Integration of Nodal and BMP Signaling by Mutual Signaling Effector Antagonism

Gary Huiming Soh,1 Autumn Penecilla Pomreinke,1 and Patrick Müller1,2,3,*

1Systems Biology of Development Group, Friedrich Miescher Laboratory of the Max Planck Society, Max-Planck-Ring 9, 72076 Tübingen, Germany
2Modeling Tumorigenesis Group, Translational Oncology Division, Eberhard Karls University Tübingen, Otfried-Müller-Straße 10, 72076 Tübingen, Germany
3Lead Contact
*Correspondence: patrick.mueller@tuebingen.mpg.de
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SUMMARY

Opposing sources of bone morphogenetic protein (BMP) and Nodal signaling molecules are sufficient to induce the formation of a full axis in zebrafish embryos. To address how these signals orchestrate patterning, we transplant sources of fluoroescingly tagged Nodal and BMP into zebrafish embryos, robustly inducing the formation of secondary axes. Nodal and BMP signal non-cell-autonomously and form similar protein gradients in this context, but the signaling range of Nodal (pSmad2) is shorter than the BMP range (pSmad5). This yields a localized region of pSmad2 activity around the Nodal source, overlapping with a broad domain of pSmad5 activity across the embryo. Cell fates induced in various regions stereotypically correlate with pSmad2-to-pSmad5 ratios and can even be induced BMP- and Nodal-independently with different ratios of constitutively active Smad2 and Smad5. Strikingly, we find that Smad2 and Smad5 antagonize each other for specific cell fates, providing a mechanism for how cells integrate and discriminate between overlapping signals during development.

INTRODUCTION

During development, cells need to know their location and fate in order to form an embryo. The required positional information can be conveyed by gradients of secreted signaling molecules that diffuse from a localized source to induce exposure-dependent cell responses (reviewed in Müller et al., 2013; Rogers and Schier, 2011). The earliest cell-fate decisions during vertebrate development are controlled by the signaling molecules Nodal and BMP, which form orthogonal overlapping activity gradients in zebrafish embryos (Figure 1A). Nodal induces the formation of the germ layers, which are subdivided into ventral and dorsal territories by BMP signaling (reviewed in Rogers and Müller, 2019). Nodal and BMP are secreted transforming growth factor β (TGF-β) superfamily ligands (Zhou et al., 1993; Wozney et al., 1988), which signal through a hetero-tetrameric complex composed of ligand-specific serine/threonine kinase receptors (Wrana et al., 1992) as well as co-receptors (Shen and Schier, 2000). Nodal signaling leads to the phosphorylation of the latent cytoplasmic signaling effectors Smad2/3, whereas BMP signaling causes the phosphorylation of Smad1/5/8. These pSmads then accumulate in the nucleus, where they regulate the expression of target genes (Heldin et al., 1997).

During zebrafish germ-layer patterning, the two Nodals Squint and Cyclops are produced at the embryonic margin and induce endoderm and mesoderm formation at a distance from the source (Bisgrove et al., 2017; Montague and Schier, 2017; Pelliccia et al., 2017; Chen and Schier, 2001; Feldman et al., 1998). Fgf8, a Nodal target gene, further extends the range of mesoderm (van Boxtel et al., 2018; van Boxtel et al., 2015; Mathieu et al., 2004; Rodaway et al., 1999). At the same time, the two BMPs Bmp2b and Bmp7 are produced predominantly on the ventral side to control dorsal-ventral patterning (Pomreinke et al., 2017; Zinski et al., 2017; Ramel and Hill, 2013). Bmp2b and Bmp7 form heterodimers, and homodimers of Bmp2b and Bmp7 alone do not elicit signaling (Little and Mullins, 2000). Additionally, Bmp2b is produced in the dorsal organizer to moderate the production of Chordin, a BMP inhibitory protein (Xue et al., 2014).

Strikingly, Nodal and BMP signaling together are sufficient to trigger all processes required to form an embryo. This was spectacularly demonstrated by generating ectopic juxtaposed sources of Nodal and BMP to induce a secondary embryonic axis in zebrafish (Figure 1A) (Xu et al., 2014). The ratio of Nodal to BMP signaling was suggested to be the determining factor in specifying the necessary cell fates for the embryonic axis. Nodal by itself creates axial structures, high Nodal-to-BMP ratios induce posterior head structures, low Nodal-to-BMP ratios organize the tail, and intermediate ratios generate the middle trunk (Fauny et al., 2009). However, it is unknown how Nodal and BMP gradients form, it is currently debated whether Nodal and BMP signal over long distances (Rogers and Müller, 2019; Pomreinke et al., 2017; Zinski et al., 2017; van Boxtel et al., 2015; Ramel and Hill, 2013; Müller et al., 2012; Chen and Schier, 2001), and the molecular mechanisms that allow cells to respond to different ratios of Nodal and BMP signaling are unclear.

To address these questions, we transplanted sources expressing fluorescently tagged Nodal and BMP into zebrafish embryos and generated secondary axes with high efficiency. Interestingly, Nodal and BMP formed protein gradients with similar shape and
amplitude in these secondary axis formation assays, but BMP had a long signaling range whereas Nodal induced pSmad signaling only locally around the transplanted clone. We found that the difference in signaling ranges can be explained by differential signaling activities of BMP and Nodal. Strikingly, specific ratios of constitutively active Smad2 and Smad5 were also able to generate a variety of embryonic structures, showing that the organizing ability of different Nodal/BMP ratios is mediated by different ratios of Smad2 and Smad5. We discovered that Smad2 and Smad5 selectively antagonize each other for certain cell fates while acting synergistically for others, which allows cells to respond differently to varying Nodal/BMP ratios. This selective

Figure 1. Secondary Axis Inducing Nodal and BMP Double Clones Produce a Localized Region of pSmad2 Activity Overlapping with a Broad Domain of pSmad5 Activity

(A) Nodal and BMP form orthogonal overlapping gradients in zebrafish embryos. Transplanting ectopic sources of Nodal and BMP induces the formation of a secondary axis, which contains both anterior and posterior structures such as the hindbrain, otic vesicles, notochord, and tail.

(B) Double clones of Bmp2b/7-sfGFP and Squint-mVenus imaged 30 min and 180 min post-transplantation. The first row depicts confocal microscopy images of Bmp2b/7-sfGFP (red) and Squint-mVenus (green). The second row shows light-sheet microscopy images of embryos immunostained with anti-pSmad2 (green) or anti-pSmad5 (red) antibodies as well as a cross-reactive anti-GFP antibody to detect Bmp2b/7-sfGFP and Squint-mVenus (blue). The third row shows comparable wild-type embryos. Nodal clones are traced in cyan and BMP clones are traced in white. Scale bar, 150 μm.

(C) Higher magnification of images shown in (B) with separate fluorescent channels. Scale bar, 150 μm.

(D) Images showing Nodal/BMP double clones with different spacings of transplanted cells taken immediately after transplantation. Scale bar, 150 μm.

(E) Nodal/BMP double clones were transplanted with different spacings into blastula-stage zebrafish embryos: narrow (~0 μm between clones, n = 60), moderate (40–50 μm between clones, n = 44), wide (120–150 μm between clones, n = 29), and very wide (>170 μm between clones, n = 20). Narrow to wide spacings support the formation of secondary axes, whereas secondary axis formation fails with extremely wide spacing between Nodal and BMP clones. Quantification was performed at 24 h post-transplantation.
mutual antagonism might represent a general mechanism for how cells integrate and discriminate between two overlapping signals during development.

RESULTS

BMP and Nodal Induce pSmad Signaling with Different Ranges

To visualize the organizing signaling gradients during secondary axis formation, we optimized a protocol to generate secondary axes by transplanting juxtaposed sources of fluorescently tagged Nodal and BMP into zebrafish embryos (Figure 1A). In contrast to the original blastomere injection approach (Xu et al., 2014), this method allows for precise control over the timing, placement, and spacing of Nodal- and BMP-producing sources. We tagged the signaling molecules with various fluorophores and tested different ratios of the fusion proteins to assess their efficiency in generating secondary axes after transplantation. Many combinations of fluorophores showed good activity in generating secondary axes (Table S1), similar to the previously reported efficiency using untagged Nodal and BMP (Xu et al., 2014). Interestingly, both zebrafish Nodals (Squint and Cyclops) were able to generate secondary axes (only Cyclops was used in the previous work of Xu et al., 2014), albeit at different amounts of the injected mRNAs. Although mCherry-tagged versions also induced secondary axes, the required relative molar amounts differed drastically from those of the untagged versions (Table S1). Furthermore, immunoblots of extracellular extracts showed that the fusion proteins were properly processed without releasing free fluorophores (Figure S1). We therefore decided to use Squint-mVenus and a 1:1 mixture of Bmp2b-sfGFP plus Bmp7-sfGFP (Bmp2b/7-sfGFP) for all subsequent axis-induction assays.

By generating localized sources of Squint-mVenus and Bmp2b/7-sfGFP, we found that these signaling molecules formed extracellular protein gradients within 30 min (Figures 1B and 1C). The gradients remained largely unchanged over the following 2 h (Figures 1B and 1C), similar to previous descriptions of Bmp2b-sfGFP and Squint-sfGFP gradient formation (Pomreinke et al., 2017; Müller et al., 2012). Interestingly, at 30 min post-transplantation, Nodal signaling (as assessed by pSmad2 induction; Figures 1B and 1C) was mostly limited to regions near the Nodal source, whereas BMP signaling (as assessed by pSmad5 induction; Figures 1B and 1C) had already spread extensively across the embryo. At 180 min, the pSmad2 signal was extended but still largely restricted to regions near the Nodal source, whereas pSmad5 remained more widely distributed (Figures 1B and 1C).

The wide and flat distribution of BMP signaling implied that the previously postulated close juxtaposition of opposing Nodal and BMP clones (Xu et al., 2014) might not be necessary for secondary axis induction and that localized Nodal signaling might be sufficient as long as there is some additional BMP signaling in the embryo. To test this prediction, we varied the spacing between Nodal and BMP sources and found that secondary axes could be generated for a wide range of different spacings (Figures 1D and 1E). The formation of secondary axes only failed when we placed the Nodal source much farther away from the BMP source than the range spanned by the pSmad5 gradient (~220 μm; Figure 1E). These results suggest that the BMP source generates a much more extensive signaling gradient than the Nodal source, even though the extracellular distributions of Nodal and BMP appear to be similar.

Secondary Axis Formation Does Not Depend on Relay Signaling

It had previously been suggested that secondary axis formation requires relay signaling through endogenous TGF-β superfamily ligands (de Oliveira-Melo et al., 2018; Xu et al., 2014). To test this idea and measure signaling kinetics in the absence of endogenous signals, we transplanted clones secreting BMP and Nodal into wild-type and mutant zebrafish embryos that lack the endogenous signaling molecules. We first transplanted clones secreting zebrafish BMP into wild-type embryos and compared the induction kinetics of pSmad5 to Mzswo (maternal-zygotic swirl-/- mutant) embryos that had received Mzswo clones ectopically producing BMP. Mzswo embryos lack functional Bmp2b (Schmid et al., 2000; Kishimoto et al., 1997) and are ideally suited to analyze the kinetics of pSmad5 induction in the absence of endogenous BMP signaling during embryonic development. Local BMP sources in wild-type embryos caused ventralization (Figure 2A, middle left), indicative of increased BMP activity in the entire embryo. The ubiquitous induction of BMP signaling was corroborated by the effects of small BMP clones in Mzswo embryos (Figure 2A, middle right) that were well rescued except for tail defects, indicating that the BMP clone indeed induced long-range signaling. Consistent with these phenotypes, BMP clones induced signaling rapidly, and pSmad5 signal filled up the entire field in both wild-type and Mzswo embryos (Figures 2B, 2C, S2, and S3A). The ability of the BMP source to induce pSmad5 signaling in Mzswo embryos demonstrates that a relay through the induction of endogenous bmp2b is not required. Strikingly, local BMP juxtaposed to Nodal clones even induced secondary axes in Mzswo embryos (Figure 2A, bottom right), indicating that BMP can work non-cell-autonomously and without relay signaling in this context.

To test the role of potential relays and quantify the dynamics of Nodal signaling, we transplanted cells producing Squint-mVenus into wild-type or Mzsqt;cyc mutant host embryos (clones transplanted into Mzsqt;cyc host embryos were Mzsqt;cyc mutant as well). Mzsqt;cyc mutants lack all maternal and zygotic Nodal ligands (Feldman et al., 1998) and serve as a background to analyze Nodal signaling in the absence of endogenous Nodal signals. Squint-mVenus clones in wild-type and Mzsqt;cyc embryos generated ectopic axial trunk structures (Figure 2D, middle). Strikingly, Nodal and BMP double clones were able to generate a secondary axis with anterior mesodermal structures such as anterior somites (Figure 2D, bottom right, blue arrowhead), which are normally absent in Nodal-deficient Mzsqt;cyc embryos (Figure 2D, top right, cyan arrowhead). The primary axis in these embryos lacked anterior mesodermal structures (Figure 2D, bottom right), as is expected for Mzsqt;cyc embryos (Feldman et al., 1998). In agreement with the restricted effect of anterior mesoderm rescue, pSmad2 staining was found locally around the Nodal
clones (Figure 2E), pSmad2 signal in MZsqt;cyc embryos extended up to several cell diameters away from the clone (Figures 2E and S3B), demonstrating that Nodal acts non-cell autonomously and without the need for a relay-based mechanism in this context as well. However, the range of Nodal-induced pSmad2 was significantly shorter than the range of BMP-induced pSmad5 (Figures 2C, 2F, and S3B). Importantly, untagged Squint and Bmp2b/7 generated a similarly large difference in the spatial ranges of Nodal and BMP signaling (Figures S2A and S2B), ruling out the possibility that the fluorescent tags are causal for the range differences.

Nodal and BMP Have Different Signaling Ranges despite Similar Ligand Distributions

Our finding that secondary axis formation is independent of relay signaling and purely relies on exogenously supplied signals provides an ideal system to test whether differences in signal gradient formation kinetics can explain the different signaling activity ranges. To relate the signal gradients to the signaling ranges, we developed a method to quantify the absolute concentrations of labeled Nodal and BMP in living zebrafish embryos based on their fluorescence intensities. We first purified recombinant sfGFP and mVenus proteins and established calibration curves relating the molar concentrations to their fluorescence intensities (Figure S4). We then used these calibration curves to determine the concentrations of fluorescently tagged Nodal and BMP expressed from local sources in zebrafish embryos. We found that the BMP gradient spanned a concentration range from 20 nM to 7 nM over 150 μm at 30 min post-transplantation (Figure 2G). At this time point, the pSmad5 gradient was similarly broad (Figure 2C), even when 5-fold less bmp2b/7-sfGFP mRNA was used (Figure S2C). Interestingly, although the concentration of BMP slightly increased over time (Figure 2G), most likely due to a larger effect of BMP production compared to its degradation, pSmad5 intensity concomitantly decreased (Figures 2C, S2A, and S2C). The decrease in pSmad5 intensity was also observed in chordin morphants (Figure S2D), arguing against the possibility of BMP signaling dampening by this major BMP antagonist (Fisher and Halpern, 1999; Blader et al., 2017; Schulte-Merker et al., 1997) in this context. In contrast, other Chordin-independent BMP-feedback inhibitors such as Bambia and Smad7 might be responsible for the down-regulation of BMP signaling over time (Pogoda and Meyer, 2002; Tsang et al., 2000).

The Squint-mVenus gradient produced from a localized clone formed with similar concentration distributions and dynamics as the BMP protein gradient at early time points but sharply dropped by 180 min post-transplantation (Figure 2H), possibly due to unstable mRNA, decreased translation, reduced secretion, or rapid internalization. In contrast to the drop in the Nodal gradient amplitude (Figure 2H), the levels of pSmad2 increased over time (Figure 2F).

Together, these results show that although Nodal and BMP form similar proteins distributions, their respective pSmad gradients are radically different, similar to the distinct distributions of pSmads induced by endogenous signaling molecules (Figure S2E). Therefore, the drastic differences in the pSmad gradients cannot be explained by the small differences in the amount of secreted Nodal and BMP proteins or by their similar effective diffusion coefficients (~3 μm²/s) (Bläisle et al., 2018; Pomreinke et al., 2017; Zinski et al., 2017; Müller et al., 2012).

Different Signaling Ranges Arise from Differences in Signaling Activity

Nodal signaling is antagonized by the feedback-induced Nodal inhibitors Lefty1 and Lefty2 during early zebrafish development (Rogers and Müller, 2019; Rogers et al., 2017; Agathon et al., 2001; Meno et al., 1999; Thissle and Thissen, 1999). To test whether the shorter signaling range of Nodal compared to BMP is due to inhibition by Lefty1 or Lefty2, we assessed signaling in Squint-mVenus clone experiments in which both the donor and the recipient embryo were MZlefty1;lefty2 double mutants lacking all maternal and zygotic Lefty activity (Rogers et al., 2017). Interestingly, in the absence of Lefty antagonism, the pSmad2 signal was extended (Figures S2F and S3C) but still much shorter than the range of pSmad5 induced by BMP clones.
(compare to Figure 2C). These results show that signaling range modulation by Lefty cannot explain the drastic difference between Nodal distribution and signaling in this context.

We therefore hypothesized that the different signaling ranges (pSmad5 and pSmad2) from similar input gradients (BMP and Nodal) might result from different signaling activation kinetics. According to the law of mass action and Hill kinetics, signals with higher sensitivity can induce activation faster, leading to a longer signaling range, whereas signals with low sensitivity might require extended exposure until activation is induced in a threshold-type manner, leading to a shorter signaling range (Michaelis et al., 2011). To test this idea, we took advantage of the recent discovery that a single source of mouse BMP4 can generate a secondary zebrafish axis (de Olivera-Melo et al., 2018), which suggested the possibility that mouse BMP4 might carry both BMP and Nodal secondary axes in zebrafish embryos (Figure 3A), ruling out the possibility that potential contaminations of commercial mouse secondary axes in zebrafish embryos (Figure 3A), which suggested the possibility that mouse BMP4 might carry both BMP and Nodal superfamily ligands are responsible for secondary axis formation.

To assess the plausibility that a single source of mouse BMP4 might generate different pSmad distributions, we developed a mathematical model based on Hill kinetics that we parameterized with the diffusion coefficient and protein half-life previously measured for zebrafish BMP (Pomreinke et al., 2017; Zinski et al., 2017) (Figure 3B). In this model, the differential readout of the mouse BMP4 gradient by pSmad5 and pSmad2 is dependent on a single parameter, i.e., the steepness of the pSmad activation term ($k_o$ for pSmad5, $k_o$ for pSmad2) that convolves the affinity of the BMP4 ligand for the BMP and Nodal receptors as well as the pSmad activation kinetics (Figure 3B). Simulations with smaller $k_o$ compared to $k_o$ values predicted that pSmad2 should be activated close to the mouse BMP4 source, whereas pSmad5 should have a wider range (Figure 3B).

In agreement with this model prediction, we found that pSmad2 and pSmad5 were indeed activated at different ranges by mouse BMP4. Local sources of mouse BMP4 in zebrafish embryos induced locally restricted pSmad2 but widespread pSmad5 (Figures 3C and 3D), providing further support that opposing sources of Nodal and BMP are not strictly needed for secondary axis formation. To rule out the possibility that pSmad2 activation is due to indirect induction of endogenous Nodal signals (de Olivera-Melo et al., 2018), we generated clones expressing mouse bmp4 in Nodal-deficient MZsqt;cdc mutant zebrafish embryos. We found that mouse BMP4 can indeed directly induce both pSmad2 and pSmad5 non-cell-autonomously (Figures 3E and 3F). Together, these results support our model that the exact same signal gradient can induce signaling effector activation at different ranges solely due to differences in signaling activity.

Our model implies that the action range of a signaling molecule with high signaling activity should be limited by its diffusion coefficient, whereas the action range of a signaling molecule with low signaling activity should be limited by its signaling activity rather than its diffusivity. To test this prediction, we sought to artificially reduce the diffusion coefficients of Nodal and BMP and measure how this affects their signaling ranges. We perturbed the protein distributions of Nodal and BMP using morphotrap, transmembrane proteins with extracellularly facing anti-GFP nanobodies that can drastically reduce the diffusivity of extracellular proteins tagged with GFP derivatives (Mörsdorf and Müller, 2019; Almuedo-Castillo et al., 2018; Harmansa et al., 2017). The protein distributions of Bmp2b/7-sfGFP and Squint-mVenus expressed from localized sources were strongly restricted in the presence of the morphotrap (Figure 3G). The sharp Bmp2b/7-sfGFP distribution led to a strongly restricted pSmad5 signal around the clone, whereas the already narrow range of pSmad2 was only marginally affected when the Squint-mVenus distribution was perturbed (Figures 3G and S2G). These findings provide additional support for our model that BMP has a longer signaling range than Nodal due to its higher signaling activity.

Strikingly, morphotrap-mediated range-restricted Nodal and BMP still supported the formation of secondary axes when the clones were closely juxtaposed (~0 µm between clones), whereas secondary axes could no longer be induced when the clones were far apart (>120–150 µm) from each other (Figure 3H, top). To test whether the long-range activity of Nodal or BMP is required in this context, we next perturbed the ranges of Nodal or BMP individually. Interestingly, morphotrap-mediated range restriction of Nodal was without consequence for narrowly or widely spaced clones (Figure 3H, middle), whereas secondary axis formation was abrogated when range-restricted BMP clones were placed far away from normal Nodal clones (Figure 3H, bottom). These results show that differences in the Nodal and BMP signaling ranges are functionally relevant for the formation of secondary axes. We note, however, that in normal embryos BMPs do not form a discrete source but are expressed in a broad domain, which gives rise to a broad signaling domain of pSmad5. Nodal by contrast is localized to the margin in a much more restricted domain, and pSmad2 is likewise activated in a much more restricted domain (reviewed in Rogers and Müller, 2019).

In conclusion, we showed that Nodal and BMP can induce signaling with different ranges, differential signaling activity can explain the differences in signaling ranges, and differential signaling ranges are relevant for secondary axis formation.

**Different Structures Can Be Induced by Specific Amounts of Active Smad2 and Smad5**

Our results suggest that the formation of a secondary axis requires a broad distribution of BMP and highly localized Nodal signaling. However, in addition to the spatial distributions, the relative signaling levels may also important for secondary axis formation (Fauny et al., 2009). By varying the relative levels of BMP and Nodal, we found that it is the ratio of Nodal to BMP rather than the absolute signaling level that determines whether a secondary axis can form. Lowering Nodal levels with respect to BMP levels precluded secondary axis induction (Figure S5A), whereas a commensurate reduction in both Nodal and BMP levels restored secondary axis formation (Figure S5A), correlating with a specific distribution of pSmad2 to pSmad5 ratios (Figures S5B and S5C).

To test whether the observed pSmad2-to-pSmad5 signaling effector ratio is causal for secondary axis induction, we
generated embryos in which we activated specific ratios of Smads in a localized region independently of the extracellular signaling molecules. By exchanging the three C-terminal serines with aspartates, we generated constitutively active Smad2 (Smad2-CA) and Smad5 (Smad5-CA) signaling effectors, which can activate the transcription of their respective target genes.
We found that expression of smad2-CA and smad5-CA in a localized region (Figure 4A) can generate various ectopic embryonic structures. Injecting smad2-CA alone generated an ectopic trunk structure containing axial tissues expressing the floorplate marker shha (Krauss et al., 1993) (Figure 4B, black arrowhead), just like in the case of an ectopic source of Nodal (Figure 4B) (Fauny et al., 2009). When we injected smad2-CA mixed with increasing amounts of smad5-CA mRNA (Figures S6).
Synergistically by both Smad2-CA and Smad5-CA at a moderate amount of Smad2-CA (Figure 5D). In contrast, high amounts of Smad2-CA led to reduced eve1 expression (Figure 5D), consistent with the absence of dorsal eve1 expression (Joly et al., 1993) where Nodal signaling is active over a long period of time (van Boxtel et al., 2018; Dubrulle et al., 2019). These results suggest that the selective mutual antagonism of Smad2 and Smad5 allows cells to respond specifically to different ratios of Smad2 and Smad5.

Our selective mutual antagonism mechanism predicted specific expression patterns of gsc, foxi1, and eve1 in the Nodal/BMP double clone secondary axis formation assay. gsc is induced by high Smad2 activity and suppressed by Smad5 activity (Figure 5B) and should therefore be expressed near the Nodal source opposite to the BMP clone, foxi1 is induced by high Smad5 activity and suppressed by Smad2 activity (Figure 5C) and should therefore be expressed near the BMP source opposite to the Nodal clone, and eve1 has a biphasic activation profile (Figure 5D) and should therefore be expressed in a complex pattern. To test these predictions, we subjected embryos carrying Nodal/BMP double clones to in situ hybridization with various probes followed by pSmad2 and pSmad5 immunostaining (Figure 5E). In agreement with the predictions of our selective mutual antagonism model, we found that gsc was expressed in the presence of pSmad2, but not when pSmad2 overlapped with pSmad5 (Figure 5E). In contrast, foxi1 was induced in regions of pSmad5 activity, but expression was reduced when pSmad5 overlapped with pSmad2 (Figure 5E). Strikingly, eve1 was induced in the predicted complex domain; eve1 was induced where pSmad2 overlapped with pSmad5, but it was not detected at the highest pSmad2 activity in the overlapping region (Figure 5E).

In conclusion, we found that the organizing activities of Nodal and BMP are mediated by specific amounts of active Smad2 and Smad5, whose selective mutual antagonism allows cells to respond specifically to different Nodal/BMP input ratios.

**DISCUSSION**

Understanding the dynamics of axis formation during early vertebrate development has largely been hampered by the lack of tools to relate the input from signaling gradients to the patterning output in terms of signaling effector activation and target gene expression. Transgenic animals expressing fluorescent fusions of the relevant signaling molecules under the control of endogenous regulatory elements are currently not available, and the timing and amplitudes of signaling gradients cannot be easily manipulated with good spatiotemporal control. Here, we used our optimized secondary zebrafish axis induction assay as an experimentally tractable model system to understand signaling input-output relationships and to decipher how Nodal and BMP signaling are integrated to form a secondary embryo.

Using active fluorescent fusions of Nodal and BMP expressed from clonal sources, we found that the signaling molecules form similar protein gradients of comparable shape and amplitude in zebrafish embryos. The similar protein gradients are in stark contrast to the differential distributions of the signaling effectors. The Nodal source generates a localized pSmad2 gradient that is overlaid by a broader pSmad5...
gradient induced by the BMP source. Taking advantage of the dual BMP/Nodal activity of mouse BMP4, we experimentally confirmed the prediction of our model that different signaling ranges of a single protein gradient can be explained by differences in signaling activity. In addition to differences in diffusion/clearance-based signal dispersal (Rogers and Müller, 2019), differences in signaling activity might therefore represent an additional knob to tune the ranges of signaling molecules and may play a role in restricting Nodal signaling to the margin. Consistent with this hypothesis, similar differences in TGF-β superfamily signaling dynamics were recently identified in cultured cells (Miller et al., 2019; Yoney et al., 2018). For example, Activin exogenously added to mouse embryonic stem cells was found to activate Smad2 rapidly and had a long signaling range, whereas BMP4 activated Smad1 more slowly and had a shorter signaling range (Yoney et al., 2018). In agreement with law of mass action considerations (Michaelis et al., 2011), it may thus be a general feature of developmental signaling systems that ligands that rapidly activate their effectors have a longer range, whereas ligands that slowly activate their effectors have a shorter range.

The difference in Nodal and BMP signaling ranges arises due to differences in signaling activation kinetics and yields a field of various positional information values in terms of pSmad2-to-pSmad5 ratios. Previous work has shown that ectopic expression of different amounts of Nodal and BMP induces the
formation of specific embryonic structures (Fauny et al., 2009). Here, we found that ectopic expression of different amounts of constitutively active Smads is sufficient to generate these structures, indicating that varying ratios of Smads are the major factors that confer the inductive capabilities of Nodal and BMP. Mutual antagonism as well as limited synergism between activated Smad2 and Smad5 can lead to distinct combinations of target gene expression sets that correlate with their spatial expression domains (Figures 5 and 6), and an important future goal will be to decipher the molecular mechanism by which pSmad ratios are integrated at the level of signaling or at target gene promoters (Figure 6).

Similar cases of mutual antagonism also exist for other signaling pairs, such as Bicoid and Caudal (reviewed in Briscoe and Small, 2015). However, since Bicoid represses Caudal translation via direct binding to caudal mRNA (Niessing et al., 2002), their mutual antagonism is not selective. The selective antagonism mechanism might be needed for Nodal and BMP because they form overlapping orthogonal gradients (reviewed in Rogers and Müller, 2019) instead of anti-parallel gradients. The overlapping nature of the Nodal and BMP gradients leads to an area with both high pSmad2 and pSmad5 activity, areas with either high pSmad2 or high pSmad5 alone, as well as areas without pSmad2 or pSmad5. However, cell fates in areas with high pSmad2 alone or high pSmad5 alone are different from those in areas with both high pSmad2 and high pSmad5. Therefore, the selective antagonism mechanism not only allows cells to sense the ratio of Nodal and BMP but also can work when these gradients extensively overlap at the ventral margin. It is possible that similarly easily implemented mechanisms might generally be involved in the interpretation of other overlapping gradients.

In summary, we used the Nodal/BMP-mediated secondary axis formation assay as a model system to understand how the integration of signaling gradients leads to the activation of signaling effectors and subsequent patterning. In this context, we found that Nodal and BMP activate effector Smads non-cell autonomously and induce signaling at different spatial ranges due to differences in their signaling activities. This yields a field of positional information values in terms of differential signaling effector ratios. Varying ratios of constitutively active Smads can induce different embryonic structures, and selective mutual antagonism of activated Smad2 and Smad5 allows cells to respond to different ratios of Nodal and BMP signaling. It is tempting to speculate that, similar to the Yamanaka factors that can convert differentiated cells into pluripotent cells (Takahashi and Yamanaka, 2006), it might be possible in the future to use the inductive properties of different ratios of constitutively active Smads to induce the formation of desired embryonic structures from pluripotent stem cells for regenerative medicine.

STAR+METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.03.051.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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Meno, C., Gritsman, K., Onahi, S., Otfuji, Y., Heckescher, E., Moghida, K., Shi-


# STAR★METHODS

## KEY RESOURCES TABLE

<table>
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<tr>
<th>REAGENT or RESOURCE</th>
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<td><strong>Antibodies</strong></td>
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LEAD CONTACT AND MATERIALS AVAILABILITY

All reagents generated in this study are available without restriction from the Lead Contact, Patrick Müller (patrick.mueller@tuebingen.mpg.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Zebrafish lines
Zebrafish husbandry was executed in accordance with the guidelines of the State of Baden-Württemberg (Germany) and approved by the Regierungspräsidium Tübingen (35/9185.46-5, 35/9185.81-5). The TE strain was used for experiments with wild-type zebrafish embryos. Maternal-zygotic double-homozygous sqtcz35 (Feldman et al., 1998) and cycm294 (Sam-path et al., 1998) mutants (MZsqt;cyc) as well as maternal-zygotic swrtc300a (Mullins et al., 1996) (MZswr) mutants were generated by germline replacement (Ciruna et al., 2002). Maternal-zygotic double-homozygous lef ty12146;lefty21146 mutants (MZlefty1;lefty2) were generated using a rescue approach with 4.8 μM of the Nodal inhibitor SB-505124 (Rogers et al., 2017).
METHOD DETAILS

Plasmids and in vitro synthesis of RNA

All plasmids for in vitro synthesis of mRNA were generated by inserting the sequence of interest into the EcoRI and XhoI sites of the pCS2(+) vector, containing the consensus Kozak sequence GCCACC directly in front of the start codon (Müller et al., 2012).

To generate pCS2-Bmp7-sfGFP, sequences encoding sfGFP flanked by LGDPPVAT linkers were inserted two amino acids downstream of the RSVR Furin cleavage site (Hawley et al., 1995). pCS2-Bmp2b-sfGFP was described previously (Pomreinke et al., 2017). pCS2-Bmp2b-mCherry was derived from pCS2-Bmp2b-sfGFP by exchanging the sfGFP-coding sequence with mCherry-encoding sequences. pCS2-Squint-mVenus and pCS2-Squint-mCherry were derived from pCS2-Squint-GFP (Müller et al., 2012) by exchanging the GFP-coding sequence with mVenus- and mCherry-encoding sequences, respectively. pCS2-Cyclops-mVenus and pCS2-Cyclops-mCherry were derived from pCS2-Cyclops-GFP (Müller et al., 2012) by exchanging the GFP-coding sequence with mVenus- and mCherry-encoding sequences, respectively.

Constitutively active Smads were generated by replacing the three C-terminal serine codons with aspartate codons. Smad5 was cloned from zebrafish shield-stage cDNA into the pCS2(+) vector with the primers GCGCGAATTCGCCACCATGACCTCCATGTCC CATGTCCTG and GCGCTCTAGATTACGAGACAGAGATGCG. The Q5 site-directed mutagenesis kit (New-England Biolabs) was then used to replace the C-terminal serine codons with the primers GACGGTGACTAATCTAGAACTATAGTG and ATCGATGGGGTT CAGAG. Smad2-CA was directly amplified and modified from zebrafish shield-stage cDNA with the primers TGCTTCGAATTCCATGTCATCGCAGCGTACGGAGGG and GACATTTCTGACTTGTTGCTTTCAC and ACTATAGTTCTAGATTAGTCCATGTCATCGCAGCGTACGGAGGG. The amplicon was cloned into the pCS2(+) vector.

mRNA for microinjection was generated using the SP6 mMessage mMachine kit (Thermo Fisher Scientific) according to the manufacturer’s instructions after plasmid linearization with NotI-HF (New England Biolabs, Cat#R3189). Plasmids containing sequence fragments of eve1, fox1, shha, krox20 and hoxc13b were generated using Zero Blunt TOPO PCR Cloning (Invitrogen, Thermo Fisher Scientific) and the following primers: CTGGTTCCAGAACCGGAGA and GGAAAGCATATGTA CATGGGGTTGAT for eve1, GTGAGCCAGCAGACCGAG and CTGGTTGGTGTGCAGATGCAG for fox1, ATACTGGCGCTGTTAGCGC and ACATTTCCTGACACCTTGCTTGC for shha,CACAACTTCCAGTGCAGA and GACCCGCTTGACTGCTCA for hoxc13b. AAGCCAGATGACCCACTAC and ACACAACGTTATAGTGGGAG for fox1, TCTAGGGTGGTCACTAGCAGA and ATTTACTGACACCTTGCTTGC for fox1, shha, hoxc13b. Linear fragments for eve1, fox1, shha, krox20 and hoxc13b were produced by PCR with M13 forward and M13 reverse primers. To generate templates for gsc and sox32 probes, plasmids were linearized with EcoRI and NotI (New England Biolabs) respectively (Müller et al., 2012). RNA probes for in situ hybridization were synthesized from these linearized plasmids using SP6 or T7 polymerase and DIG-modified ribonucleotides (Roche). RNA probes were purified using RNeasy kits (QIAGEN).

Recombinant proteins

sfGFP and mVenus were expressed in One Shot TOP10 E. coli using the plasmids pBAD-sfGFP (Addgene plasmid #54519 (Pédelacq et al., 2006)) and pBAD-mVenus (Addgene plasmid #54845 (Nagai et al., 2002)) after overnight induction with 10 mg/ml arabinose at 16 C. The fluorescent proteins were then purified by ethanol extraction as previously described (Samarkina et al., 2009). Briefly, E. coli cells were lysed by sonication in 20 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, pH 7.8, and sodium chloride and ammonium sulfate were added to the lysate to a final concentration of 0.41 M and 2.63 M, respectively. 1.2 volumes of 96% ethanol were then added to the lysate, and the mixture was vigorously shaken. After centrifugation, the fluorescent proteins became partitioned into the upper organic phase. The upper phase was recovered, and 0.25 volumes of n-Butanol were added. The mixture was then centrifuged, causing the fluorescent proteins to be partitioned into the lower aqueous phase from which they were recovered. The purified fluorescent proteins were then concentrated and buffer-exchanged into phosphate buffered saline (PBS) via ultrafiltration with Pierce protein concentrators (10K MWCO, Thermo Fisher Scientific). Fluorescent proteins were quantified using a Nanodrop 1000 (Thermo Fisher Scientific) by measuring their absorption spectra. The concentration was calculated according to the Beer–Lambert law using the measured peak absorption and the molar extinction coefficients taken from FPbase (Lambert, 2019).

Immunoblotting

Extra- and intracellularly enriched embryo extracts were prepared as described previously (Pomreinke et al., 2017; Müller et al., 2012). Protein samples were resolved on 12% polyacrylamide gels and transferred to polyvinylidene fluoride membranes using a semi-dry blotting system (Bio-Rad). The blots were blocked in 5% low fat milk in PBS containing 0.1% Tween 20 (PBST) for 1 hour, before being incubated overnight at 4 C with a dilution of 1:5000 rabbit anti-GFP antibody (Life Technologies) in 1% low fat milk in PBST. The blots were washed 3 times for 10 min each with PBST and then incubated for 1 h at room temperature with a dilution of 1:5000 goat anti-rabbit horseradish peroxidase (Jackson ImmunoResearch) in 1% low fat milk in PBST. Finally, the blots were washed 3 times for 10 min each with PBST, and the signal was developed with SuperSignal West Dura extended duration substrate (Thermo Fisher Scientific) for imaging with a chemiluminescence imaging system (Fusion 483 Solo, Vilber Lourmat).
Injections and transplantsations

For transplantation experiments, embryos were dechorionated using 0.1 mg/ml Pronase (Roche) in 5 mL embryo medium and rinsed in embryo medium to remove the Pronase (Rogers et al., 2015). Embryos were then injected with 2 nL of injection mix at the 1- or 2-cell stage and incubated at 28°C until transplantation. Unfertilized or injured embryos were discarded. Transplantation was done when the embryos reached sphere stage. A cylinder of cells, approximately 80 μm in diameter and 100 μm in length, was transplanted from sphere-stage donor embryos expressing squint-mVenus or bmp2b-sfGFP + bmp7-sfGFP (termed bmp2b/b7-sfGFP in the following) into uninjected sphere-stage sibling hosts. Combinations of 100 pg squint-mVenus mRNA or 100 pg + 100 pg bmp2b/7-sfGFP mRNA were used for most experiments, except for Figure S5, where 20 pg squint-mVenus mRNA along with 20 pg + 20 pg bmp2b/7-sfGFP mRNA and 20 pg squint-mVenus mRNA along with 100 pg + 100 pg bmp2b/7-sfGFP mRNA were additionally used. For transplantations with mouse BMP4, 40 pg of mouse bmp4 mRNA was used. Cells for mock transplantations and those with mouse BMP4 were additionally labeled by injecting 100 pg of 10 kDa cascade blue-dextran (Thermo Fisher Scientific) at the one-cell stage. Following transplantation, the embryos were placed in Ringer’s solution (116 mM NaCl, 2.8 mM KCl, 1 mM CaCl₂, 5 mM HEPES) for 15 min to recover and then incubated at 28°C until further processing.

To test the activity of constitutively active Smads, different amounts of smad2-CA or smad5-CA mRNA as detailed in the figures were injected into one-cell stage embryos, which were dechorionated at shield stage before fixation.

For the generation of ectopic structures with constitutively active Smads, embryos were dechorionated. Three adjacent blastomeres in embryos at the 64-cell stage were then injected with 20 pg smad2-CA, 20 pg smad2-CA + 20 pg smad5-CA, or 20 pg smad2-CA + 80 pg smad5-CA mRNA per blastomere. For Nodal and BMP receptor inhibition, 40 μM of SB-505124 (Sigma-Aldrich) and 10 μM of Dorsomorphin (Abcam) were used, respectively.

Combined whole-mount immunofluorescence and in situ hybridization

Embryos were fixed in 4% formaldehyde in PBS overnight at 4°C, dehydrated in 100% methanol and stored at −20°C until further processing. Chromogenic in situ hybridization was carried out as described before (Thiese and Thiese, 2008). Fluorescent in situ hybridization (FISH) was executed as described before (Almuedo-Castillo et al., 2018). If immunostainings were subsequently done, embryos were also incubated with 20 mM HCl for 25 min to inactivate the horse radish peroxidase (Liu et al., 2006).

For whole-mount immunostainings, embryos were washed three times with PBST and then permeabilized with cold acetone at −20°C for 20 min. Blocking and antibody incubations were performed in 10% FBS in PBST, and all washes were done with PBST. To carry out the dual pSmad2 and pSmad5 stainings, embryos were first blocked and then incubated with a 1:5000 dilution of a rabbit anti-pSmad2 antibody (Cell Signaling Technology) at 4°C overnight followed by 8 washes for 15 min each. The samples were then blocked and incubated with 1:500 goat anti-rabbit horseradish peroxidase (Jackson ImmunoResearch) at 4°C for 20 min. Blocking and antibody incubations were performed in 10% FBS in PBST, and all washes were done with PBST. To inhibit the activity of constitutively active Smads, different amounts of smad2-CA or smad5-CA mRNA were additionally used. Cells for mock transplantations and those with mouse BMP4 were additionally labeled by injecting 100 pg of 10 kDa cascade blue-dextran (Thermo Fisher Scientific) at the one-cell stage. Following transplantation, the embryos were placed in Ringer’s solution (116 mM NaCl, 2.8 mM KCl, 1 mM CaCl₂, 5 mM HEPES) for 15 min to recover and then incubated at 28°C until further processing.

Light-sheet microscopy

Fluorescence images of fixed samples were obtained using a Lightsheet Z.1 microscope (ZEISS). Samples were mounted in 1% low-melting agarose (Lonza, Cat#50080) in embryo medium using a size 3 glass capillary sample holder (ZEISS) and a needle to orient the embryos. The samples were imaged as a series of z stacks with the following objectives and imaging conditions: W Plan-Apochromat 20 × objective, 0.5 × zoom, separate exposure, 80 ms exposure time, 6.4 μm average light-sheet thickness, 10 μm intervals between z-slices. For samples stained by in situ hybridization alone, a 488 nm laser (100 mW) was used at 6% power for foxi1, 1% power for gsc, 8% power for eve1. For samples with double pSmad staining, a 488 nm laser (100 mW) was used at 1% power, a 561 nm laser (20 mW) at 5% power, and a 638 nm laser (75 mW) at 8% power. For samples with in situ hybridization signals and double pSmad staining, a 561 nm laser (20 mW) was used at 5% power, and a 405 nm laser (20 mW) was used at 10% power in addition to the other three lasers. Images were acquired with 1920 pixels × 1920 pixels (877.13 μm × 811.13 μm) dimensions. The multispectral image was then converted into a single channel image by linear unmixing using ZEN Black (ZEISS) (Figure S2H).

Confocal microscopy

Live imaging of embryos was executed on an LSM 780 NLO confocal microscope (ZEISS) using an LC C-Apochromat 40 × /1.1 NA water immersion objective. Embryos were mounted in 1% low-melting point agarose in glass bottom Petri dishes (MatTek Corporation) and covered with embryo medium. Embryos were maintained at 28°C during the experiments using a heated chamber and imaged 30, 60, 120 and 180 min post-transplantation. The fluorophores were excited with a 50 mW argon laser, sfGFP was excited at 488 nm with 16% laser power, and mVenues was excited at 514 nm with 35% power. The emission was collected as a multispectral image using a 32-channel GaAsP QUASAR array. Images were acquired with 512 × 512 pixels (425.10 × 425.10 μm) dimensions. The multispectral image was then converted into a single channel image by linear unmixing using ZEN Black (ZEISS) (Figure S2H).
QUANTIFICATION AND STATISTICAL ANALYSIS

Image analysis
Fiji was used for all image analyses (Schindelin et al., 2012). Nodal and BMP gradients were quantified similar to previous approaches (Pomreinke et al., 2017; Müller et al., 2012). A median filter of 1 pixel radius was applied to the images for denoising. This was followed by a maximum intensity projection of 14 z-slices.

Nodal, BMP, pSmad2 and pSmad5 gradients were quantified in a rectangular 66 µm wide and 150 µm long region. The “plot profile” function in Fiji was used, which averages the data along the width. Background levels were determined by measuring the average intensity from untransplanted embryos, and the background was subtracted from the gradient profiles. Prism (GraphPad Software) was used for data plotting. All error bars indicate 95% confidence intervals.

The pSmad ratio images were generated by first subtracting background intensities from each channel separately followed by a Gaussian blur with a sigma of 10. The Gaussian blur was performed to visualize an area of pSmad2 or pSmad5 activity instead of specific nuclei and to minimize aberrantly high ratios resulting from division by very low pSmad5 intensities. After Gaussian blurring, the pSmad2 channel was divided by the pSmad5 channel to generate the final image.

To determine the target gene response with varying amounts of Smad2-CA + Smad5-CA, the average intensity in a central circular region with half of the embryo radius was measured.

Mathematical modeling
The finite element method was used for two-dimensional numerical simulations of differential signaling activation kinetics from a single input gradient. The zebrafish animal pole was modeled as a circle with a radius of 300 µm, and the mouse BMP4 (mBMP4) expressing clone was placed concentrically into the embryo disc with a radius of 10 µm. mBMP4 gradient formation was simulated using the following partial differentiation equation:

$$\frac{\partial m \text{BMP}4}{\partial t} = D \nabla^2 m \text{BMP}4 + k_1(x) - k_2 m \text{BMP}4$$

where $D = 3 \, \mu m^2/s$ represents the diffusion coefficient of mBMP4 (based on measurements of zebrafish Bmp2b (Pomreinke et al., 2017; Zinski et al., 2017)), $k_1 = 1/s$ represents the spatially restricted production rate constant of mBMP4 exclusively within the clone, and $k_2 = 10^{-4}/s$ (based on measurements of zebrafish Bmp2b (Pomreinke et al., 2017)) represents the spatially uniform clearance rate constant.

The readout of the resulting mBMP4 gradient by pSmad5 and pSmad2 was modeled as

$$\frac{\partial p \text{Smad}5}{\partial t} = k_3 \frac{m \text{BMP}4}{k_4 + m \text{BMP}4} - k_5 p \text{Smad}5$$

$$\frac{\partial p \text{Smad}2}{\partial t} = k_3 \frac{m \text{BMP}4}{k_4 + m \text{BMP}4} - k_6 p \text{Smad}2$$

where $k_3 = k_4 = 10^{-4}/s$ represent the degradation rate constants of the pSmads and $k_5 = k_6 = 1/s$ represent their production rate constants. $k_3 = 100$ and $k_4 = 10^6$ represent the different signaling thresholds that activate pSmad5 and pSmad2, respectively.

The solution at each time step in the discretized geometry was determined using a sparse LU factorization algorithm (UMFPACK), and the time stepping was computed using a backward Euler step method (COMSOL Multiphysics 3.5a). Simulations were executed for a total of 7200 s.

While $D$ and $k_2$ are based on directly measured quantities, the values for $k_1, k_3, k_4, k_5, k_6$ and $k_7$ have not been experimentally determined. $k_1, k_3$ and $k_4$ control the amplitude of the gradients – but not their shape – and were therefore set to an arbitrary value of 1/s. Equal values of $k_3$ and $k_4$ were chosen to reflect the timescale of gradient formation. A large difference between $k_5$ and $k_6$ values was chosen to illustrate that a single signaling molecule gradient can generate vastly different activity gradients based on differential signaling kinetics.

DATA AND CODE AVAILABILITY

The raw images and data used in this work are available from the Lead Contact upon request.