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Review article

Nodal and BMP dispersal during early zebrafish development

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A B S T R A C T

The secreted TGF-β superfamily signals Nodal and BMP coordinate the patterning of vertebrate embryos. Nodal specifies endoderm and mesoderm during germ layer formation, and BMP specifies ventral fates and patterns the dorsal/ventral axis. Five major models have been proposed to explain how the correct distributions of Nodal and BMP are achieved within tissues to orchestrate embryogenesis: source/sink, transcriptional determination, relay, self-regulation, and shuttling. Here, we discuss recent experiments probing these signal dispersal models, focusing on early zebrafish development.

1. Introduction

In the contemporary view of embryogenesis, signaling molecules must be distributed in the embryo at the right place and the right time to specify different cell fates and coordinate patterning. This view arose from several observations (De Robertis, 2006; Fukuda and Kikuchi, 2005; Gurdon and Bourillot, 2001; Rogers and Schier, 2011; Schier and Talbot, 2005). First, fate mapping demonstrated that distinct cell types originate from stereotypical embryonic locations. Second, exposure to signaling molecules can induce different cell fates, and signaling molecules are usually expressed locally in embryonic regions that give rise to these fates. Third, removal of signaling molecules and excess signaling both alter cell fate specification and lead to mis-patterned embryos. Finally, secreted signaling molecules and their extracellular antagonists were shown to explain the decades-old observation that some embryonic regions can induce specific cell fates in surrounding tissues. Taken together, these observations indicate that achieving the correct distribution – typically a gradient – of signaling molecules is crucial for developmental patterning.

Secreted signaling molecules are largely responsible for organizing the vertebrate body plan during development (Schier and Talbot, 2005; Tuazon and Mullins, 2015). Among these are the TGF-β superfamily signals Nodal and BMP, which together instigate and coordinate embryonic patterning (Box 1) (Hill, 2017; Langdon and Mullins, 2011; Massague, 2012; Plouhinec and De Robertis, 2009; Ramel and Hill, 2012; Schier, 2009; Schier and Talbot, 2005; Smith, 2009; Wu and Hill, 2009). Nodal specifies endoderm and mesoderm and patterns the germ layers, and BMP specifies ventral fates and patterns the dorsal/ventral axis. Strikingly, these two signaling molecules appear to be sufficient to orchestrate patterning: In zebrafish embryos, an entire secondary axis can be generated by introducing juxtaposed clones expressing Nodal and BMP, which activate and regulate additional signaling pathways required for patterning (Xu et al., 2014).

In this essay, we discuss current models of signal distribution during early embryogenesis, focusing mainly on zebrafish Nodal and BMP because of their remarkable ability to coordinate the majority of axis formation. We examine major evidence supporting and challenging different models of Nodal and BMP dispersal, and suggest approaches to resolve open questions.[1]

2. Models of signal dispersal

A pioneering and influential signal dispersal model was proposed in 1970 by Francis Crick. In the *source/sink model*, a localized source produces a signaling molecule at a constant rate, which then diffuses through the tissue and is destroyed by a localized "sink" positioned at a distance from the source (Fig. 1a) (Crick, 1970). The resulting spatial gradient of signaling molecules generates a spatial gradient in signaling activity, causing cells to acquire different position-dependent fates (Rogers and Schier, 2011). Eighteen years later, Wolfgang Driever and Christiane Nüsslein-Volhard published their celebrated studies on Bicoid, a *Drosophila* patterning protein with a graded distribution thought to be generated by diffusion from a local source combined with spatially uniform degradation – a modified version of Crick's proposal (Driever and Nüsslein-Volhard, 1988a, 1988b). Other signaling molecules were subsequently identified across the animal kingdom and suggested to form gradients via a source/sink mechanism, including Nodal and BMP (Chen and Schier, 2001; Harmansa et al., 2015;

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[1] Signal dispersal models involving long filopodial extensions called cytonemes have recently been discussed elsewhere (Kornberg, 2017). In zebrafish, cytonemes have been implicated in the transport of the hydrophobic signaling molecule Wnt (Stanganello et al., 2015).

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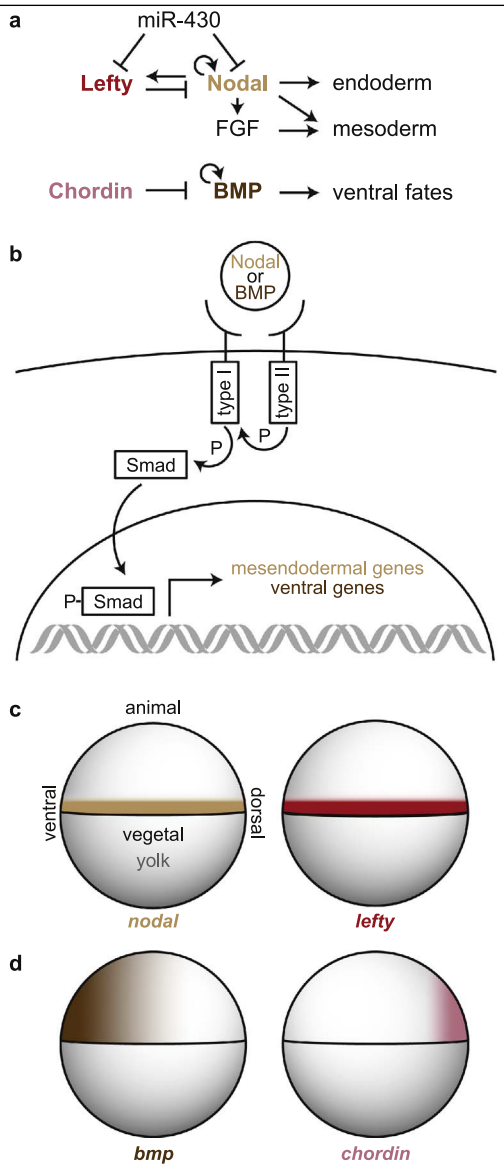
Box 1.TGF-β superfamily patterning systems in zebrafish.



The secreted TGF-β superfamily signals No dal and BMP orchestrate the patterning of germ layers and the dorsal/ventral axis, respectively (Hill, 2017; Langdon and Mullins, 2011; Massague, 2012; Plouhinec and De Robertis, 2009; Ramel and Hill, 2012; Schier, 2009; Schier and Talbot, 2005; Smith, 2009; Wu and Hill, 2009). Nodal ((a), beige) induces endoderm and mesoderm, its own expression, the secreted Nodal inhibitor Lefty (red), and the mesoderm activator Fibroblast Growth Factor (FGF) (Branford and Yost, 2002; De Robertis and Moriyama, 2016; Gritsman et al., 1999; Mathieu et al., 2004; Rodaway et al., 1999; van Boxtel et al., 2015). In zebrafish, two *nodal* genes (*squint* and *cyclops*) and two *lefty* genes (*lefty1* and *lefty2*) are expressed during germ layer development (Schier, 2009; Schier and Talbot, 2005). An additional TGF-β superfamily member, Vg1 (not shown), forms heteromers with Nodal and is required for full endogenous Nodal activity (Bisgrove et al., 2017; Montague and Schier, 2017; Pelliccia et al., 2017). Translation of *squint*, *lefty1*, and *lefty2* is inhibited by the microRNA miR-430; *cyclops* translation is unaffected by miR-430 (Choi et al., 2007; van Boxtel et al., 2015). BMP ((a), dark brown) induces ventral fates and is repressed by its extracellular inhibitor Chordin (pink). BMP signaling is required for maintenance of *bmp* expression at later developmental stages (Hammerschmidt et al., 1996; Kishimoto et al., 1997; Nguyen et al., 1998; Schmid et al., 2000), although auto-induction does not appear to be relevant for the establishment of the *bmp* mRNA gradient (Fürthauer et al., 2004; Ramel and Hill, 2013; Zinski et al., 2017). Binding of TGF-β superfamily signals to type I and type II serine/threonine kinase receptors induces phosphorylation of cytoplasmic Smad proteins, which translocate to the nucleus and associate with additional transcription factors to activate target gene expression (b) (Shi and Massague, 2003). Nodal signaling activates phosphorylation of Smad2/3, while BMP activates Smad1/5/9 phosphorylation (Itoh et al., 2000; Schier and Talbot, 2005; Shi and Massague, 2003). During zebrafish embryogenesis at blastula and early gastrula stages, *lefty* and *nodal* are first expressed on the dorsal side and then in the entire embryonic margin, patterning mesendoderm (c) (Schier and Talbot, 2005). *bmps* are expressed in a ventrally-peaking gradient along the dorsal-ventral axis, and *chordin* is expressed dorsally (d) (Tuazon and Mullins, 2015). *bmp2b*, *bmp4*, and the BMP-like ligand *admp* are also expressed dorsally (not shown, Fürthauer et al., 2004; Gritsman et al., 1999; Kishimoto et al., 1997; Lele et al., 2001; Nguyen et al., 1998; Schmid et al., 2000; Willot et al., 2002; Xue et al., 2014).

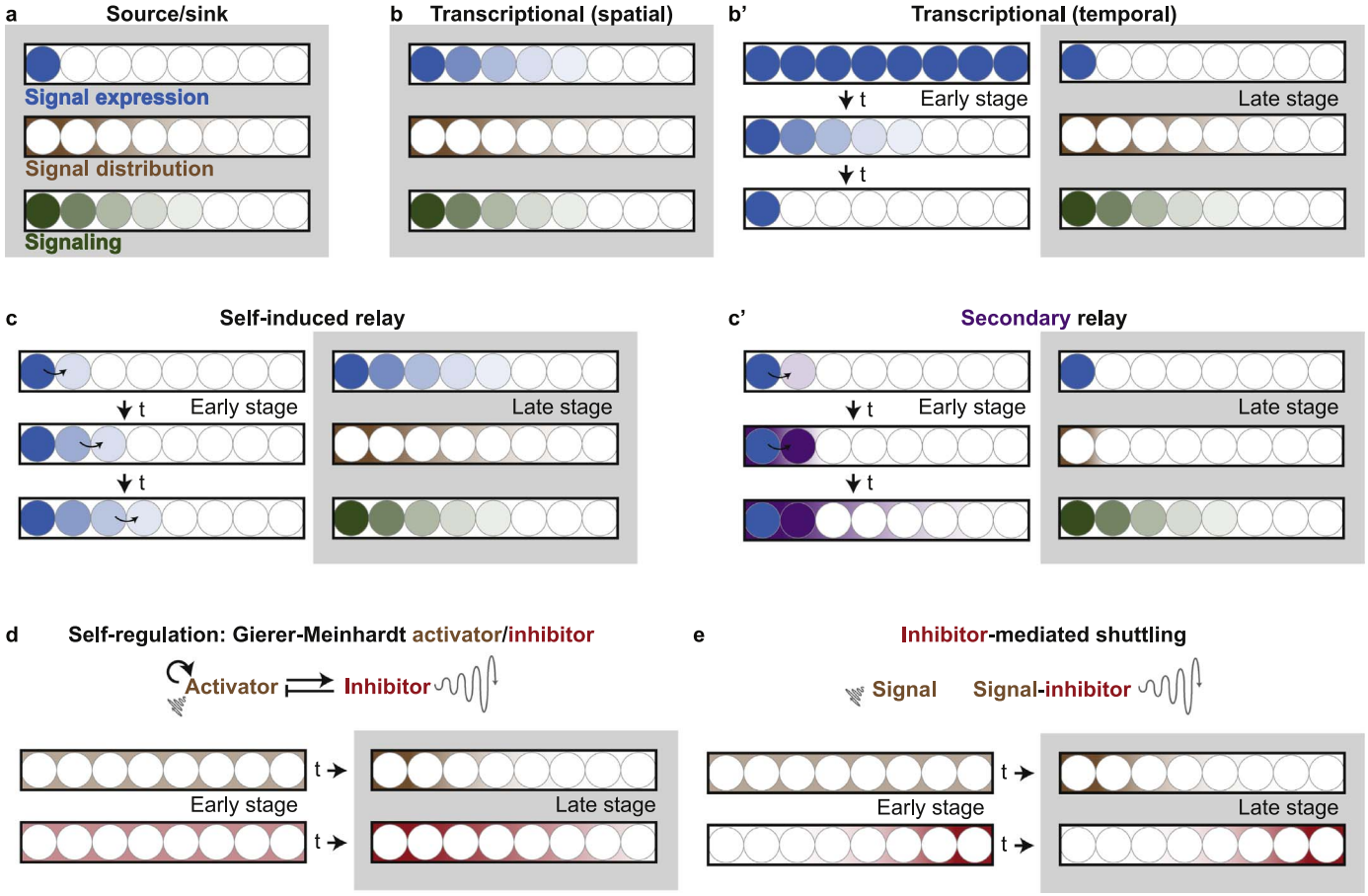


Fig. 1. Signal dispersal models. Models are illustrated in a simplified row of cells (circles). Gray boxes show distributions at later developmental stages established by earlier events as indicated. (a) Source/sink model. Signal (brown) is produced in a localized source (blue) within a tissue, diffuses through the extracellular space, and activates signaling (green). In this example, the sink is uniformly distributed (not shown). Signal distribution and signaling extend beyond the expression domain. This model is also referred to as the "Synthesis-Diffusion-Clearance" (SDC) (Rogers and Schier, 2011) or "Synthesis-Diffusion-Degradation" (SDD) (Gregor et al., 2007) model. (b-b') Transcriptional determination models. In both the spatial and temporal models, movement of signal away from its source is negligible or not biologically relevant. (b) In the spatial transcriptional determination model, a signal's distribution follows its expression domain. Signal distribution and signaling do not extend significantly beyond the expression domain. (b') In the temporal transcriptional determination model, initially uniform signal expression refines to a localized region over time. Cells "remember" and respond to previous signal exposure, or signal may persist extracellularly after cells cease expression as illustrated here, depending on signal stability and expression kinetics. At later stages, signaling extends beyond the signal expression domain, since all responding cells previously expressed the signal. (c) Positive autoregulation extends a signal's expression domain in the self-induced relay model. Signal distribution and signaling do not extend beyond the expression domain. (c') A signal may induce a longer-range secondary signal (purple) that activates signaling beyond the original signal's expression domain. (d) In Gierer and Meinhardt's self-regulating "reaction-diffusion" system, a poorly diffusive activator (brown) induces itself and a faster diffusing inhibitor (red). Gray arrows indicate relative mobility and range. This system can create activator gradients that adjust to embryo size (not shown). The specific resulting distributions of activator and inhibitor depend on their reaction rates and diffusion coefficients. (e) "Shuttling" of signal away from a localized inhibitor source leads to an accumulation of signal at a distance from the inhibitor source. Signal-inhibitor complexes increase the signal's effective mobility or stability (see text for details). Here, we depict the diffusion-based shuttling model.

Kicheva et al., 2007; Müller et al., 2012, 2013; Pomreinke et al., 2017; Yu et al., 2009; Zinski et al., 2017).

At the other extreme, the *transcriptional determination model* proposes that movement of signal away from a source is negligible or not relevant for patterning, and that signal distribution is instead defined by the signal's expression domain (Fig. 1b-b') (Alexandre et al., 2014; Dubrulle and Pourquie, 2004; Hashimoto-Partyka et al., 2003; Jones et al., 1995; Ramel and Hill, 2013; Spirov et al., 2009; van Boxtel et al., 2015). This could be achieved by a spatial mRNA gradient that generates a corresponding protein gradient (Fig. 1b). Alternatively, the expression domain of a broadly expressed signal could shrink to a localized region over time, resulting in spatial ligand or signaling gradients at later developmental stages (Fig. 1b'). Such a temporal mRNA gradient has recently been proposed to control the distribution of Wingless, a secreted signal required for wing patterning in *Drosophila*. Experiments with immobilized Wingless demonstrated that its diffusion is dispensable for wing patterning (Alexandre et al., 2014). Instead, the initially ubiquitous expression of Wingless refines to a localized source over time, suggesting that activation of signaling outside of this source at later stages reflects cellular "memory" of prior exposure.

The *relay model* (Fig. 1c) is an extension of the spatial transcriptional determination model (Fig. 1b). In this model, a signal expands its range using self-induction to sequentially activate its own expression in neighboring cells. For example, in zebrafish Nodal signaling mutants, *nodal* expression is initiated but not maintained (Meno et al., 1999), demonstrating that positive feedback helps regulate the extent of signaling. Both the relay model and the spatial transcriptional determination model predict that signal distribution and signaling domains do not extend significantly beyond a signal's expression domain. Alternatively, the effective range of a signal may be extended beyond its expression domain by induction of a second signal that has a longer range and shares target genes (Fig. 1c').

A complete model of signal distribution must account for the fact that embryogenesis is robust. For example, in 1903 Hans Spemann demonstrated that the dorsal half of a bisected salamander embryo can generate normally patterned but smaller larvae (De Robertis, 2006). *Self-regulation models* can explain how embryos adjust to this dramatic alteration in size. In 1952, Alan Turing mathematically described a system of two diffusing molecules that could generate spatial patterns by virtue of their interactions (Turing, 1952), an idea

further developed by Alfred Gierer and Hans Meinhardt in the 1970s (Gierer and Meinhardt, 1972). In the most prominent incarnation of this self-organizing system, a mobile "activator" molecule induces its own production and that of a faster diffusing "inhibitor" (Fig. 1d). Interestingly, depending on the reaction rates and diffusivities, activator gradients that adjust to tissue size can form from an initially near-homogeneous distribution (Gierer and Meinhardt, 1972; Müller and Nüsslein-Volhard, 2016). However, purely self-organizing systems arising from homogeneous initial conditions appear to be rare in development. Instead, pre-existing biases such as localized deposition of maternal determinants often strongly influence embryonic patterning systems (Meinhardt, 2008). For example, maternally deposited factors establish localized *nodal* expression at the zebrafish margin (Hong et al., 2011; Kelly et al., 2000; Schier and Talbot, 2005; Xu et al., 2012). The term "self-regulation" more accurately describes the contemporary view, in which systems of interacting molecules that can adjust their own distributions are imposed on top of external biases. Further mathematical exploration has produced additional modifications to Gierer and Meinhardt's original proposal. When interactions with non-mobile factors such as receptors are taken into account, differential diffusivity between activator and inhibitor is not required for pattern formation, although greater diffusivity differences translate into more robust systems (Marcon et al., 2016).

Finally, the *shuttling model* hinges on the idea that signal can be redistributed or "shuttled" within an embryo by interacting with a diffusing inhibitor that affects the signal's mobility or stability (Fig. 1e). BMP and its inhibitor Chordin in particular have been proposed to function as a shuttling system in a variety of developmental contexts from flies to frogs (Ben-Zvi et al., 2014, 2008; Eldar et al., 2002; Genikhovich et al., 2015; Holley et al., 1996; Lapraz et al., 2009; Matsuda and Shimmi, 2012; Mizutani et al., 2005; Peluso et al., 2011; Shilo et al., 2013; Shimmi et al., 2005; Wang and Ferguson, 2005; Zhang et al., 2007). Similar to self-regulating systems (François et al., 2009), this popular model with appropriate signaling feedback can theoretically explain aspects of developmental robustness, such as the ability of bisected frog embryos to generate complete, well-patterned tadpoles (Ben-Zvi et al., 2008).

In the following, we discuss the major findings of studies that have examined and extended models of Nodal and BMP dispersal during early zebrafish development.

3. Nodal signal dispersal

Consistent with the source/sink (Fig. 1a), relay (Fig. 1c,c'), and self-regulation (Fig. 1d) models, Nodal expressed ectopically from a localized source can activate target genes in surrounding tissues in zebrafish embryos (Box 2a) (Chen and Schier, 2001; Jing et al., 2006; Müller et al., 2012; Tian et al., 2008). Subsequently, a relay-based mechanism (Fig. 1c,c') was ruled out in this context when it was demonstrated that Nodal-secreting clones in Nodal-insensitive mutants can induce signaling in distant wild type cells (Box 2a') (Chen and Schier, 2001). Thus, in ectopic expression assays, Nodal must move away from its source to activate target expression in cells outside of the clone.

Ectopic expression assays also demonstrated that the zebrafish Nodal ligand Squint activates target genes over a greater distance than its paralog Cyclops (Chen and Schier, 2001; Jing et al., 2006). The observed differences in activity range could theoretically be caused by lower stability of Cyclops protein (Fig. 2a) (Jing et al., 2006; Le Good et al., 2005), which may be destabilized by its prodomain (Tian et al., 2008; Wang et al., 2016). Half-lives of 12 and 719 h (i.e., 0.5 and 30 days) were measured in cell culture for Cyclops and Squint protein, respectively (Wang et al., 2016). Gradient formation simulations using half-lives of 115 min for Squint and 23 min for Cyclops yielded shorter-range Cyclops gradients (Wang et al., 2016). The value used for Squint in these simulations originated from a study that followed the decay of photoconverted Squint-Dendra2 (Müller et al., 2012), and the value used for Cyclops was estimated (Wang et al., 2016). However, in order to compare half-lives, the same assay should be employed to measure the stability of both proteins in their native environment. Using a photoconversion-based assay in living zebrafish embryos, half-lives of ~2 h were measured for both Squint- and Cyclops-Dendra2, revealing that differences in stability do not explain range differences in this context (Müller et al., 2012; Rogers et al., 2015).

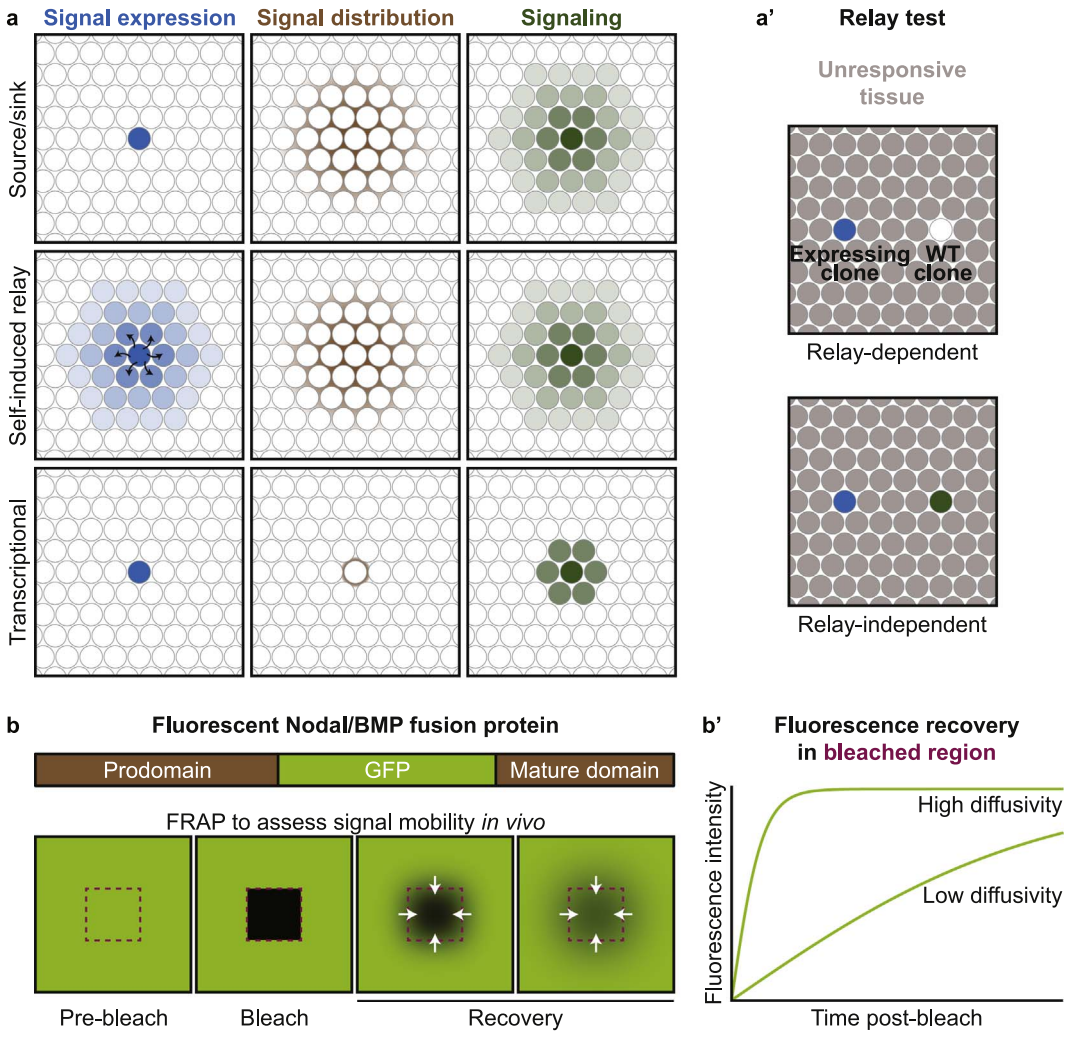
Instead, fluorescence recovery after photobleaching (FRAP) experiments (Box 2b,b') showed that Cyclops has a lower effective diffusion coefficient than Squint (1 $\mu m^2/s$ compared to 2–3 $\mu m^2/s$ (Bläßle et al., 2018; Müller et al., 2012)), suggesting that differences in diffusivity rather than stability explain the shorter activity range of Cyclops. Based on these biophysical measurements, when expressed from a localized source Cyclops and Squint should form short- and medium-range gradients, respectively, covering several cell diameters over a biologically relevant period of 1–2 h. Ectopic expression assays (Box 2a) using Cyclops- and Squint-GFP confirmed these predictions (Müller et al., 2012).

Why is Cyclops less mobile than Squint? Differences in signal diffusivity could be caused by differential interactions with extracellular membrane-bound binding partners that affect the spread of a signal through a tissue (Fig. 2) (Müller et al., 2013; Rogers and Schier, 2011). Cyclops and Squint were recently shown to bind the Nodal receptor Acvr2b with dissociation constants of 120 and 60 nM, respectively (Wang et al., 2016). This is in principle compatible with receptor-mediated hindered diffusion, although additional diffusion regulators must be postulated to explain the lower diffusivity of Cyclops given the stronger binding affinity of Squint. However, in order for receptors to function as diffusion regulators, binding must be reversible and fast relative to ligand diffusion, and receptors must be present at appropriate concentrations to influence global ligand diffusion (Crank, 1979; Miura et al., 2009; Müller et al., 2012, 2013). It is currently unclear whether Nodal-receptor on/off binding kinetics and the endogenous receptor concentration fulfill these requirements. Based on the measured Nodal-receptor affinities and assumptions about the abundance and function of receptors as diffusion regulators (Wang et al., 2016), Nodal diffusion would be implausibly slow – even slower than the movement of cells and more than an order of magnitude smaller than their experimentally measured diffusivities (Müller et al., 2012).

FRAP experiments have also demonstrated that Nodal and its secreted inhibitor Lefty satisfy Gierer and Meinhardt's original requirements for a self-regulating system (Fig. 1d): In zebrafish embryos, Nodal diffuses more slowly than its inhibitor Lefty (1–3 $\mu m^2/s$ compared to 10–20 $\mu m^2/s$), and Nodal induces both itself and Lefty (Box 1a) (Feldman et al., 2002; Meno et al., 1999; Müller et al., 2012). Interestingly, when one of these requirements – induction of the inhibitor by the activator – was experimentally removed, embryos were still viable, but more sensitive to signaling fluctuations (Rogers et al., 2017). Although the Nodal/Lefty system has features consistent with a classical Gierer-Meinhardt-type self-regulating system that confers robustness against signaling fluctuations, the relevance of this during normal embryogenesis is currently unclear.

Much of the work supporting a role for diffusion in Nodal gradient formation is based on ectopic expression of tagged ligands (Box 2). The extent to which this reflects formation of the endogenous Nodal signaling gradient is an area of active investigation, and there is evidence supporting roles for relay-based mechanisms during endogenous patterning. Consistent with a self-induced relay-based mechanism (Fig. 1c), Nodal can induce its own expression (Schier, 2009), and *nodal* expression is not maintained in Nodal signaling mutants (Meno et al., 1999). Furthermore, the endogenous *nodal* expression domain overlaps almost completely with the distributions of the Nodal signal transducer pSmad2 and a transcriptional reporter of Nodal signaling (van Boxtel et al., 2015). The relay-mediated expansion of the *nodal* expression domain and signaling gradient has been proposed to occur during a < 2 h

Box 2. Selected experimental approaches to test signal dispersal models.



Signal distribution models have been tested by experiments in which clones of cells (circles) express signal ectopically. Predictions of signal expression (blue), signal distribution (brown), and signaling (green) in the tissue surrounding the ectopic clone of cells are illustrated in (a). The source/sink and relay mechanisms support signaling over a distance of several cells. In contrast, in the transcriptional determination mechanism depicted here, the ectopic clone is capable of inducing signaling only cell-autonomously and in directly neighboring cells. To test whether signal must be relayed through neighboring cells or via a secondary signaling molecule (a'), a clone expressing signal (blue) can be generated in tissue that is unresponsive to signaling (gray). If signaling is induced in a second clone of wild type cells located at a distance from the first clone, signaling does not require relay (lower panel). This approach demonstrated relay-independent Nodal signaling in zebrafish (Chen and Schier, 2001). Fluorescent fusion proteins (b) have been used to assess signal mobility, stability, and distribution *in vivo*. Functional fusions incorporate fluorescent proteins such as GFP between the pro- and mature domains of Nodals and BMPs; GFP remains associated with the mature domain after cleavage of the prodomain. In Fluorescence Recovery After Photobleaching (FRAP) assays, fluorescently tagged signal (light green) is expressed in a tissue, a laser is used to photobleach a region of the tissue (black, outlined in magenta), and the recovery of fluorescence as unbleached molecules diffuse into the bleached region (white arrows) is monitored (b) (Fradin, 2017; Lippincott-Schwartz et al., 2003; Loren et al., 2015; Müller et al., 2013). Fluorescence recovers rapidly when fusion proteins are highly mobile and slowly when fusions have low mobility (b'). In zebrafish, FRAP has been used to measure the effective diffusion coefficients of fluorescently tagged BMP2b (2–4 $\mu m^2/s$), its inhibitor Chordin (~6 $\mu m^2/s$), the Nodal signals Squint (2–3 $\mu m^2/s$) and Cyclops (~1 $\mu m^2/s$), their inhibitors Lefty1 (~11 $\mu m^2/s$) and Lefty2 (~19 $\mu m^2/s$), and the mesoderm activator FGF8 (~2 $\mu m^2/s$) (Bläßle et al., 2018; Müller et al., 2012, 2013; Pomreinke et al., 2017; Zinski et al., 2017).

"window of opportunity" that is ultimately ended by a burst of production of the inhibitor Lefty. However, in the absence of Lefty activity, *nodal* expression is only modestly expanded (Rogers et al., 2017; van Boxtel et al., 2015), suggesting that additional repressors could act to halt *nodal* spread. The microRNA miR-430 was implicated (van Boxtel et al., 2015), since it is known to translationally repress the Nodal signal *squint* (but not its paralog *cyclops*) and both *leftys* (Box 1a) (Choi et al., 2007). When *leftys* and miR-430 were knocked down, *squint* expression expanded nearly to the opposite pole of the zebrafish embryo. miR-430 was thus hypothesized to have two roles in restricting Nodal expansion: direct inhibition of *squint* translation, and ending *nodal* expansion with a burst of Lefty production by lifting repression of *lefty* translation (van Boxtel et al., 2015).

In conflict with the pure self-induced relay model (Fig. 1c), some

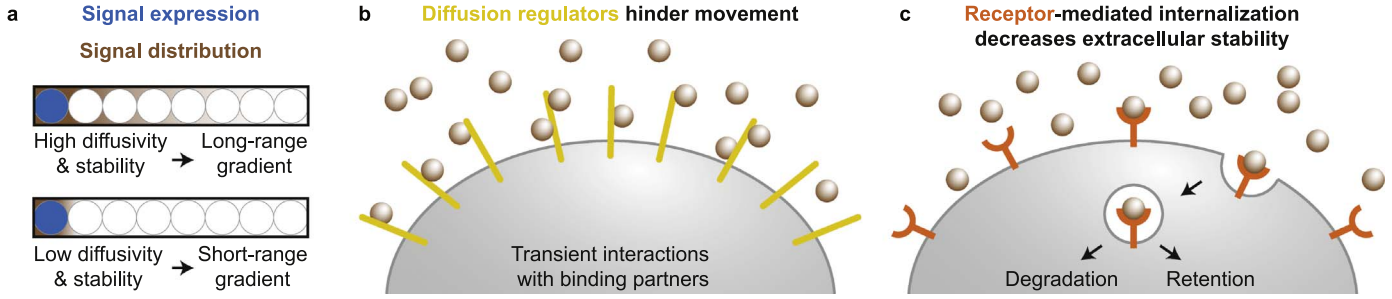


Fig. 2. Factors affecting signaling range in the source/sink model. (a) The effects of diffusivity and stability on signal distribution (brown) according to the source/sink model are illustrated in a simplified row of cells (circles). The signal is expressed from a localized source (blue). Higher stability and mobility lead to a broader signal distribution. (b-c) Interactions with extracellular binding partners can affect signal dispersal by altering signal stability or mobility. (b) Transient binding to "diffusion regulators" (yellow) may hinder signal (brown) movement through a tissue and reduce its signaling range. Higher affinity interactions with diffusion regulators have been proposed to explain the shorter range of the Nodal signal Cyclops compared to its paralog Squint (Müller et al., 2012, 2013), although the molecular identities of proposed diffusion regulators remain unknown. (c) Receptor (orange) binding can instigate internalization, irreversible trapping, and removal of signal from the diffusible signal pool, effectively destabilizing mobile signal and shortening its range. Signal may be retained inside the cell and continue signaling in specialized vesicles (Jullien and Gurdon, 2005; Vizán et al., 2013), or undergo degradation. If receptor binding is reversible, receptors could also act as diffusion regulators.

Nodal target genes are activated beyond the *nodal* expression domain (Gritsman et al., 2000). To explain this observation, it was proposed that Nodal's effective range is extended via a secondary relay mechanism (Fig. 1c'). Nodal induces the mesoderm activator FGF (Box 1a), a signal that shares target genes with Nodal and has been suggested to signal over a longer range (Bennett et al., 2007; Gritsman et al., 1999; Hill, 2017; Mathieu et al., 2004; Rodaway et al., 1999; Scholpp and Brand, 2004; van Boxtel et al., 2015, 2018). Accordingly, expression of several of these target genes expands or shrinks when FGF is overexpressed or inhibited, respectively (Rodaway et al., 1999; van Boxtel et al., 2015). Nodal-induced FGF may therefore be responsible for expression of Nodal targets outside of the *nodal* expression domain.

However, several observations also support a role for Nodal diffusion in the spreading of its endogenous signaling activity. First, despite the modest expansion of the *nodal* expression domain in the absence of Lefty (Rogers et al., 2017; van Boxtel et al., 2015), Nodal signaling and target gene expression are dramatically expanded (Agathon et al., 2001; Rogers et al., 2017). Nodal that has diffused away from its source may now be capable of activating signaling in the absence of Lefty. Second, assuming normal initiation of *nodal* expression (Feldman et al., 1998) at the margin in embryos lacking both Lefty and miR-430, the expansion of *nodal* expression nearly to the animal pole – a distance of approximately 20–30 cells (Keller et al., 2008) – would occur in less than two hours. In a one-dimensional column of 20–30 cells, each cell would have approximately 4–6 min to sense, transcribe, translate, and secrete enough Nodal to induce its neighbor. This seems implausibly fast. For example, transcripts of the Nodal targets *ntl* and *gsc* only accumulate 30 and 60 min after Nodal exposure, respectively (Dubrulle et al., 2015). Conversely, while Nodal can theoretically diffuse to the animal pole within two hours (Müller et al., 2012), it is unclear whether sufficient amounts could be delivered in this time frame to activate target genes. Rather, a combination of increased Nodal production together with Nodal diffusion might better explain this result. Third, *lefty* mutants can be rescued to adulthood by exposure to low, constant levels of a Nodal inhibitor drug starting at the 8-cell stage, indicating that a sudden burst of Nodal inhibition is not required for successful development (Rogers et al., 2017).

Together, the evidence indicates that self-induced relay, secondary relay via FGF induction, self-regulation, and diffusion-based mechanisms all contribute to endogenous Nodal-mediated patterning. Ectopic expression experiments with tagged Nodal demonstrated significant mobility, movement away from localized sources, and the ability to signal through Nodal-unresponsive tissue. However, the endogenous *nodal* expression and signaling domains closely overlap, Nodal signaling is required to establish the full extent of the *nodal* expression domain, and FGF signaling expands Nodal target gene activation. Further work is needed to understand how these and other mechanisms such as feedback inhibition regulate the endogenous distribution of Nodal signaling and lead to robust germ layer patterning.

4. BMP signal dispersal

Different mechanisms have been proposed to mediate BMP gradient formation during zebrafish dorsal/ventral patterning, including spatial transcriptional determination (Fig. 1b), diffusion (Fig. 1a), and shuttling (Fig. 1e). Below, we describe recent studies examining these models.

Similar to Nodal, comparisons of *bmp2b* expression and the BMP signaling domain using either a BMP-sensitive transcriptional reporter or the BMP signal transducer pSmad1/5/9 (Box 1b) as readouts revealed a close relationship in early zebrafish embryos (Ramel and Hill, 2013). *bmp2b* expression and the BMP signaling domain both start as low, nearly uniform distributions, and over time refine to a ventral-to-dorsal gradient (Pomreinke et al., 2017; Ramel and Hill, 2013; Zinski et al., 2017). The endogenous distribution of the BMP2b prodomain also overlaps well with the *bmp2b* transcriptional domain (Ramel and Hill, 2013), although the location of the active mature signal is still unknown. Dorsal inhibition of *bmp* transcription is mediated by repressors such as Dharma and FGF (Fürthauer et al., 2004; Koos and Ho, 1999; Leung et al., 2003; Ramel and Hill, 2013), and secreted BMP inhibitors such as Chordin (Box 1) further restrict the BMP signaling range (Schier and Talbot, 2005; Schulte-Merker et al., 1997).

Despite the broad BMP expression domain, BMP diffusion likely contributes to gradient formation in zebrafish. FRAP experiments (Box 2b,b') measured diffusivities of ~2–4 $\mu m^2/s$ for fluorescent BMPs (Pomreinke et al., 2017; Zinski et al., 2017), comparable to the Nodal ligand Squint (Müller et al., 2012). Consistent with its measured mobility and stability, tagged BMP forms protein gradients spanning several cell diameters when expressed from a localized source (Pomreinke et al., 2017). Computational modeling demonstrated that hypothetical dorsal/ventral patterning systems in zebrafish are more robust when BMP can diffuse (Zinski et al., 2017). A combinatorial "graded source/sink" mechanism, in which BMP diffusion is layered on top of a transcriptional gradient with Chordin acting as a dorsal sink, has been proposed to underlie the spreading of BMP ligand in zebrafish (Pomreinke et al., 2017; Zinski et al., 2017). Interestingly, in the absence of Chordin, BMP signaling on the extreme dorsal side does not increase as much as expected if Chordin were the only dorsally-acting BMP inhibitor (Pomreinke et al., 2017). Thus, additional extracellular regulators such as Follistatin and Noggin may be involved in dampen-

ing BMP signaling dorsally (Dal-Pra et al., 2006; Pomreinke et al., 2017; Zinski et al., 2017).

Although the graded source/sink model is a useful basic description of this dorsal/ventral patterning system, Chordin has been suggested to have additional roles beyond simply inhibiting BMP. The range of a signal can be affected by modification of its diffusivity or stability (Fig. 2a), and Chordin has been proposed to modulate the distribution of BMP using diffusion- or stability-based shuttling (Ben-Zvi et al., 2014, 2008; Eldar et al., 2002; Genikhovich et al., 2015; Holley et al., 1996; Lapraz et al., 2009; Matsuda and Shimmi, 2012; Mizutani et al., 2005; Peluso et al., 2011; Shilo et al., 2013; Shimmi et al., 2005; Wang and Ferguson, 2005; Zhang et al., 2007). Binding of Chordin to BMP inhibits signaling, which can be subsequently relieved if Chordin is cleaved by a uniformly distributed extracellular protease (Blader et al., 1997). In the diffusion-based shuttling model, Chordin-bound BMP is much more diffusive than unbound BMP (Fig. 1e). For a given BMP molecule, the closer it is to the Chordin source, the more likely it is to be bound by Chordin and thus mobile, and the farther away it is from the Chordin source, the more likely it is to be unbound and immobile. Over time this results in the accumulation of BMP at a distance from the Chordin source. In the stability-based shuttling model, Chordin, Chordin-bound BMP, and free BMP are equally mobile, but BMP transiently bound to Chordin escapes receptor-mediated degradation and is therefore more stable than unbound BMP (Fig. 2c) (Genikhovich et al., 2015; Mizutani et al., 2005). In this way, localized Chordin expression could create spatial differences in BMP stability that alter its distribution within a tissue.

Both the diffusion- and stability-based shuttling models are consistent with observations in early *Drosophila* embryos: They can explain how the very sharp BMP signaling peak is generated, and are consistent with the observed distribution of BMP signaling and BMP-GFP in the absence of Chordin (Ross et al., 2001; Rushlow et al., 2001; Wang and Ferguson, 2005). However, diffusion-based shuttling does not appear to have a role during early zebrafish dorsal/ventral patterning. If shuttling contributed to the formation of the BMP signaling peak, the peak would be expected to decrease in *chordin* mutants. This is the case in *Drosophila* (Ross et al., 2001; Rushlow et al., 2001), but not in zebrafish *chordin* mutants or frog *chordin* morphants – signaling is expanded, but the ventral peak is not significantly decreased (Plouhinec et al., 2013; Pomreinke et al., 2017; Zinski et al., 2017). FRAP experiments (Box 2b,b') in zebrafish demonstrated that the diffusivity of Chordin is not much higher than the diffusivity of BMP (~6 $\mu m^2/s$ compared to 2–4 $\mu m^2/s$) (Pomreinke et al., 2017; Zinski et al., 2017), and BMP mobility did not change in *chordin* morphants (Zinski et al., 2017). Importantly, Chordin overexpression did not increase BMP diffusivity, although the diffusion-based shuttling model predicts that BMP-Chordin heteromers should be much more mobile than BMP alone (Pomreinke et al., 2017). Finally, Chordin ectopically expressed from a localized clone did not significantly alter the distribution of BMP-GFP expressed from a juxtaposed clone (Pomreinke et al., 2017).

Together, these results indicate that Chordin-mediated diffusion-based BMP shuttling does not occur during early zebrafish development. In frog embryos, shuttling models can theoretically explain size-dependent tissue scaling (Ben-Zvi et al., 2014, 2008), but – in conflict with the prediction of the shuttling model – the endogenous ventral BMP signaling peak is not affected by Chordin removal (Plouhinec et al., 2013). Further *in vivo* analysis will therefore be necessary to assess the role of shuttling in the scaling of frog embryos (François et al., 2009). In contrast, there is clear evidence that the sharp BMP signaling gradient in fly embryos is generated by shuttling (Ross et al., 2001; Rushlow et al., 2001; Wang and Ferguson, 2005). However, the zebrafish BMP signaling peak is not as pronounced as that observed in fly embryos (Zinski et al., 2017), and it is not clear how robust zebrafish are to changes in size along the dorsal/ventral axis.

The most parsimonious current model of BMP signal dispersal during zebrafish dorsal/ventral patterning involves interactions between mobile BMP and Chordin in a graded source/sink mechanism. However, this model does not take into account other important extracellular feedback regulators such as the BMP-induced protein Sizzled, which limits the range of BMP signaling by effectively stabilizing Chordin protein (Plouhinec and De Robertis, 2009; Umulis et al., 2009). Further examination of dorsal/ventral patterning in zebrafish will be required to determine how additional secreted BMP signaling regulators contribute to the graded source/sink mechanism to ensure reproducible embryogenesis.

5. Addressing remaining questions

Decades of research have led to five major models of Nodal and BMP dispersal during embryogenesis: source/sink, transcriptional determination, relay, self-regulation, and shuttling (Fig. 1). Different mechanisms may be employed in different developmental contexts – for example, BMP in the larval fly wing disc most likely moves away from its source via a source/sink mechanism (Harmansa et al., 2015), whereas in frogs, BMP4 does not appear to signal over long distances (Hashimoto-Partyka et al., 2003; Jones et al., 1996). Multiple mechanisms may act together simultaneously: Transcriptional gradients, relay, and diffusion-based mechanisms are all likely to contribute to Nodal gradient formation during germ layer patterning. However, several open questions about Nodal and BMP distribution during vertebrate embryogenesis remain.

Although the source/sink model (Fig. 1a) is supported by the observation that Nodal and BMP can diffuse from a source and form gradients in zebrafish embryos, these results are based on ectopic expression, often of tagged signals (Chen and Schier, 2001; Jing et al., 2006; Le Good et al., 2005; Müller et al., 2012; Pomreinke et al., 2017; Tian et al., 2008; Wang et al., 2016). Better reagents to examine the endogenous distributions of these proteins – such as antibodies targeting their mature domains and transgenics with fluorescent fusions under the control of endogenous regulatory elements (Durdu et al., 2014) – will be useful. The functional requirement for diffusion in vertebrates should be directly tested by immobilizing signals and their antagonists *in vivo*. This can be achieved by tethering signals to signal-producing cells (Fig. 3), an approach that has been successful in distinguishing between source/sink and transcriptional determination models in invertebrates (Alexandre et al., 2014; Brankatschk and Dickson, 2006; Harmansa et al., 2015; Strigini and Cohen, 1997; Zecca et al., 1996). For example, membrane-tethering experiments demonstrated that Wingless diffusion is dispensable for *Drosophila* wing disc patterning, and suggested a temporal transcriptional mechanism (Fig. 1b') (Alexandre et al., 2014). In contrast, lineage-tracing studies in the wing disc showed that cells that have never expressed BMP still activate BMP signaling, and immobilized BMP was unable to support patterning in the wing disc, ruling out the temporal transcriptional model and supporting a source/sink-based mechanism in this context (Evans et al., 2009; Harmansa et al., 2015; Weigmann and Cohen, 1999).

The mechanisms that regulate Nodal and BMP mobility and stability should also be explored further in vertebrates (Fig. 2). Functional studies assessing Nodal and BMP mobility, stability, and distribution (Box 2) when levels of potential binding partners (such as receptors) are manipulated will be important. Further, explaining range differences will require the identification and characterization of additional modulators of ligand mobility as well as stability and signaling, such as Vg1 (Ambrosio et al., 2008; Bisgrove et al., 2017; Deglincerti et al., 2015; Duchesne et al., 2012; Marjoram and Wright, 2011; Montague and Schier, 2017; Morris et al., 2007; Müller et al.,

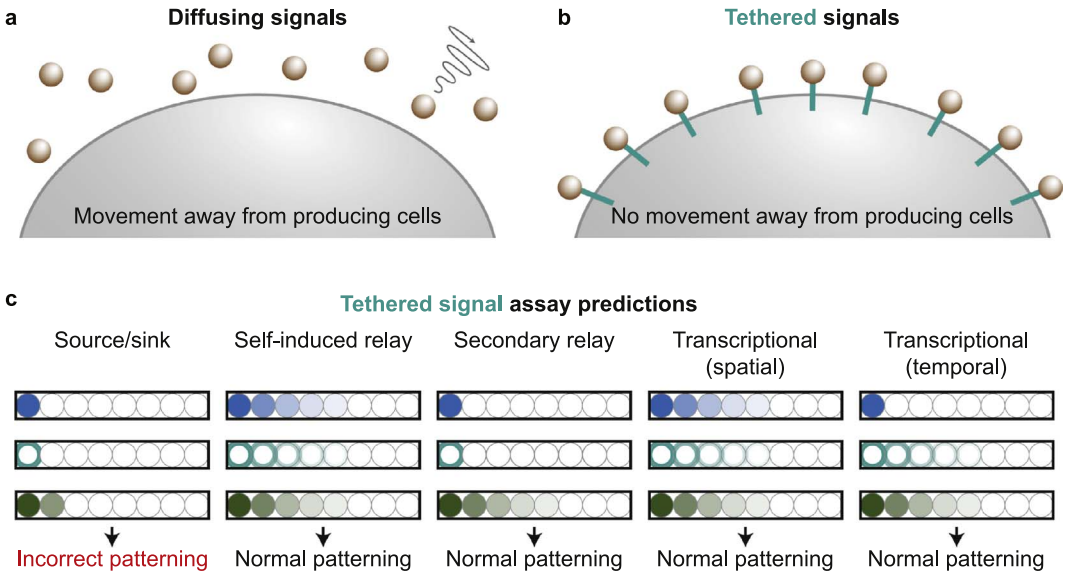


Fig. 3. Membrane tethering to test the requirement for diffusion. Signal tethering assays similar to those used in *Drosophila* (Alexandre et al., 2014; Harmansa et al., 2015) could be used to test the source/sink model in vertebrates. (a) Secreted signals (brown) that may normally diffuse away from producing cells (gray) can be experimentally tethered (b) to the membrane of producing cells using e.g. transmembrane domains (turquoise) that restrict signal mobility. (c) When signal is membrane-tethered (turquoise), the source/sink model predicts that signal will not be found beyond its expression domain (blue), signaling (green) will not be activated in distant cells, and patterning will fail. The other models predict a minimal impact on patterning since long-range movement of signal is not required. Note that the exact distribution of ligand in these scenarios will depend on signal stability and expression kinetics.

2013; Oki et al., 2007; Pelliccia et al., 2017; Tanaka et al., 2007; Wang et al., 2016; Yu et al., 2009). The importance of signaling range during patterning should also be more carefully examined: Although intuitively the range of a signal should be tightly regulated to ensure robust patterning, this idea needs to be corroborated *in vivo*. For example, *squint*$^{-/-}$ mutants are viable but temperature-sensitive (Pei et al., 2007), suggesting that the short-range, less mobile Nodal signal Cyclops can be sufficient for successful patterning unless development is challenged.

The importance of self-induced relay at the level of transcription could be examined using new genetic engineering approaches (Auer et al., 2014; Gaudelli et al., 2017; Komor et al., 2016; Zhang et al., 2017) to disrupt Nodal-responsive elements in endogenous *nodal* promoters. Additionally, the expression of *nodal* in embryos lacking both *lefty* and miR-430 should be examined at early developmental stages. Perhaps *nodal* is expressed earlier than usual in this context or development is delayed, and the timing of induction via a self-induced relay mechanism is more plausible.

Questions also remain about the role of Nodal-induced FGF in extending the range of Nodal signaling in zebrafish (Box 1a, Fig. 1c'). FGF signaling should be assessed in *lefty* mutants to determine whether increased FGF signaling could contribute to expanded Nodal target gene expression (Rogers et al., 2017). It is also unclear why FGF apparently has a longer signaling range than Nodal (Chen and Schier, 2001; Scholpp and Brand, 2004), given their similar diffusivities and expression domains (Bennett et al., 2007; Gritsman et al., 1999; Müller et al., 2012, 2013; Thisse et al., 2001; van Boxtel et al., 2015, 2018). Careful experiments directly comparing FGF and Nodal sources, protein distributions, production levels, and relative activities will be needed to explain endogenous range differences.

How Nodal and BMP signaling work together to pattern tissues is an important area of active investigation with many unresolved questions. Nodal induces the BMP inhibitor Chordin as well as the BMP ligands BMP4 and ADMP dorsally (Bennett et al., 2007; Gritsman et al., 1999). How does Nodal/BMP signaling overlap contribute to the coordination of patterning, and why is BMP activity also required on the dorsal side (Xue et al., 2014)? Furthermore, Nodal and BMP might form heteromers that mutually interfere (Yang et al., 2010), possibly affecting signaling interpretation. Future work should develop these observations into functional descriptions of the cooperative mechanisms governing embryogenesis.

Ultimately, successful models of embryogenesis also need to account for its observed robustness: Bisected embryos can develop normally (De Robertis, 2006); zebrafish *squint*$^{-/-}$, fly *bicoid*$^{+/-}$, and fly *chordin*$^{+/-}$ mutants experience altered signaling or gene expression during embryogenesis but are viable (Biehs et al., 1996; Dougan et al., 2003; Driever and Nüsslein-Volhard, 1988a; Mizutani et al., 2005); zebrafish *chordin*$^{-/-}$ mutants can be rescued by uniform expression of *chordin* (Fisher and Halpern, 1999; Schulte-Merker et al., 1997); secondary embryonic axes can be induced by transplantation of different embryonic regions or ectopic BMP/Nodal expression (De Robertis, 2006; Fauny et al., 2009; Xu et al., 2014); and dissociated amphibian embryos and aggregates of stem cells can give rise to structured tissues and organoids, respectively (Nieuwkoop, 1992; Turner et al., 2017). These phenomena hint at control mechanisms involving self-regulation that allow patterning to adjust to changes in embryo size, signaling levels, gene expression, and other intrinsic or extrinsic noise. Existing and future models involving additional players and self-regulatory mechanisms will be intriguing to explore further with quantitative methods.

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