriptional elongation and pre-mRNA splicing [14,15]. We therefore favor the possibility that SNW1 might regulate gene expression, at this level, of components required for BMP receptor activation, which could include the ligands themselves, proteins involved in their activation, extracellular matrix components, or co-receptors.

The Essential Role of SNW1 in Regulating BMP Activity May Be Evolutionarily Conserved

Since the function of SNW1 is conserved between Xenopus and zebrafish and its protein sequence is highly conserved from C. elegans to human, its function may also be conserved in other organisms. Indeed, we have evidence that SNW1/Bx42 RNA interference expression in the Drosophila wing disc perturbs P-Mad (Drosophila phosphorylated Smad1) levels (unpublished data), consistent with reported effects of SNW1/Bx42 RNA interference reducing the expression of Dpp target genes, Spalt and Optomotor Blind in the same system [35]. In sum, we have identified a novel role for SNW1 in neural plate border sharpening and neural crest fate. Since the neural crest is a vertebrate-specific embryonic tissue type, it is not surprising that this functional effect is a consequence of a more fundamental and likely conserved role that SNW1 plays in regulating BMP activity.
### A

|          | p-Smad1 | DAPI | GFP tracer |
|----------|---------|------|------------|
| Control  | D       | D    | D          |
| 10/10    | D       | D    | D          |
| Noggin DNA | D       | D    | D          |
| 8/10     | D       | D    | D          |

Stage 13

### B

- MoSNW1a: - + -
- Noggin DNA: - - +
- p-Smad1
- Tubulin

Stage 14

### C

| Slug | Sox2 | Phenotype/GFP |
|------|------|---------------|
| Control 10/10 | 10/10 | 27/27         |
| MoSNW1a 10/10 | 9/10  | 32/37         |
| Noggin DNA 8/10 | 10/10 | 17/20         |

Stage 14

### D

| Slug Fluorescein |
|-----------------|
| Un.injected    |
| 30/30          |
| 27/30          |
| Targeted inj at 1632-cell stage |
| Un.injected + Fdx |
| 22/27          |
| 25/25          |
| 8 pg BMP2b DNA + Fdx |
| Un.injected    |
| 21/23          |
| MoSNW1a        |
| 19/25          |
Figure 8. Reduction of BMP signaling by expression of Noggin in the dorsal ectoderm mimics SNW1 depletion. (A–C) Embryos were either uninjected, injected with 20 ng of MoSNW1a at the one-cell stage, or injected with 5 pg of a Noggin expression plasmid and 100 pg of GFP mRNA as a tracer in one dorsal A tier blastomere at the 16-cell stage. Samples were fixed at stage 13 for immunostaining for p-Smad1 (A), pseudo-colored magenta). Samples were taken at stage 14 for Western blot analysis using antibodies against p-Smad1 and Tubulin, the latter serving as a loading control (B), and samples were fixed at stage 14 for WISH using probes against Slug and Sox2 and at stage 28 for phenotype analysis (C). In (A) the nuclei are visualized with DAPI (pseudo-colored cyan) and the Noggin-injected cells with GFP. The dorsal side (D) is marked. In (B) two irrelevant lanes have been removed as indicated by the gap. In the bottom right panel of (C), the GFP marks the region of the embryo expressing exogenous Noggin. (D) Xenopus embryos were either uninjected or injected at the one-cell stage with 20 ng of MoSNW1a. At the 16- to 32-cell stage both groups of embryos were injected in one of the A1/D2/A2/B2 blastomeres with either 8 pg of plasmid expressing zebrafish BMP2b or 16 pg of the same plasmid together with Fdx as a tracer, as indicated. Embryos were fixed at stage 14, and neural crest was detected by staining for Slug by WISH. The Fdx was visualized with an antibody against fluorescein.

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GATGTCGCTTACAAG-3' and 5'-CAGAGAAGATGTCCTACTCC-3'. The PCR product was cloned into pGEM-T (Promega) and sequence verified.

Generation of the Transgenic BRE-mRFP Zebrafish Line
A detailed report of the transgenic BRE-mRFP line will be published elsewhere. Briefly, the construct used to generate pG3L3-BRE-mRFP was the pGL3-BRE-Luciferase plasmid [41], in which we replaced the luciferase gene with mRFP. The BRE-mRFP sequence was further subcloned into the TOL2 vector to generate a stable transgenic line as described [42–44]. An mRFP antisense in situ probe was generated by cloning a full-length mRFP cDNA into pGEM-T (Promega).

WISH, Double In Situ Hybridization, and Immunostaining
WISH was performed in Xenopus and zebrafish essentially as previously described [45,46]. The probes are described below. The chromogenic reaction was carried out using BM purple alkaline phosphatase substrate (Roche) or NBT/BCIP (Sigma). Fdx (Molecular Probes) was visualized with an anti-fluorescein antibody (11426338910; Roche). The double in situ protocol in zebrafish was modified from [47] using digoxigenin- and fluorescein-labeled antisense probes. The chromogenic substrates used were NBT/BCIP (Sigma) in combination with BCIP (Roche) or INT/BCIP (Roche). The use of BCIP as the first chromogenic substrate results in turquoise staining. However, when used after NBT/BCIP, BCIP gives rise to grey-purple staining. INT/BCIP was used as the second chromogenic substrate for Hgg1 detection. For double in situ hybridization in Xenopus, embryos were incubated with two differentially labeled probes during hybridization. The first probe was developed as described for single in situ hybridizations. For the second probe, embryos were washed three times in methanol, then rehydrated in decreasing concentrations of ethanol until 100% PBS-Tween was reached, before repeating the antibody incubation and staining steps. The second chromogenic substrate used was either BCIP alone or Magenta Phos (Sigma). Immunostaining of Xenopus embryos was carried out as previously described [48] using a 1:200 dilution of p-Smad1 antibody (9511; Cell Signaling Technology). Immunostaining of zebrafish embryos was as previously described [4].

WISH Probes
Digoxigenin-UTP (Roche)-labeled antisense RNA probes were synthesized using cDNA templates encoding Snail [49], Slug [16], Sox9 [17], Twist [18], FoxD3 [50], Sox2 [16], Sox3 [51], Wnt8 [52], Noggin [34], Xbra [35], Xnot [54], Chordin [55], Goosecoid [56], BMP4 [57], Sizzled [38], Zic3 [59], Otx2 [60], MyoD [61], Epidermal keratin [62], Sox10 [63], FoxD3 (zebrafish); [64], MyoD (zebrafish); [65], N-cadherin [66], Otx2 (zebrafish); [67], Gad4 [68], Even-skipped-like 1 [69], Nodal tail [70], BMP26 [71], BMP7 [72], and Hgg1 [73]. The antisense probe against Mxl was generated from the plasmid XMs1 in pGEM4Z and protects the region of Mxl encoding amino acids 40–129. The antisense probe against zebrafish BMP4 was generated from the plasmid pCS2P-zBMP4 (a gift from Arne Lekven) and protects full-length BMP4. The probe against Xenopus SWI7 was generated from pGEM-T-XISNWI and protected the region of SWI7 encoding amino acids 102–300. The probe against zebrafish SWI7 was generated from pGEM-T-zSNW1 and protects full-length SWI7.

Alcian Blue Staining
Alcian blue staining of cartilage was carried out as previously described [8].

Western Blotting
Xenopus embryos or animal caps were snap frozen at the required stage, and extracts were prepared as previously described [74]. Mammalian cell extracts were prepared as described in [31]. For zebrafish protein extracts, typically ten embryos (including chorion) were snap frozen and subsequently processed using the same protocol as for Xenopus embryos, except that the embryos were lysed using a plastic pestle and a pellet pestle motor (Kontes). Western blotting was performed using standard techniques and the following antibodies: from Santa Cruz Biotechnology, anti-SNW1 (H-300), anti-Smad4 (B8), anti-MCM6 (sc-30139), anti-Ski (H-329); from Abcam, anti-SNW1 (ab67715; for Figure 7A), anti-Tubulin (ab6160); from Cell Signaling Technology, anti-p-Smad1 (9511); from Millipore, anti-p-Smad2 (clone A5); from Zymed, anti-Smad1 (38-5400); and from BD Biosciences, anti-Smad2/3. Each lane on the Western blots is an average of 10–15 embryos, with 1–1.5 embryo equivalents of extract loaded.

RNA Extraction from Xenopus Embryos, RT-PCR, and qPCR
Extraction of total RNA from Xenopus embryos was carried out using Trizol reagent (Invitrogen) followed by a cleanup and on-column DNase treatment using the RNaseasy Mini Kit (Qiagen). The Affinity-Script QPCR cDNA Synthesis Kit (Stratagene) was used for cDNA synthesis following the manufacturer’s protocol. Semi-quantitative PCR from cDNA templates was carried out using GoTag (Promega) on a GeneAmp PCR system 9700 (Applied Biosystems), and qPCR was performed using an ABI 7500 Fast System (Applied Biosystems) with SYBR Green Master Mix (Applied Biosystems). The primers were as follows: SWI7, 5’-TGATGCTATTGCTGTCAGAGG-3’ and 5’-CTTGTGGGGACAGGTATTGT-3’; Chordin, 5’-CATGTCGTTTCAAGAGTCAA-3’ and 5’-GATGCTATTGCTGTCAGAGG-3’; Goosecoid, 5’-TGCCGTAGTATGTGTGTAGCTG-3’; Sizzled, 5’-TGCGTGATGTCTGTATTGCTGAGAGTACTC-3’ and 5’-ATCTTGGTGTGAGGTCTGCAAA-3’; Slug, 5’-CAAGTGTTAATTTGATCTGAGACGG-3’ and 5’-ACATTCTTTTCTGCTGAGATGTTGCTGCAAA-3’. Each lane on the Western blots is an average of 10–15 embryos, with 1–1.5 embryo equivalents of extract loaded.

MDA-MB-231 Cell Culture and siRNA Transfection
MDA-MB-231, MDA-MB-231 CAGA2-p-Luc/TK-Renilla and MDA-MB-231 BRE-Luc/TK-Renilla cell lines were cultured in DMEM supplemented with 10% FBS. siRNA transfection was
carried out using INTERFERin siRNA transfection reagent (PolyPlus Transfection) with 1 nM of siRNA. Cells were incubated after transfection for 72 h before further processing. siRNAs were purchased from Dharmacon. RISC-Free siControl (D-001220-01-05); Human Smad4 SMARTpool (M-005902-01); Human SNW1 SMARTpool (L-012446-00); Human SNW1-5 (J-012446-05); Human SNW1-6 (J-012446-06); Human SNW1-7 (J-012446-07); Human SNW1-8 (J-012446-08); and ON-TARGETplus Control Non-targeting siRNA 1 (D-001810-01).

Luciferase Assays and Co-Immunoprecipitations

MDA-MB-231 cells stably expressing Renilla from the Herpes Simplex Virus Thymidine Kinase (HSVtk) promoter (Promega) and Luciferase from either the TGF-β-dependent promoter CAGA12 [76] or the BMP-dependent promoter BRE [41] were used to study signal-induced transcription. Luciferase and Renilla were assayed as previously described [31]. Co-immunoprecipitations (IPs) were carried out as previously described [31].

Figure 9. A model of SNW1 function. A schematic diagram summarizing our findings in both Xenopus and zebrafish embryos. A dorsal-anterior view of a schematized embryo is shown. doi:10.1371/journal.pbio.1000593.g009
In Vitro Translation

The T<sub>7</sub>T Coupled Reticulocyte Lysate System (Promega) was used for direct translation of in vitro transcribed mRNA, and translation was carried out according to the manufacturer’s protocol. <sup>35</sup>S-labeled methionine and unlabeled amino acids were added to a 20 μl of reticulocyte lysate reaction along with 4 μg of mRNA template (same mRNAVolume ratio as injected into embryos). For reactions where MOs were added to block translation, 400 ng of MO was added (same MO:Volume ratio as MOs injected into embryos). Samples were separated on a 15% SDS gel, which was Coomassie stained before being dried and exposed to Hyperfilm (Amersham) to detect protein bands positive for <sup>35</sup>S-Met.

Supporting Information

Figure S1 SNW1 is a highly conserved protein. Multiple sequence alignment of SNW1 protein sequences from various model organisms using ClustalW. The *X. laevis* SNW1 protein (NP_001089903) is 98% identical to the *X. tropicalis* SNW1 (NP_001017145), 88% identical to *Homo sapiens* SNW1 (NP_056377), 87% identical to *Mus musculus* SNW1 (NP_079783), 87% identical to *Gallus gallus* SNW1 (NP_421294), 83% identical to the *Danio rerio* SNW1 (AAI07987), 62% identical to the *Drosophila melanogaster* ortholog Bsc42 (NP_511093), 53% identical to the *C. elegans* ortholog SKP-1 (NP_505950), and only 22% identical to Saccharomyces cerevisiae ortholog PRP45 (NP_009370). SNW1 is 87% identical to *C. elegans* ortholog Bx42 (NP_511093), 53% identical to the *Homo sapiens* protein, 83% identical to the *X. laevis* protein (outlined in red), which is central to the SNW domain (outlined in orange). In some organisms SNW1 is also known as SKIP [32]. In all organisms except yeast, SNW1 has a C-terminal nuclear localization signal (NLS; outlined in green), suggesting that it is a nuclear protein.

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Figure S2 SNW1 is ubiquitously expressed in the *Xenopus* embryo. (A) Total RNA was extracted from eggs (E) or embryos at different stages as indicated and analyzed by semi-quantitative RT-PCR for SNW1 andSlug, with *Omnithine decarboxylase* (ODC), which is ubiquitously expressed at a constant level [77] as a loading control. The neural-crescent-specific marker Slug is a positive control for a gene induced at neural stages. To demonstrate specificity of the products, the reactions were performed with (+RT) or without (-RT) reverse transcriptase. (B) Total RNA was extracted from whole embryos or the dorsal or ventral halves of bisected embryos at the stages indicated and analyzed by qPCR for SNW1, and the dorsally and ventrally expressed controls *Chordin* (*Chd*) and *Sizzled* (*Szl*), respectively.

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Figure S3 Depletion of SNW1 results in a dorsализed phenotype, reduces the number of *Snail*-positive neural crest cells, disrupts migration of neural crest cells into the branchial arches at the tailbud stages, and results in loss of cranial cartilage. (A) MOs used to deplete SNW1 in *Xenopus* and zebrafish. The two translation-blocking MOs targeting *X. laevis* (X) SNW1 are shown and aligned with both the *X. laevis* and *X. tropicalis* (Xl) sequences for SNW1. MoSNW1a overlaps the ATG (highlighted in green), while MoSNW1b binds in the 5’UTR. The MOs are 100% complementary to the *X. laevis* SNW1 sequence, whereas there are five non-conservative mismatches against the *X. tropicalis* sequence for each MO. The sequence targeted by the zebrafish SNW1 splice-blocking MO is shown. The exon is shown in uppercase and the intron in lowercase. (B) In vitro translation using rabbit reticulocyte lysate of either *X. laevis* or *X. tropicalis* SNW1 in the absence or presence of MoSNW1a or MoSNW1b. Translation of *X. laevis* SNW1 is inhibited by both MOs, with MoSNW1a being the more efficient. Neither MO inhibits translation of *X. tropicalis* SNW1. SNW1 levels were detected by labeling with <sup>35</sup>S-methionine and autoradiography. Equal amounts of translation reaction were loaded in each lane. (C) One-cell embryos were injected with 40 ng of control MO (C), 20 ng of MoSNW1a (Ma), or 40 ng of MoSNW1b (Mb). Whole embryo extracts were prepared at the stages indicated and analyzed by Western blotting for SNW1, and Tubulin as a loading control. Both MOs are effective in blocking zygotic SNW1 translation, as evidenced by a reduction in SNW1 protein levels only from stage 12 onwards, with Ma being more efficient than Mb, consistent with (B). (D) One-cell embryos were injected as in (C). Embryos were fixed at stage 28 and analyzed by WISH for *Snail*, which reveals that cranial neural crest induction is disrupted. (E) Injection of MoSNW1a in one cell of a two-cell embryo reduces cranial cartilage formation on the injected side (arrows). Two-cell *Xenopus* embryos were injected in one cell with either 20 ng of control MO or 20 ng of MoSNW1a, along with 250 pg of GFP mRNA as a tracer. In (D) and (E) the number of embryos out of the total analyzed that showed the presented phenotype is given.

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Figure S4 Overexpression of SNW1 can rescue the effects of SNW1 depletion in *Xenopus* and zebrafish. (A) The effects of SNW1 knockdown in *X. laevis* embryos can be rescued by overexpression of *X. tropicalis* SNW1. Embryos injected with 20 ng of control MO, 20 ng of MoSNW1a, 500 pg of *X. tropicalis* SNW1 mRNA, or both MoSNW1a and *X. tropicalis* SNW1 mRNA at the one-cell stage. Neural crest induction was assayed by WISH for *Sagl* and *Sox9* at stage 16. The phenotype was analyzed at stage 30. The number of embryos out of the total analyzed that showed the presented staining pattern/phenotype is given. (B) *Zebrafish* embryos were injected with 15 ng of either control MO or MoZFSNW1. They were photographed for phenotype analysis at 20 hpf. The morphant embryos present a dorsализed-like phenotype, but also display necrosis in the head, which is not rescued by p53 MO co-injection (data not shown) [78]. (C) Overexpression of *X. laevis* SNW1 partially rescues the effects of SNW1 knockdown in zebrafish. Embryos were either un.injected or injected with 7.5 ng of MoZFSNW1, 125 pg of *X. laevis* SNW1 mRNA, or both. Embryos were cultured until 40 hpf, when they were analyzed for phenotype. MO-injected embryos and embryos injected with both the MO and the rescue mRNA were scored for a dorsialized phenotype (looking only at the extent of posterior structures) as in [71] (right-hand graph).

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Figure S5 SNW1 knockdown has no effect on mesoderm induction or gastrulation and does not affect Activin-dependent induction of mesodermal tissue in *Xenopus* animal caps. (A) One-cell *Xenopus* embryos were injected with 20 ng of MoSNW1a. Uninjected control and MoSNW1a-injected embryos were fixed at stage 12 for WISH using probes against *Xbra*, *Goosecoid*, and *Sizzled*. (B) Animal caps dissected from MoSNW1a-injected embryos elonagte similarly in response to 20 ng/ml Activin (PeproTech) as caps cut from control embryos. Thus mesoderm induction is not affected by injection of MoSNW1a. (C) *Zebrafish* embryos were injected with 15 ng of either control MO or MoZFSNW1. The embryos were
fixed at 12 hpf and stained for the mesoderm markers No tail a (axial and tail), Even-skipped-like 1 (paraxial and tail), and Cdx4 (posterior axial and tail as well as some ectoderm). In SNW1 morphants, the expression of the mesoderm markers is largely unchanged, but there is a slight reduction in their expression in the tail mesoderm. Otx2 is a marker for anterior neural ectoderm and is normal albeit slightly expanded in SNW1 morphants. Otx2 and Cdx4 expression also indicate that anterior/posterior patterning is preserved in the absence of SNW1. In all cases the number of embryos out of the total analyzed that showed the presented staining pattern is given.

Figure S6 The BRE-mRFP transgenic zebrafish line is a reporter for BMP activity. (A) 24-h transgenic embryos imaged using bright field or fluorescence microscopy, or processed for in situ hybridization using a probe against mRFP. mRFP protein or mRNA is indicative of BMP activity. Indeed, in the anterior/head region, mRFP is detected in known sites of BMP expression and/or activity such as the dorsal retina and lens (arrow; [79]) and the otic placode (arrowhead; [80]). mRFP is also strongly detected in the epiphysis/pineal gland, consistent with data obtained in Xenopus (asterisk; [81]). In the posterior/tail region, mRFP as a readout of BMP signaling is present for instance in the dorsal ectoderm, where BMP ligands are expressed (arrow; [82]), the cloaca (arrowhead; [83]), and the somites (S; [82]). (B) mRFP in situ hybridization on transgenic embryos at 85% epiboly. mRFP transcripts are detected in the ventral ectoderm and the ventral lateral plate and intermediate mesoderm, where BMP signaling is known to be active [26]. The mRFP pattern is consistent with p-Smad1/5 staining at the same stage (arrowheads; see also [4]). (C) Noggin overexpression inhibits mRFP transcription downstream of the BRE promoter. As expected, injection of X. laevis Noggin mRNA results in reduced BMP signaling [26], which leads to lower amounts of mRFP transcripts in 70% epiboly embryos. Arrows and arrowhead indicate the extent of the mRFP expression domain for comparison between uninjected and injected embryos. In (B) and (C), V, ventral; D, dorsal. The number of embryos out of the total analyzed that showed the presented staining pattern is given.

Figure S7 BMP2b is the BMP family member in zebrafish that accounts for the BMP activity at the epidermis/neural ectoderm border that is dependent on SNW1. (A) Comparison of BMP2b, BMP4, and BMP7 expression in zebrafish embryos at 11 hpf [2–3 somite stage] using WISH. BMP2b transcripts are enriched at the epidermis/neural ectoderm border (arrows). In contrast, BMP4 is enriched in the anterior prechordal plate and the tailbud, while BMP7 appears to be expressed in the endoderm. (B) Transgenic BRE-mRFP embryos were injected with 15 ng of control MO or MoZFSNW1. They were fixed at 11 hpf, when WISH was performed for mRFP. SNW1 knockdown results in the strong loss of BMP activity at the epidermis/neural ectoderm border, as seen with mRFP WISH in BRE-mRFP embryos. Notably, some of the posterior mRFP staining is preserved in the SNW1 morphant, which is likely because of BMP4 activity. BMP2b expression, however, appears unaltered in SNW1 morphants, suggesting that SNW1 does not induce BMP activity at the epidermis/neural ectoderm border through the transcriptional regulation of BMP2b. Note that the same batch of injected embryos was stained for mRFP or BMP2b.

Figure S8 Localized BMP activity is detected at the neural plate border region in Xenopus embryos, and this is dependent on SNW1. Fix with or without 20 ng of MoSNW1a was injected into one cell of two-cell embryos. They were fixed at stage 13, bisected transversely through the neural crest region, and immunostained with antibodies against p-Smad1 and fluorescent. Nuclei were stained with DAPI. The specific p-Smad1 staining is nuclear and punctate. White arrowheads indicate a domain of p-Smad1 staining in the neural crest region that is lost on the injected side when SNW1 is depleted with the MO.

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: MYW MCR M MH. Analyzed the data: MYW MCR CSH. Performed the experiments: MYW MCR M. Wrote the paper: MYW MCR CSH.
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