HHS Public Access

Author manuscript

Semin Cell Dev Biol. Author manuscript; available in PMC 2016 June 27.

Published in final edited form as:

Semin Cell Dev Biol. 2015 June ; 42: 118–133. doi:10.1016/j.semcdb.2015.06.003.

Temporally coordinated signals progressively pattern the anteroposterior and dorsoventral body axes

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Abstract

The vertebrate body plan is established through the precise spatiotemporal coordination morphogen signaling pathways that pattern the anteroposterior (AP) and dorsoventral (DV) axes. Patterning along the AP axis is directed by posteriorizing signals Wnt, fibroblast growth factor (FGF), Nodal, and retinoic acid (RA), while patterning along the DV axis is directed by bone morphogenetic proteins (BMP) ventralizing signals. This review addresses the current understanding of how Wnt, FGF, RA and BMP pattern distinct AP and DV cell fates during early development and how their signaling mechanisms are coordinated to concomitantly pattern AP and DV tissues.

Keywords

Morphogens; Gastrulation; BMP; Wnt; FGF; Nodal

1. Introduction

The anteroposterior (AP) and dorsoventral (DV) axes are the foundation for the bilateral body plan. In vertebrates both the AP and DV axes are generated by distinct signaling proteins that act as morphogens. Morphogens specify discrete cell fates over a distance in a concentration-dependent manner and generate activity gradients of high, intermediate, and low to specify distinct cell fates [1]. During axial patterning, these gradients span the entire length of an embryo and the amount of each signal at precise AP and DV locations patterns the entire organism. Accordingly, spatial regulation of morphogen signaling is critical to ensure the correct morphogen levels in particular positions. Moreover, this precise distribution of morphogen signaling across each axis cannot be established instantaneously and entire body axes cannot be patterned all at once. Correct morphogen signaling levels must be maintained throughout the patterning process and cells must also be equipped to know exactly when to respond to morphogen signals to adopt their correct fate [2]. Therefore, temporal regulation of morphogen signaling and target cell competence is also

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essential to pattern a complete body axis. Furthermore, patterning of all AP and DV tissues spans late blastula, gastrula, and somitogenesis stages, which are distinct and dynamic physical environments. Thus, spatial and temporal regulation of morphogen signaling must also be coordinated to navigate the challenges of early embryo development. Critically, although separate mechanisms exist for patterning the AP and DV axes, both axes are patterned concomitantly; these orthogonal patterning mechanisms must work in harmony across both space and time to properly pattern the organism.

This review will address the current understanding of *Xenopus* and zebrafish AP and DV axial patterning as separate processes during gastrulation, as well as more recent advances in uncovering the mechanisms that coordinate AP and DV patterning. Finally, this review will discuss current and novel techniques for manipulating spatial and temporal aspects of patterning, including the associated caveats and prospects for future development and application of these techniques.

2. Combinatorial Wnt, FGF, Nodal, and RA morphogenetic signaling patterns the AP axis

Although initial AP polarity in amphibians and fish is determined by the maternally established animal-vegetal axis of the egg, patterning of distinct AP cell fates in all vertebrates is controlled during late blastula and gastrula stages. By the end of gastrulation in *Xenopus*, zebrafish, chick, and mouse, a clear division of anterior and posterior cell fates has been established [3–11]. AP patterning is mediated by Wnt, fibroblast growth factor (FGF), Nodal, and retinoic acid (RA) signaling. Specifically, Wnt, FGF, Nodal, and RA specify posterior cell fates and the specification of anterior cell fates relies on the graded inhibition of these signals (Fig. 1a – c). During blastula and gastrula stages Wnt, FGF, and Nodal establish the broad regions of the AP body axis (the head, trunk, and tail as most posterior) (Fig. 1a – b). Additionally, Nodal patterns the mesendoderm while Wnt, FGF, and RA specify distinct AP cell fates in the neural plate, dividing it into four distinct regions to establish the central nervous system (CNS). These four rostral (anterior) to caudal (posterior) subdivisions are the forebrain, midbrain, hindbrain, which is further subdivided into rhombomeres (numbered 1 – 7 from rostral to caudal in the zebrafish), and spinal cord (Fig. 1a) [12]. Although the complete specification of CNS fates extends beyond gastrulation, these subdivisions of the CNS can be used as a reliable readout of AP axial patterning. The roles of Wnt, FGF, Nodal, and RA signaling in patterning the AP body axis and/or the CNS are described below.

2.1. A Wnt gradient specifies posterior cell fates

Wnts are secreted cysteine-rich glycoproteins that bind to the Frizzled (Fz) family of receptors with the assistance of co-receptors such as low-density lipoprotein receptor-related proteins (LRPs) and heparin-sulphate proteoglycans (HSPGs) [13]. During AP patterning, Wnt signaling activates the canonical Wnt/β-catenin pathway and promotes the expression of posterior genes [14,15]. During early and mid-blastula stages in the frog and zebrafish embryo, maternal Wnt signaling is localized dorsally and establishes the dorsal organizer, which establishes DV asymmetry (Section 3) [14]. However, during late blastula and

gastrula stages zygotic Wnt signaling is excluded from the dorsal organizer, and localized to the ventrolateral embryo margin; this change in localization during gastrulation coincides with a dramatic change in Wnt function. Zygotic Wnt functions in posterior tissue development (Fig. 1b): increasing zygotic Wnt signaling results in the loss of head structures [16–20], whereas embryos deficient in Wnt signaling exhibit a dramatic loss of the tail and a reciprocally enlarged the head [21–25]. AP patterning by Wnt also depends on Wnt antagonists, including secreted Frizzled-related proteins (sFRPs) and Dickkopf (Dkk), which are localized anteriorly (Fig. 1b) [15,17,26]. sFRPs are secreted proteins that contain domains homologous to the Wnt binding site of Fz receptors and thus bind Wnt and prevent Fz activation, while Dkk proteins are membrane-bound and bind LRP co-receptors to prevent the propagation of Wnt signaling [13].

Wnt signaling is also key to AP neural patterning, specifying caudal CNS cell fates [19,20,23,27–33]. Studies of AP patterning in the CNS beautifully show that Wnt acts as a morphogen to specify caudal cell fates in a concentration-dependent manner [23,27,31–34]. Remarkably, grafting Wnt-expressing cells or beads near forebrain progenitors [33,35] or incubating *Xenopus* animal cap explants (the most anterior tissue) with Wnt [31] induces caudal cell fates that vary depending on the amount of Wnt expressed. This supports a prominent role for Wnt signaling in establishing the broad subdomains of the AP axis since Wnt can directly convey posterior positional information to specify the proportion and distribution of caudal cell fates in multiple regions of the developing CNS. Importantly, the most rostral cell fates, like the forebrain, require Wnt signal inhibition, which underscores the equal importance of Wnt antagonism in AP patterning (Fig. 1b) [30,36].

Although as a morphogen Wnt must function over a distance, Wnt is post-translationally modified with lipids that make it hydrophobic, insoluble, and poorly mobile, thus limiting its ability to form a signaling gradient by free diffusion [37]. Recent studies of fluorescently tagged Wnt in live zebrafish embryos offer an alternative mechanism for generating a gradient: short, actin-based filopodia can transport Wnt to the contact point between neighboring cells and activate Wnt signaling, increasing its effective signaling range [38].

2.2. An FGF gradient specifies posterior cell fates

FGFs are secreted growth factors that bind and activate FGF receptors (FGFRs). FGFRs are receptor tyrosine kinases (RTKs) and FGF binding results in receptor dimerization and intracellular *trans* phosphorylation [39]. Activated FGFRs recruit and activate a wide range of effectors, including Grb2 and Ras, which ultimately activate MAPK (mitogen activate protein kinase). Activated MAPK phosphorylates various transcription factors to regulate gene expression [39]. In zebrafish, FGF signaling is first induced during early blastula stages by maternal Wnt signaling [14]. This initial FGF expression localizes to the dorsal margin and contributes to inducing the dorsal organizer in DV axis formation (Section 3). However, similar to Wnt, FGF expression expands throughout the margin during gastrulation. This change in FGF expression coincides with a distinct role for FGF to promote posterior tissues development (Fig. 1b) [40]. Loss of FGF activity results in the loss of trunk and tail [41–43], while gain of FGF activity causes the loss of head tissues [44,45]. FGF signaling is inhibited by Sprouty proteins, which interfere with the activation of the MAPK signaling cascade

(Fig. 1b) [46]. Interestingly, since Sprouty can be localized in the cytosol or at the membrane, the mechanism of Sprouty inhibition of MAPK is context dependent and remains to be characterized during AP patterning [46,47].

During CNS development, FGF maintains the midbrain-hindbrain boundary [48,49] and induces caudal cell fates like the hindbrain and spinal cord [50–55]. Unlike Wnt signaling, FGF is not sufficient to ectopically induce caudal cell fates in the forebrain [35] or in animal explants [30]. Although this supports a more prominent role for Wnt signaling in specifying the broad subdivisions of the CNS [12], it is notable that FGF is required to generate a permissive environment for the caudalizing activity of Wnt [30]. The complex relationship between FGF and Wnt during neural patterning remains to be fully characterized, but a recent study suggests that Wnt may regulate Sprouty expression, providing a mechanism to coordinate Wnt and FGF signaling [55].

Evidence indicates that FGF functions as a morphogen, differentially activating posterior genes in a concentration-dependent manner [52,55,56]. Studies of tagged FGF and single molecule fluorescence correlation spectroscopy (FCS) suggest that the FGF gradient is generated by free diffusion of the ligand and receptor-mediated endocytosis [56,57].

2.3. A Nodal gradient specifies mesendoderm and posterior cell fates

Nodal proteins are secreted ligands belonging to the TGFβ superfamily. Nodal signaling is mediated by EGF-CFC (epidermal growth factor-Cripto-1/FRL-1/Cryptic) co-receptors and type I and type II Activin receptors, which are serine/threonine kinases [58,59]. Receptor activation results in the phosphorylation and nuclear accumulation of the Smad2 and Smad3 transcription factors, which then direct the transcriptional activity of target genes [60–62]. The Nodal ligands identified in vertebrates are named as follows: Nodal in mouse; Nodal-related 1 (Ndr1, previously known as Squint), Ndr2 (previously known as Cyclops), and Ndr3 (previously known as Southpaw) in zebrafish; and *Xenopus* Nodal-related (Xnr) 1, 2,4,5, and 6 [62]. In early zebrafish and *Xenopus* embryos, Nodals are expressed around the margin and enriched dorsally and have region- and stage-specific functions [22,25,63–67]. Dorsally, Nodal is induced by maternal Wnt signaling during blastula stages [68] and functions in formation of the dorsal organizer (Section 3) [58,63,69–71]. At the margin during gastrulation, Nodal is essential to induce and pattern the mesendoderm [58,63,65–67,70,72–75]. In the mesendoderm, Nodal acts as a morphogen to differentially specify cell fates in a concentration-dependent manner: high levels of Nodal activity specify endoderm while lower levels specify mesoderm [71,73,75–77].

Nodal also directs AP axial patterning in the zebrafish by specifying posterior cell fates, such as the trunk and tail (Fig. 1b) [60,78,79]. Mis-expressing Nodal and BMP in the most anterior domain of the embryo, the animal pole, ectopically induces trunk and tail tissues [80,81]. The posterior fate of the induced tissue depends on the amount of BMP expressed, suggesting that the relative ratio of BMP to Nodal signaling in the margin directs trunk and tail patterning: an equal BMP/Nodal ratio specifies trunk, while a higher ratio specifies tail [81]. Since specific levels of BMP relative to Nodal are required, it remains unclear whether Nodal is functioning as a morphogen in this context. Furthermore, the ability of Nodal to induce trunk and tail tissues may be indirect: Nodal has been shown to induce Wnt and FGF

expression, which promote posterior cell fates (Fig. 1b) [34,82]. Although these studies suggest a role for Nodal in AP patterning, Nodal is also patterning the mesendoderm during the same time period. These two roles of Nodal need further investigation: do they cooperate or inform each other? are they independent and, if so, what mechanisms enable that independence? is Nodal acting as a morphogen in one context but not the other?

In the zebrafish gastrula, the endogenous Nodal signaling gradient has been visualized by fluorescence of Smad2 and Smad3 (intracellular readouts of Nodal activity) and is highest at the margin and decreases anteriorly (animally) [83]. Importantly, the Nodal signaling gradient is shaped by the Nodal antagonist, Lefty, which binds to Nodal and EGF-CFC coreceptors to attenuate Nodal signaling [77,78,84–87]. Furthermore, Nodal and Lefty, as an activator/inhibitor pair, exhibit characteristics described in reaction-diffusion models of pattern formation; mainly that Nodal acts at a short-to midrange distance and induces its own expression and the expression of its inhibitor, Lefty, which acts at a long-range distance [75,88,89]. Recent studies of zebrafish Nodal and Lefty indicate that they have different *in vivo* diffusion rates (Lefty has a longer range than Nodal), providing strong support to the reaction-diffusion model of pattern formation [86,90].

2.4. An RA gradient specifies posterior CNS

RA, synthesized through the oxidation of retinol (vitamin A), acts as the ligand for nuclear RA receptors (RAR) [91]. Activated RARs dimerize with retinoid X receptors (RXR), then bind specific DNA motifs to regulate gene expression [91,92]. Although embryos lacking RA signaling still develop posterior tissues and, therefore, RA does not play a role in the broad specification of the body plan, RA signaling is essential during gastrulation to correctly pattern the hindbrain [93–96]. Specifically, RA induces genes that specify the identity of more caudal hindbrain segments (rhombomeres 4 – 7 in the zebrafish) [97–99] and thus does not play a role in delineating the broad AP subdivisions of the CNS (Fig. 1a) [12,97,100].

In the context of the hindbrain, RA acts as a morphogen to directly specify distinct posterior cell fates in a concentration-dependent manner (Fig. 1b) [99]. Importantly, discrete levels of RA signaling depend on the active degradation of RA anteriorly by Cyp26 proteins, which degrade RA into its polar metabolites (Fig. 1b) [99,101,102]. *cyp26* can be both induced by RA and suppressed by Wnt and FGF signaling, suggesting that Cyp26 integrates the three posterior neural signals to pattern the hindbrain [99,100]. Additionally, cellular retinoic acid-binding proteins (Crabps), which transport RA to Cy26 enzymes for degradation, maintain the robustness of the RA gradient [103]. Recently, a gradient of free, unbound RA has been directly observed in live zebrafish embryos by measuring fluorescence resonance energy transfer (FRET) of novel genetically encoded probes for RA (GEPRAs) [104]. The observed RA gradient is highest in the trunk and then declines in a graded fashion both anteriorly and posteriorly, generating a two-tailed gradient [104].

3. A BMP signaling gradient patterns the DV axis

In *Xenopus* and zebrafish, the initial DV axis is established by maternal Wnt/β-catenin signaling localized to prospective dorsal cells, which activate dorsal gene expression

[14,105–108]. This initial DV polarity depends on the vegetal localization of dorsal determinants in the egg and their asymmetric transport via microtubules to the future dorsal side during cleavage stages. In zebrafish, dorsal activation of maternal Wnt/β-catenin signaling activates the expression of dorsal genes such as *bozozok (boz*) and *fgf* that establish and maintain the dorsal organizer after the mid-blastula transition (MBT) [14]. Nodal also contributes to dorsal organizer formation by inducing the expression of other dorsal genes, such as *goosecoid* (*gsc*) [63,71]. The organizer protects dorsal cell fates by inducing *chordin* (*chd*), *noggin* (*nog*), and *follistatin* (*flst*) dorsally (Section 3.2) and by repressing the activity of *vox/vent/ved*, which are transcriptional repressors of dorsal genes (Section 3.3) [109–116]. Given the correct maternal establishment of the dorsal organizer, the patterning of cell fates along the DV axis is achieved by a gradient of BMP signaling, as described below.

3.1. A BMP gradient specifies ventrolateral cell fates

BMPs are secreted growth factors belonging to the TGFβ superfamily that act as a morphogen to pattern DV tissues along the embryonic axis. While there are numerous distinct BMP ligands, the prominent BMPs during DV patterning are BMP2/4 and BMP7 [117]. BMPs are secreted as either covalently linked homodimers or heterodimers and bind a serine/threonine kinase receptor complex composed of two type I (Bmpr1 and/or Acvr1l) and two type II (Bmpr*2* and/or Acvr*2*) receptors [118–122]. In zebrafish, a BMP2/7 heterodimer functions as the obligate ligand, signaling through Bmpr1 and Acvr1l and still unknown type II receptors [118]. The activated type I receptors phosphorylate the C-terminus of the Smad1/5 transcription factor (pSmad1/5)[1], resulting in its nuclear accumulation [118,123,124]. In the zebrafish embryo, while initial *bmp* expression is widespread and present dorsally, it becomes restricted the ventral half of the embryo during DV patterning [130,131]. BMP signaling is essential to specify ventral cell fates: loss of BMP signaling results in complete loss of ventral tissues like epidermis, pronephros, blood and tail with the concurrent expansion of dorsal tissues like neural and anterior somites (Fig. 1a). There is also a dorsally produced BMP, the antidorsalizing morphogenetic protein (ADMP), which synergizes with ventrally produced BMPs to restrict neural tissue to the dorsal side of the embryo [132,133].

BMP acts as a morphogen to directly specify distinct cell fates at discrete DV positions through precise levels of BMP signaling. In the developing zebrafish embryo, high BMP signaling activity specifies ventral cell fates (e.g. epidermis, blood, tail), intermediate BMP signaling levels specify lateral fates (e.g. neural crest), and no BMP signaling results in dorsal fates (e.g. neural, somites) (Fig. 1a and c) [134–139]. The BMP signaling gradient has been visualized by immunofluorescent staining for nuclear pSmad1/5, a direct intracellular readout of BMP signaling. Use of this approach in *Xenopus* [140–143] and zebrafish [129,139,144,145] reveals a gradient of pSmad1/5 at mid-blastula stages that intensifies during gastrulation, exhibiting high pSmad1/5 intensity ventrally and little to no pSmad1/5 dorsally. However, in zebrafish there are differing reports for nuclear pSmad1/5 in the dorsal organizer: some observe no dorsal pSmad1/5 intensity [129,139,144], while another

[1]In *Xenopus*, Smad1 is the primary transducer of BMP signaling during gastrulation, while in zebrafish *smad5* functions predominantly over *smad1* [125–128]. Since Smad1 and Smad5 have equivalent phosphorylation sites and ventralizing activity [129], they will be referred to as Smad1/5 in this review.

observes pSmad1/5 in the dorsal organizer [145]. Although all groups use the same antibody and dilution, there are differences in embryo pre-staining processing [145]. An epitope recovery method shows pSmad1/5 in the dorsal organizer, whereas standard methods do not. Further studies are needed to resolve the differences in pSmad1/5 detection in the organizer.

3.2. Extracellular modulators are critical to generate and regulate the BMP signaling gradient

The BMP signaling gradient is generated and further shaped extracellularly. In zebrafish embryos devoid of BMP signaling, BMP heterodimer protein can be injected directly into the extracellular space and completely rescue the pSmad1/5 gradient by the onset of gastrulation [118]. Injection of exogenous heterodimer protein circumvents other potential mechanisms for gradient formation, such as graded *bmp* expression or transcriptional inputs (BMP transcriptional feedback loops begin *after* the onset of gastrulation and, therefore, are not contributing to rescue of the BMP gradient *prior* to the onset of gastrulation; see Section 5). Rescue by the injected heterodimer protein suggests that the BMP gradient is generated largely by extracellular mechanisms. This is consistent with the rescue of severely dorsalized BMP mutants by injecting *bmp* RNA at the one-cell stage; although *bmp* RNA is initially present uniformly, the BMP gradient is still able to correctly pattern the DV axis [131,137].

From invertebrates to vertebrates, extracellular modulators are essential to establish and regulate BMP signaling levels [117,133]. Extracellular modulators also enable BMP signaling to act in distinct phases or cellular domains by providing precise spatial and temporal control of signaling, such as in the preplacodal ectoderm (Section 4.5) [146–148]. BMP signaling is required initially to specify these tissues at the onset of gastrulation, but must be completely blocked after gastrulation for their final specification. The primary extracellular modulators of BMP signaling are the BMP antagonists, which are secreted dorsally and bind and sequester BMPs to prevent ligand-receptor binding (Fig. 1c) [149–151]. The BMP antagonists include Noggin (Nog), Follistatin (Flst), and Chordin (Chd) [133,152,153]. Chd, in particular, is vital not only for generating the BMP signaling gradient, but also for continuing to shape the BMP gradient at various stages throughout DV patterning [151,154–159].

Unlike Nog and Flst, Chd itself can be modulated by additional extracellular regulators (Fig. 1c). First, the related metalloproteases Bmp1a and Tolloid (Tld) can cleave and inactivate Chd [160–163], and their inhibitor, Sizzled (Szl), blocks their protease activity, preventing Chd cleavage [163,164]. Twisted-gastrulation (Tsg), which forms a ternary complex with Chd and the BMP ligands, can either promote the cleavage of Chd by Tld or, in the absence of Tld, inhibit BMP signaling [165–170]. Finally, BMP binding endothelial regulator (BMPER, also known as Crossveinless-2) can also antagonize Chd activity and may do so in a complex with Tsg and BMP [146,171–173]. This plethora of Chd regulators suggests that delimiting Chd activity is key to shaping the BMP signaling gradient and DV patterning (Fig. 1c). Furthermore, there is emerging evidence that defining the Chd expression domain is also a crucial parameter for establishing and maintaining a correct BMP signaling gradient [142,145,174,175].

3.3. Transcriptional regulation of BMP by Wnt, FGF, and Nodal signaling

Although Wnt, FGF, and Nodal are clearly required for AP patterning (Section 2), these factors also affect BMP signaling and DV patterning through transcriptional regulatory relationships that persist from their role in establishing early DV polarity. For example *fgf*, which is initially induced by maternal Wnt/β-catenin, contributes to organizer formation by transcriptionally repressing *bmp* expression and inducing *chd* expression, thus antagonizing BMP signaling (Fig. 1d) [176–178]. Similarly, Nodal contributes to organizer formation by inducing *gsc* [63,71,179] and *chd* [180], which can inhibit BMP signaling (Fig. 1d). Notably, *chd* is required for the dorsalizing activity of the organizer [158], but *chd* expression is not fully dependent on the organizer [180].

On the other hand, the role of Wnt signaling changes when it is zygotically expressed. As opposed to its maternal role in establishing the dorsal organizer, zygotic Wnt signaling promotes *vox/vent/ved* expression to promote ventral cell fates [181,182]. *vox/vent/ved* maintain *bmp* gene expression ventrally and transcriptionally repress *boz*, *chd*, and *gsc* restricting their expression to dorsal regions (Fig. 1d) [114–116,179,183–185]. *boz*, initially induced by maternal Wnt/β-catenin, promotes dorsal cell fates by acting as a transcriptional repressor of *vox/vent/ved*, *bmp*, and zygotic *wnt*, while also stimulating *chd* expression (Fig. 1d) [176,186–189]. Interestingly, BMP can also induce *vox/vent/ved* expression and thus positively regulate its own expression (Fig. 1d) [116,184], though it acquires this ability after the BMP gradient has been established and it has begun patterning the DV axis [130,137,139]. The stage-specific contribution of these transcriptional regulatory relationships remains to be fully characterized and integrated into our understanding of DV patterning.

4. Temporally progressive patterning of the AP and DV axes

Tissues along the AP axis are patterned progressively from anterior to posterior, a feature of AP patterning that is well characterized (Fig. 1a) [190]. On the other hand, the temporal patterning of DV tissues has only recently been investigated. While it had been established in *Xenopus* and zebrafish that BMP signaling patterns most of the DV axis from mid-blastula through gastrula stages, there was only a general understanding of the broad tissue types that were being patterned (Fig. 1a) [148,191,192]. Additionally, the tail is not completely specified within that time window. Although tail progenitors are defined at the onset of gastrulation, their distinct cell fates are not specified until somitogenesis stages [80,159,192–195]. Given this broad time window for DV patterning (mid-blastula to early somitogenesis stages), the dynamic nature of the BMP morphogen gradient, and the progressive patterning of the AP axis that occurs concomitantly, the field was lacking a precise understanding of the temporal control of DV patterning. A pivotal set of experiments tackled this issue and demonstrated in zebrafish that DV patterning, similar to AP patterning, occurs in a temporally progressive manner (Fig. 2) [129,139].

4.1. The DV axis is progressively patterned from anterior to posterior

A direct approach to examine when BMP signaling patterns discrete DV tissues is to modulate BMP signaling levels over time. While Section 7.2 details multiple methods to

exert temporal control of signaling, our group used heat shock inducible transgene expression in the zebrafish. One transgene used was the wild-type BMP receptor *acvr1l* (previously called *alk8*) under the control of the *hsp70* promoter in a maternal-zygotic *acvr1l* mutant (*hsp:acvr1l*; MZ-*acvr1l*) [139]. Heat shock induction of *acvr1l* expression can rescue the severely dorsalized MZ-*acvr1l* phenotype [196]. Importantly, a normal embryo-wide BMP activity gradient is apparent 30 minutes after heat shock, demonstrating rapid control and robust rescue efficiency via *acvr1l* transgene induction [139]. Following a series of heat shock inductions at distinct time points, it was found that *acvr1l* induction as late as the late blastula or onset of gastrulation could fully rescue the gastrula pSmad1/5 gradient and the severe dorsalization of MZ-*acvr1l* mutant embryos (Fig. 2a) [139]. Surprisingly, although pSmad1/5 is evident during mid-blastula stages, this BMP signaling is not necessary for patterning or to generate a robust pSmad1/5 gradient during gastrulation since *acvr1l* first expressed at the onset of gastrulation sufficed to pattern the embryo and generate a normal signaling gradient.

However, following heat shock induction, transgene expression persists for up to several hours [159,192]. To determine when BMP signaling is required to pattern tail tissues, the *hsp:acvr1l* transgene was induced in zygotic (Z) *acvr1l* mutants, which only display dorsalized tail tissue [196]. By employing a similar developmental series of heat shock rescue experiments detailed above in *hsp:acvr1l*; Z-*acvr1l* embryos, *acvr1l* induction at the one-somite (10.5 hpf) stage fully rescued all Z-*acvr1l* mutants, whereas induction at later somitogenesis stages only partially rescued the tail dorsalization or did not rescue [139]. This indicates that BMP signaling is sufficient during post-gastrula stages to pattern the tail (Fig. 2c). Therefore, the DV axis is patterned during at least two time windows: BMP initiates patterning of the head and trunk beginning in the late blastula or at the onset of gastrulation and patterns the tail during early somitogenesis stages (Fig. 2a – c).

4.2. BMP signaling progressively patterns the ectoderm and mesoderm from anterior to posterior in a temporal fashion

The studies described above demonstrate that DV axial body patterning initiates during late blastula/early gastrula stages, while patterning of DV tissues of the tail initiates at the end of gastrulation. But these experiments left open when distinct domains along the DV axis are patterned (i.e. are head and trunk DV tissues patterned concomitantly or sequentially). To address this question, a developmental time series of BMP inhibition studies was performed. A transgene was used that expresses the BMP antagonist *chd* (Section 3.2) under the *hsp70* promoter (*hsp:chd*), which can abolish BMP signaling in a wild-type embryo within 60 minutes of heat-shock induction [129,139]. In these experiments, *hsp:chd* embryos were heat shocked at distinct 30-minute intervals from blastula through gastrula stages and then phenotyped to determine the extent of dorsalization. If a tissue remains properly patterned (i.e. not dorsalized) after heat-shock induction of Chd at a specific stage, then BMP signaling has already patterned that tissue prior to the stage of heat shock. Since neural tissues are dorsally derived and inhibited by BMP signaling (Fig. 1a and 1c), loss of BMP signaling results in their ventral expansion. Complete dorsalization causes a clear phenotype: neurectodermal markers are radially expanded and encircle the embryo (compare

Fig. 3a and 3b). Thus, the ventral expansion of neurectodermal markers was used to gauge the extent of dorsalization.

Heat shock of *hsp:chd* embryos at a mid-blastula stage (3 hpf) and the subsequent loss of BMP signaling by 4 hpf caused complete dorsalization (Fig. 3b) [129]. Remarkably, loss of BMP signaling at time points at and after 4.5 hpf (resulting from heat shock at and after 3.5 hpf) resulted in the dorsal restriction of neurectodermal markers in a progressive (from rostral to caudal), time-dependent fashion (Fig. 3b – 3d′, asterisks). Loss of BMP signaling at a late blastula stage (4.5 hpf) caused the radial expression of all markers except for the most rostral marker, *six3* (forebrain), which was restricted dorsally (Fig. 3b′) [129]. Therefore, BMP signaling acts prior to 4.5 hpf to properly pattern the forebrain, while it functions after 4.5 hpf to pattern more caudal tissues. Loss of BMP signaling at an early gastrula stage (6 hpf) caused the radial expansion of all markers except for *six3* and *pax2.1* (midbrain-hindbrain boundary, MHB), which were both restricted dorsally (Fig. 3c), demonstrating that BMP signaling patterns the MHB between 4.5 and 6 hpf (Fig. 3c, red asterisk) [139]. Strikingly, hindbrain rhombomeres R3 and R5 (marked by *krox20*) are patterned in 30-minute intervals (Fig. 3c′ – 3d): R3 requires BMP signaling prior to 6.5 hpf (Fig. 3c′, light blue asterisk) and R5 prior to 7 hpf (Fig. 3d, dark blue asterisk) [139]. Finally, the most caudal hindbrain marker, *hoxb1b*, requires BMP signaling prior to 8.5 hpf (Fig. 3d′, purple asterisk) [129]. It is important to note that at the later developmental stages BMP signaling is required during specific intervals, as opposed to being required for a longer duration [129].

Notably, BMP signaling also patterns the mesoderm in a progressive, temporal manner. In the *hsp:chd* experiments discussed above, the expression of mesodermal markers were also characterized in parallel to those of the neurectoderm [139]. Like the neurectoderm, the DV fates of the mesoderm are progressively patterned from anterior to posterior: the anterior pronephros requires BMP signaling prior to 6 hpf, the posterior pronephros prior to 6.5 hpf, and blood precursors prior to 7 hpf (Fig. 1a) [139].

Overall, the studies summarized here and in the previous section demonstrate that BMP signaling specifies the entire DV axis in a time-dependent, progressive fashion.

4.3. An identical patterning clock coordinates DV and AP progressive patterning

The discovery that DV axial patterning progresses along the AP axis analogous to AP patterning (Sections 4.1 – 4.2) and the fact that both axes are patterned during gastrulation prompt the question of whether DV and AP patterning are coordinated in time and/or space or are regulated by independent temporal mechanisms. Recently, this question was addressed by simultaneously manipulating DV and AP patterning, which demonstrated that the patterning of both axes is temporally coordinated [129]. These experiments relied on markers with expression domains dually specified by BMP and either FGF, Wnt, or RA, respectively. For example, *otx2* is a marker of anterior neurectoderm (forebrain and midbrain) [197] that is restricted anteriorly by FGF and Wnt signaling and dorsally by BMP signaling, which together define its expression domain (Fig. 4a, left panel). In contrast, *hoxb1b* is a marker of caudal hindbrain that requires posterior FGF, Wnt, or RA signaling in

conjunction with dorsal restriction by BMP signaling, to define its posterior-dorsal expression domain (Fig. 4c, left panel).

If AP and DV patterning are temporally coordinated, then alterations in AP patterning would similarly alter the temporal patterning of DV tissues. To evaluate this, embryos were either anteriorized by inhibiting FGF or Wnt, or posteriorized by overexpressing FGF, Wnt, or RA (Fig. 4a and 4c, right panels). These AP alterations were performed in *hsp70:chd* embryos (described in Section 4.2) to enable concurrent temporal manipulation of BMP signaling and DV patterning. Since BMP is required at late blastula stages (4.5 – 5 hpf), heat shock at 4 hpf in an already anteriorized or posteriorized embryo resulted in the ventral expansion of the anterior or posterior marker, respectively (Fig. 4b and 4d, left panels). These compound phenotypes can be described as anteriorized-dorsalized or posteriorized-dorsalized.

The key question was whether the anteriorized or posteriorized regions in the compound phenotypes would be patterned by BMP signaling simultaneously at the time point when BMP normally patterns the marker (Fig. 4b and 4d), or independently at the time point when BMP normally patterns each position along the AP axis (Fig. 4b′ and 4d′). If patterning of the anteriorized or posteriorized regions occurs based on the normal timing of the marker, then DV and AP patterning are coordinated (Fig. 4b and 4d); conversely, if DV and AP patterning are not coordinated, then BMP would pattern the anteriorized or posteriorized regions of the marker at independent time points (Fig. 4b′ and 4d′). Strikingly, the compound phenotype was always patterned simultaneously at the time point when BMP normally patterns the marker, showing that AP and DV patterning are temporally coordinated throughout gastrulation along these orthogonal axes [129]. This intimate coordinated patterning enables cells to adopt both an AP and DV identity simultaneously, integrating the positional information of two orthogonal axes to progressively pattern the embryo from head to tail (Fig. 1a – c).

4.4. The Smad1/5 linker region coordinates AP and DV patterning

Several *in vitro* and *in vivo* studies offer a potential mechanism to coordinate AP and DV patterning: the phosphorylation state of Smad1. Although C-terminally phosphorylated Smad1/5 (referred to as Ct-pSmad1/5 in this section) is the primary downstream nuclear effector of BMP signaling (Section 3.1), FGF and Wnt signaling components can also alter the phosphorylation state of Smad1/5, specifically in the linker region between its N- and C-terminal domains (Fig. 5). MAPK, activated by FGF signaling, can phosphorylate four conserved sites in the Smad1/5 linker (pSmad1/5-L^{MAPK}) [198–200], while Wnt signaling inhibits GSK3 phosphorylation of the Smad1/5 linker (pSmad1/5-L^{GSK3}) (Fig. 5) [201]. There is a model of sequential Smad1/5 phosphorylation: first by BMPR on the C-terminus, second by MAPK in the linker, and third by GSK3 in the linker (Fig. 5a, blue arrows) [200,201]. Studies in *Xenopus* and MEFs demonstrate that pSmad1/5-L^{MAPK} represses Smad1/5 activity and that pSmad1/5-L^{GSK3} enhances this inhibition [198–200]. Specifically, pSmad1/5-L^{MAPK} and dual pSmad1/5-$L^{MAPK+GSK3}$ promote Smad1/5 polyubiquitinylation by the Smurf1 E3 ligase, which results in Smad1/5 proteosomal degradation outside the nucleus (Fig. 5a) [200–202]. Thus, the Smad1/5 response to BMP signaling, which directs DV patterning, also depends on FGF and Wnt activity, which direct AP patterning.

Since the signaling molecules that direct AP and DV patterning converge on Smad1/5 phosphorylation at distinct sites (with either inhibitory or permissive effects, respectively), differential Smad1/5 phosphorylation could coordinate the timing of DV and AP patterning (Fig. 5) [203]. Antibody staining of mid- to late gastrula stage embryos for the three distinct Smad1/5 phosphorylation states reveals that each is spatially restricted: Ct-pSmad1/5 is only observed ventrally, pSmad1/5-L^{MAPK} is localized ventral-vegetally, and pSmad1/5-L^{GSK3} is predominantly restricted to ventral-animal regions (Fig. 5) [129]. This spatial restriction is wholly dependent on the Smad1/5 phosphorylation state since Smad1/5 (phosphorylated and un-phosphorylated) is uniformly present across the embryo [129]. Furthermore, the more vegetal localization of pSmad1/5-L^{MAPK} overlaps with the region of active BMP patterning at the margin (Fig. 2), while the animal localization of pSmad1/5-L^{GSK3} does not; therefore only FGF/MAPK activity is favorably positioned to temporally regulate BMP signaling.

Strikingly, experiments with mRNA encoding a human Smad1 resistant to MAPK phosphorylation (*hSmad1-MM*) disrupt coordinated DV and AP patterning. In zebrafish embryos deficient for endogenous Smad5, mis-expressed wild-type hSmad1 fully rescues the embryo, whereas hSmad1-MM results in anterior and posterior tissue markers being patterned by BMP 30 minutes earlier than normal [129]. This indicates that pSmad1/5-L^{MAPK} regulates the timing of DV patterning; presumably, pSmad1/5-L^{MAPK} slows or inhibits the cellular response to BMP signaling by 30-minutes to ensure that AP and DV patterning occur simultaneously (Fig. 5b).

Exclusive MAPK phosphorylation of the Smad1/5 linker in the ventral-vegetal region is consistent with known FGF and Wnt activity at the margin. While FGF induces pSmad1/5-L^{MAPK}, Wnt inhibits additional pSmad1/5-L^{GSK3} possibly preventing pSmad1/5 degradation (Fig. 5b). Conversely, at the animal pole, the absence of both FGF and Wnt signaling results in pSmad1/5-L^{GSK3} localized animally (Fig. 5c). Thus, the localization of each phosphorylated Smad1/5 linker state is an amalgamation of the spatial distributions of BMP, FGF, and Wnt activity, providing a mechanism to coordinate AP and DV. This parallels regulatory mechanisms used by other TGFβ family members, which maximize different Smad2 or Smad3 phosphorylation states for distinct functions [204,205]. However, other mechanisms likely also modulate the temporal function of BMP signaling since DV tissues continue to be patterned progressively with hSmad1-MM despite being precocious by 30 minutes. For example, temporal regulation of chromatin state could also contribute to the progressive patterning of DV tissues.

4.5. BMP patterning of anterior neural tissues offers an additional setting to study spatiotemporal mechanisms that regulate BMP signaling

Although BMP signaling must be inhibited for neural tissue to be induced (Fig 1a and 1c) [206], recent studies have revealed additional spatial and temporal complexities in the role of BMP signaling in patterning distinct neural fates. First, BMP actively patterns the anterior neural ectoderm (i.e. inhibiting eye specification to protect forebrain telencephalon fates). Based on small molecule BMP receptor inhibitor studies, BMP patterns the forebrain during late blastula to early gastrula stages (though see a discussion of timing delays in Section 7.2), thus preceding the function of Wnt antagonists in forebrain specification [207]. Since

BMP also restricts forebrain markers dorsally during the same time interval (Fig. 3b – b′), studies to address the finer spatial domain of BMP activity in conjunction with the role of distinct activity levels in forebrain patterning are needed.

Second, the boundary of neural and nonneural ectoderm presents additional spatial and temporal challenges for BMP signaling regulation. Distinct levels of BMP signaling may pattern the neural crest (NC) and preplacodal ectoderm (PPE), which gives rise to sensory organs like the inner ear and olfactory epithelium (Fig. 1a) [137,148,208]. BMP signaling would need to be tightly regulated to generate such distinct domains in this very narrow region and BMPER (Section 3.2) may play a key role in this process [146]. Furthermore, PPE patterning requires two contrary phases of BMP signaling: at late blastula stages, BMP signaling specifies PPE precursors, while at late gastrula stages BMP antagonists must block BMP signaling for further PPE development [147]. Thus, patterning of the forebrain, PPE, and NC present unique environments to study spatial and temporal mechanisms (e.g. expression of competency factors) that could regulate BMP signaling and BMP antagonist activity to pattern the most anterior regions of the embryo.

5. Do the morphogenetic movements of gastrulation impact cell fate specification?

The previous section discusses recent progress in understanding basic spatiotemporal features of AP and DV patterning: both AP and DV cell fates are 1) progressively patterned along the AP axis, 2) patterned in a coordinated manner by an identical patterning clock, which is 3) mediated in part by FGF phosphorylation of the Smad1/5 linker in ventral regions of the embryo (Section 4). However, this coordinated AP and DV patterning takes place during the dynamic and rapid process of gastrulation. Gastrulation shapes the germ layers of the embryo through the conserved morphogenetic movements of cell internalization, epiboly, convergence, and extension, all of which result in dramatic cell movements and rearrangements of cellular contacts [209]. The relationship between these morphogenetic movements and concurrent AP and DV cell fate specification is key to fully understand these processes, yet this relationship is complex and requires further investigation.

5.1. Morphogenetic movements and AP and DV signaling and patterning

Evidence suggests that Wnt, Nodal, FGF, and BMP signaling can direct morphogenetic cell movements independently of their roles in cell specification [210]. For example, the BMP signaling gradient, in addition to DV fate specification, also directs domains of distinct convergent extension movements [211], possibly through the regulation of cell-cell adhesion [212]. However, since cell fate specification is difficult to truly uncouple from cell behavior experimentally, differential cell movements may still be a result of DV cell specification. Alternatively, the DV positional information supplied by the BMP gradient may independently inform cell movements [209]. Further studies are needed to distinguish between these two possibilities. There are similar studies and open questions concerning AP patterning and gastrulation associated with Nodal signaling [209].

Some cells dramatically change their position during dorsal convergence and extension and are exposed to different levels of morphogen signaling. It remains mostly unknown whether these cells are already specified, bring their fate with them, and are refractory to the new signaling environment they move through. Interestingly, BMP signaling patterns prospective head DV tissues prior to the major cell movements of dorsal convergence. Thus these rostral cells are expected to sense the same BMP signaling level during the first half of gastrulation and are specified at the time they converge dorsally (Section 4.2 and Fig. 3) [139]. In other contexts cells may be specified by morphogen signals during discrete time windows, responding to gradient thresholds, or may measure signal over a window of time as they move through a gradient. Lastly, cells may require exposure to multiple morphogen signaling levels for their specification. Further studies are required to decipher precisely the relationships between cell movements, morphogen gradients, and cell specification and the mechanisms that intertwine these processes.

5.2. How do changes in DV signaling pole proximity and gastrulation cell movements affect gradient formation?

Gastrulation from fish to mammals entails dramatic rearrangements in cellular contacts. Though the types of cell movements during gastrulation are diverse, each type consistently results in a change in cell-cell contacts, which may impact the functionality of morphogen gradients [209]. A clear example arises when considering the BMP gradient during gastrulation in the zebrafish (Fig. 2 and 6, yellow arrows). Gastrulation begins at the vegetal margin of the zebrafish embryo (50% epiboly) where the ventral-most cells, which have the highest levels of BMP signaling, are the farthest possible distance (~675 μm, embryo diameter) from the dorsal-most cells, which have no BMP signaling (Fig. 6a, compare white and black asterisks). As gastrulation and epiboly proceed, the margin progresses vegetally (posteriorly) and the ventral- and dorsal-most cells continuously move closer to each other until they eventually meet (100% epiboly) (Fig. 6b – d). The distance between these cells of opposing signals drastically decreases from ~675 μm at the onset of gastrulation to their direct apposition in the tailbud at the end of gastrulation. Thus, the cells presumed to have the highest and lowest levels of BMP signaling progressively converge until meeting in the tailbud. This dramatic increase in the proximity of cells with opposing signal may have profound effects on the shape of the BMP morphogen gradient during gastrulation [159,213].

Epiboly likely plays a key role in regulating the temporal patterning of DV tissues through FGF signaling. As epiboly proceeds, pSmad1/5-L^{MAPK} is localized to progressively more posterior (vegetal) regions (Fig. 5b), which would enable both the temporal progressivity and coordination of DV and AP tissue patterning [129]. Future studies are needed to address whether these changing signaling contexts (i.e. the proximity of cells with opposing signals or the progressively posterior restriction of pSmad1/5-L^{MAPK}) are a principal spatial mechanism to direct the shape and timing of morphogen gradients throughout gastrulation.

5.3. Morphogenetic movements reorganize the DV axis established by the onset of gastrulation

It is worth noting that, due to the massive cell movements during gastrulation, the dorsal-ventral axis defined in late blastula/early gastrula stages (Fig. 1a) is distinct from the dorsoventral axis of the post-gastrula embryo, which has a body plan that resembles the mature organism. That is, while the broad territories of the embryo can be mapped by the onset of gastrulation (Fig. 1a), these tissues are dramatically reorganized during gastrulation and neurulation [214,215]. In particular, dorsal convergence combined with extension along the AP axis results in many tissues being oriented along the AP axis after gastrulation and during somitogenesis. However, this does not mean that there is only one axis [216–219]. Prior to the onset of gastrulation, these tissues are oriented along a coordinate orthogonal to the AP axis, i.e. the DV axis in Fig. 1a (gray arrows). For example, by the onset of gastrulation the somites are oriented along the DV axis of the early gastrula (Fig. 1a) and this orientation informs the organization of the somites along the AP axis: dorsal somitic mesoderm develops into more anterior somites, while ventral somitic mesoderm develops into more posterior somites. Moreover, the epidermis is specified by BMP signaling ventrally during gastrulation, but later is present throughout all regions of the embryo; the neural crest is specified by BMP signaling in lateral regions of the gastrula embryo, but comes to lie dorsally in the neural tube following neurulation. Thus, the DV axis of the early gastrula is an independent coordinate system to that of the post-gastrula and neurula embryo.

Furthermore, visualization of morphogen gradients (Section 7.1) demonstrates that there are indeed two orthogonal axes of the embryo at the onset of gastrulation. Gradients of Nodal [83,220] and Wnt [20,221] signaling are observed along the AP axis and a gradient of BMP signaling is observed orthogonally, revealing two distinct axial coordinate systems (Fig. 1a and 1c) [129,139]. Moreover, AP patterning continues in the absence of DV patterning (e.g. in BMP loss-of-function contexts), making evident the independent patterning of these axes (Fig. 3 and 4). Thus, the DV and AP axes are essential coordinates for cell fate specification and patterning of the body plan during gastrulation. Organization of tissues along the AP axis at the end of gastrulation results from integrating orthogonal morphogen gradients with dorsal convergence and extension morphogenetic movements. Further studies are needed to understand how these distinct axes are integrated to coordinate progressive patterning of the embryo (Section 4) and how they account for cell movements.

6. What is the role of autoregulatory feedback loops in regulating and/or coordinating AP and DV patterning?

Another aspect of AP and DV patterning that remains to be fully characterized is the role of, and crosstalk between, the autoregulatory transcriptional feedback mechanisms that are activated by AP and DV signaling. Across zebrafish, *Xenopus*, and mouse, there are known feedback mechanisms that regulate FGF, Nodal, and BMP signaling. FGF and Nodal signaling transcriptionally activate their respective inhibitors. FGF signaling induces the expression of *sprouty* (*spry*) and Spry proteins comprise a major class of FGF/RTK inhibitors [46,222]. Nodal signaling induces the expression of Antivin/Lefty proteins, which antagonize Nodal signaling [84,85,223,224].

BMP signaling is regulated by multiple feedback mechanisms. High levels of BMP signaling ventrally promote *bmp*, *tld*, *tsg*, and *cv-2* expression, all BMP-promoting factors [130,170,171,213]. However, high BMP signaling also induces *szl*, which antagonizes BMP signaling by inhibiting Tld/Bmp1a cleavage of Chd [225,226]. High levels of BMP signaling also repress chd expression, restricting it to dorsal regions [227]. Additionally, there is a recent report in zebrafish that factors in extraembryonic tissues can initiate a positive feedback loop on BMP signaling [228]. BMP signaling also induces the expression of *bambi*, which encodes a transmembrane protein implicated in attenuating BMP signaling [146,229–231], although loss-of-function studies have yet to determine a role for *bambi* in DV patterning [232]. Importantly, since all of these feedback loops are active after the onset of gastrulation [127,128,130,132,137] they regulate and maintain the BMP signaling gradient rather than establish it.

Transcriptional feedback may be integral to regulate and/or shape FGF, Nodal and BMP signaling gradients. Indeed, studies applying mathematical models support a key role for both activating and inhibitory feedback loops in stabilizing and refining morphogen gradients for pattern formation [90,145,174,233–236]. However, the requirement for these feedback loops and whether they primarily confer robustness to the morphogen gradient or serve to refine the AP and DV pattern remain unknown. It would also be interesting to consider the changing environment of the gastrula embryo when modeling the role of feedback regulation. Furthermore, the coordination of feedback mechanisms either within or between signaling pathways remains unknown and could represent an additional mechanism to coordinate AP and DV patterning.

7. Current and emergent methods of spatial and temporal manipulation of AP and DV patterning

In this section we focus primarily on *in vivo* genetic and fluorescent visualization approaches, the majority of which have been developed in zebrafish.

7.1. Morphogen visualization and use of reporters

A major difficulty in studying the morphogens that pattern the AP and DV axes is that they are secreted and difficult to visualize by immunostaining at endogenous levels. Most studies of ligand expression and dynamics rely on overexpression of fluorescently labeled constructs [38,57,90,142] or the use of antibodies that recognize the immature ligand, as opposed to its fully processed form [144,145]. While the endogenous RA gradient has recently been visualized and quantified [104], that approach relies on FRET from a direct ligand-receptor interaction, which is less applicable for the Wnt, FGF, and BMP gradients since these ligands signal through more complex mechanisms (i.e. ligand bound to receptor may not be indicative of active signaling depending on the presence of co-receptors or complex stoichiometry). However, the advent of CRISPR/Cas9 genome editing offers a new approach to tag these ligands at their endogenous loci and even employ signal amplification techniques to visualize the endogenous morphogen gradient in fixed or live samples [237–239]

As secreted factors, the mRNA expression domains of these morphogens may inform gradient formation, yet this role remains uncharacterized. *In situ* transcript detection methods are qualitative and describe a transcript domain spatially, but do not provide quantitative data of transcript levels. However, recent advances in quantitative, single-molecule RNA transcript detection offer new possibilities [240–242]. For example, there are reports that *bmp* ligand expression during gastrulation is graded across the ventral half embryo, with highest expression ventrally and lowest expression laterally [144,178], while others do not report a difference in *bmp* expression [127,128,130,137,243]. Quantitative transcript detection can directly determine if graded *bmp* ligand expression could contribute to the BMP morphogen gradient.

A complementary approach to ligand visualization is the visualization of downstream readouts of the morphogen. For example, the intracellular transducer of BMP signaling is nuclear pSmad1/5, which can be directly visualized by immunofluorescence [129,139,244,245]. There are also various transgenic reporters in zebrafish for Wnt [221,246,247], FGF [248], Nodal [83], RA [99,249], and BMP signaling [144,250–252]. These reporter transgenes utilize sequences from a promoter that responds to the morphogen signal to drive reporter expression e.g. of *luciferase* or GFP. But, how rapidly the reporter is expressed after signal induction and how long the reporter signal persists after signal repression must be carefully characterized to determine the responsiveness of each reporter. To visualize morphogen signaling, which can change in a relatively short time period, it is likely best to us rapidly folding (e.g. Venus) or destabilized fluorescent proteins [83,221,246,248,252].

7.2. Methods for temporal manipulation of signaling

Our knowledge of when Wnt, FGF, RA, Nodal and BMP signals are required for AP and DV patterning comes from experiments that activate or inhibit these signals at specific developmental time points. A particularly expedient approach in *Xenopus* and zebrafish is to incubate embryos in media containing various chemical inhibitors or activators. These pharmacological treatments include SU5402 (inhibits FGFR), LiCl (inhibits the Wnt inhibitor GSK3), DEAB (inhibits RA processing), Dorsomorphin DMH1 (inhibit BMP type I receptors), and RA itself [129]. Although these chemicals have well-characterized direct effects, studies of temporal function must determine the delay between drug application and complete inhibition or activation of signaling. This delay is infrequently defined; instead it is assumed that inhibition or activation ensues immediately after drug application, which may not be the case. Surprisingly, for example, the small molecule BMP signaling inhibitor, DMH1, takes 3 hours to fully inhibit pSmad1/5 during gastrulation [129]. Determining the delay between drug removal and reversal of its impact on signaling activity is also important to define a temporal window or duration of signaling. Furthermore, one must account for the multiple functions of a signaling pathway during development. For example, Wnt establishes the dorsal organizer during mid- to late blastula stages, which is unrelated to its role directing AP patterning during gastrula stages (Sections 2.1 and 3). Therefore, any experiment that aims to understand the posteriorizing role of Wnt signaling must manipulate Wnt signaling *after* blastula stages.

Another method of temporal manipulation is to generate transgenic lines that can be induced to either inhibit or activate signaling. Previously we discussed *hsp70* promoter driven genes that inhibit or activate BMP signaling (Sections 4.1 – 4.3) [129,139,159,192,253]). When using heat shock-inducible transgenes, it is important to determine the appropriate duration of heat shock to activate or inhibit signaling, which can vary from only 10 minutes [159] to as long as an hour [139,192]. Here, too, it is important to factor in the time it takes for signaling to be effectively induced or fully repressed when defining the time of signaling function. Finally, inducing heat shock results in embryo-wide expression of the transgene. To achieve spatially restricted gene control, one may induce local heat shock by sublethal laser irradiation [254] or a microheater [255]. Alternatively, one may transplant cells from the transgenic line into a background without a heat shock transgene and heat shock the entire embryo after transplantation (cell transplantations are described below).

7.3. Methods for spatial manipulation of signaling

Morphogen signaling relies on the restricted localization and differential mobility of the morphogens themselves and their regulators. An elegant, direct, and versatile method to investigate spatial mechanisms is by generating chimeric organisms by grafting or transplanting donor cells into a host embryo [256]. First, cell transplantation or grafting assays can determine the importance of localization by altering the spatial expression of the protein of interest. For example, to determine where *tolloid* must be expressed to inhibit Chordin function, *tld*-expressing cells were transplanted into *tld* mutant embryos. Only cells transplanted into the ventral vegetal region rescued the *tld* mutant phenotype, thus revealing the region where Tld cleaves Chd to promote BMP signaling [159]. Second, cell transplantation assays can be used to further evaluate whether a signaling factor functions directly at a distance. Such studies clearly established that the zebrafish Nodal signal, Squint, functions directly on its gene targets at a distance and therefore is a morphogen [76].

Additionally, the cell transplantation approach may be extended to address questions not only of space but also of time. As noted in Section 7.2, cells from heat shock-inducible transgenic lines may be used as transplant donors to incorporate temporal and spatial control of gene expression [192,253]. Furthermore, transplantation of various regions of the zebrafish blastula-gastrula margin has revealed there are distinct cell fate organizing centers in the margin, and that these organizing centers are fully active by the onset of gastrulation, including the one that specifies tail tissue [80,81].

7.4. Conclusion

Establishing the vertebrate body plan requires the coordination and integration of AP and DV axial patterning across the entire length of the embryo and over multiple developmental stages. The spatiotemporal regulation of this process is complex, but it can reveal the essential and conserved mechanisms used to generate and maintain morphogen signaling gradients. With the advent of quantitative measurement and visualization techniques, we are closer to understanding the mechanisms that drive patterning and body plan formation.

Acknowledgments

We would like to thank Allison Jamieson-Lucy and Benjamin Tajer for comments and careful reading of the manuscript. We would also like to acknowledge grants from the National Institutes of Health R01-GM56326-16 (MCM), T32-GM007229 (FBT), and F31-GM113362-01 (FBT).

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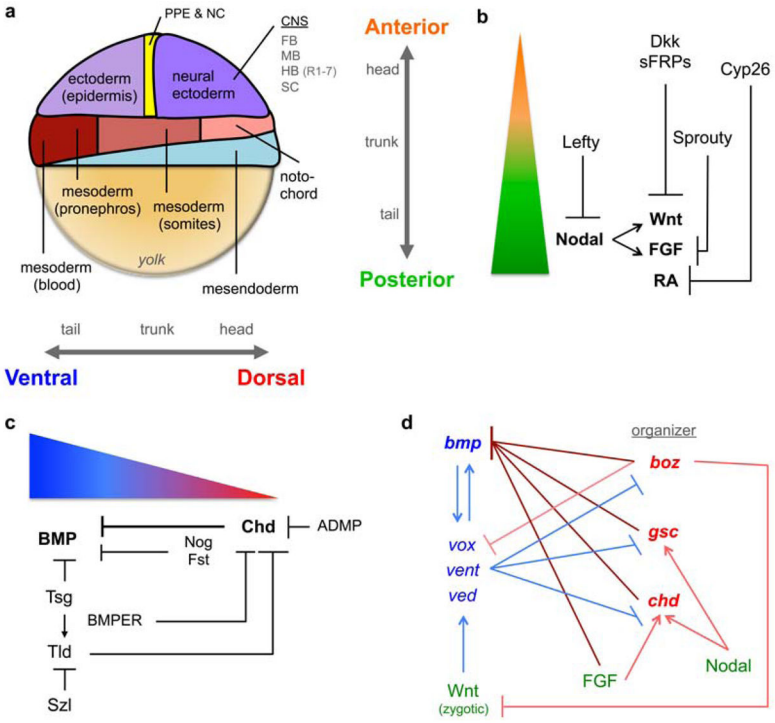

Fig. 1.

The AP and DV axes are patterned by morphogens and their regulators. (a) Fate map of a zebrafish gastrula, adapted from [215], with the orientation of the AP and DV axes. The neural ectoderm can be divided into four regions of the CNS: forebrain (FB), midbrain (MB), hindbrain (HB), which is further subdivided into rhombomeres 1 – 7 in zebrafish, and spinal cord (SC). (b) Wnt, FGF, Nodal, and RA specify posterior fates (green) in a concentration-dependent manner, while their inhibition is required for anterior fate specification (orange). (c) The BMP morphogen gradient specifies ventral cell fates (blue) at high levels and allows dorsal fate specifcation (red) at low levels. BMP signaling is regulated by extracellular factors; the DV localization of their transcriptional domain is depicted. Chd activity is key since it acts as a BMP antagonist and as the substrate for other extracellular modulators. (d) Transcriptional regulation of *bmp* and dorsal organizer genes. By activating different transcriptional repressors, zygotic Wnt promotes while FGF and Nodal antagonize BMP signaling. Blue lines indicate activity that promotes BMP signaling and red lines indicate activity that limits it (dark shades describe a direct effect, light shades an indirect effect).

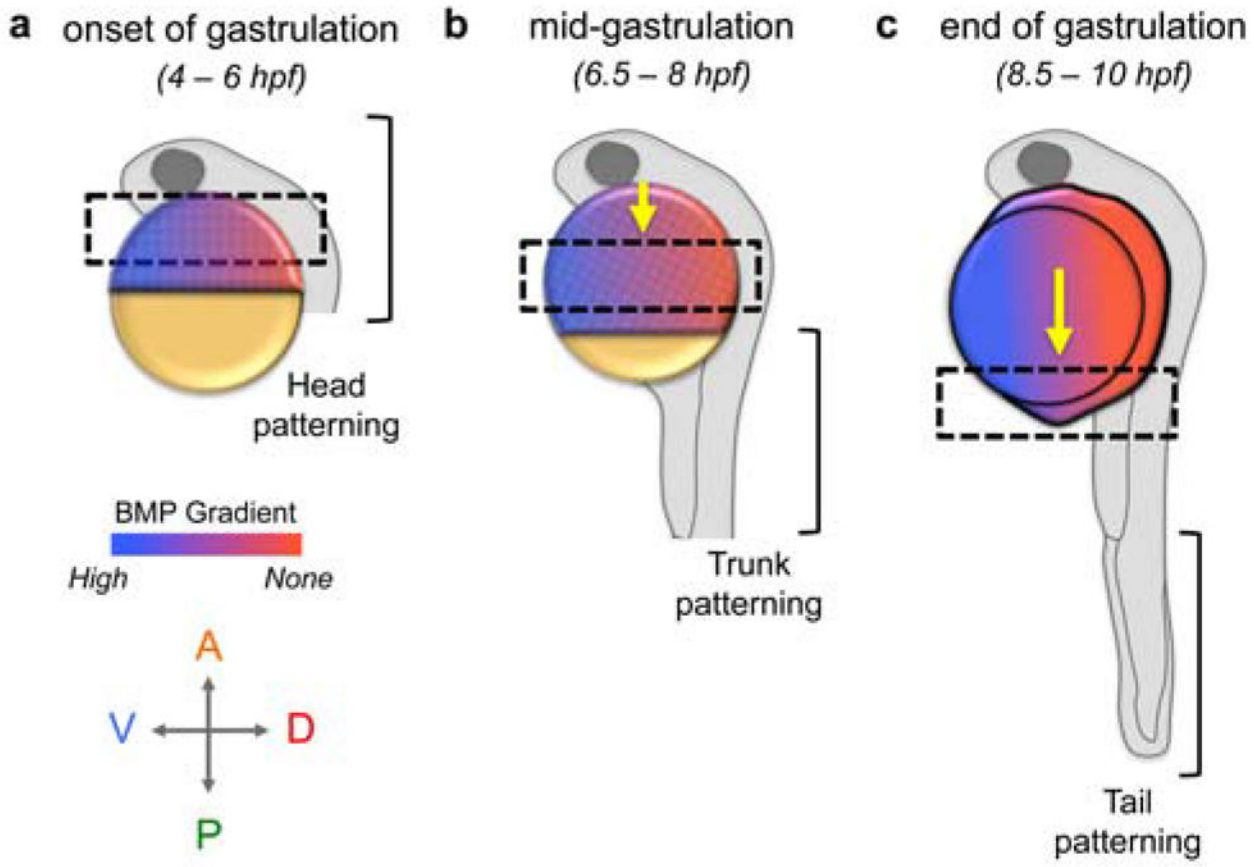

Fig. 2.

BMP signaling patterns DV cell fates progressively from anterior to posterior. AP and DV coordinates refer to the zebrafish gastrula embryos (a and b), which are depicted with cells atop the yolk. The dashed box indicates the region of active DV patterning with the corresponding portion of the body plan represented by the larval zebrafish (24 hpf). During gastrulation, cells undergo epiboly wherein the multilayered tissue thins and spreads posteriorly to completely envelop the yolk. (a) From late blastula to early gastrula stages (30 – 65% epiboly), the most anterior tissues, i.e. the head, are patterned. (b) As gastrulation proceeds, the region of active patterning progresses posteriorly (yellow arrow). At mid-gastrula stages (65 – 85% epiboly), trunk tissues are patterned. (c) From late gastrula (85 – 100% epiboly) to early somitogenesis, the most posterior tissues, i.e. the tail, are patterned.

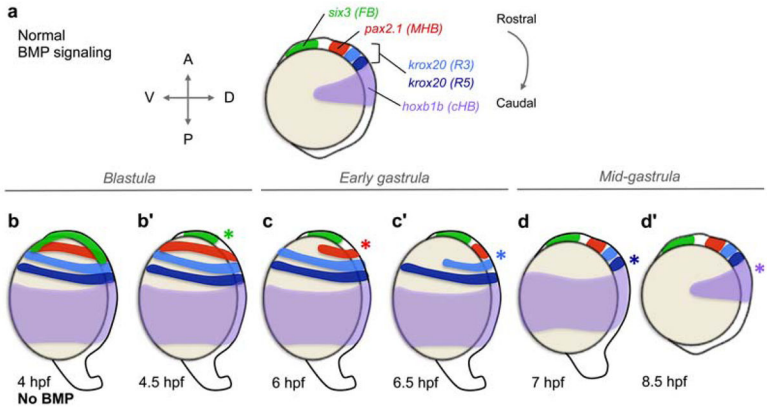

Fig. 3.

BMP signaling progressively patterns the anterior neurectoderm from rostral to caudal during gastrulation. (a) Wild-type expression pattern of anterior neurectoderm markers [FB, forebrain; MHB, midbrain-hindbrain boundary; R3, rhombomere 3; R5, rhombomere 5; cHB, caudal hindbrain]. (b – d′) Summary of experiments demonstrating that BMP signaling patterns the neurectoderm progressively, from rostral to caudal, in a time-dependent manner. (b) Loss of BMP signaling beginning at a mid-blastula stage (4 hpf) causes severe dorsalization and radial expansion of the dorsally-derived neurectoderm. (b′) Loss of BMP signaling at a late blastula stage (4.5 hpf) causes radial expansion of all markers except *six3*, which is restricted dorsally (green asterisk). Therefore, BMP patterns *six3* between 4 – 4.5 hpf. (c) Loss of BMP signaling at the onset of gastrulation (6 hpf) restricts *pax2.1* expression (red asterisk), indicating that BMP patterns the MHB between 4.5 – 6 hpf. (c′) Loss of BMP signaling at 6.5 hpf restricts R3 (light blue asterisk) and (d) loss of BMP signaling at 7 hpf additionally restricts R5 (dark blue asterisk), indicating that BMP patterns R3 and R5 in 30 minute intervals from 6 – 7 hpf. (d′) Loss of BMP signaling at 8.5 hpf restricts *hoxb1b* expression (purple asterisk), indicating that BMP patterns *hoxb1a* between 7 – 8.5 hpf.

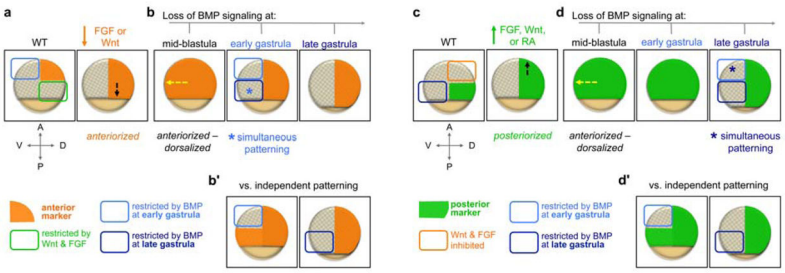

Fig. 4.

Summary of experiments demonstrating that AP and DV patterning are temporally coordinated throughout gastrulation. Marker expression is depicted in embryos at mid- or late gastrula stage. (a) BMP restricts anterior marker expression (orange) at early gastrula stages. Embryo anteriorized by inhibiting FGF or Wnt (dashed black arrow: posterior expansion of anterior marker). (b) Inhibiting BMP signaling in an anteriorized embryo at a mid-blastula stage causes a compound anteriorized-dorsalized phenotype (dashed yellow arrow: ventral expansion of the anterior marker). Since AP and DV patterning are temporally coordinated, inhibiting BMP at an early gastrula stage (when the anterior marker is normally patterned) causes complete dorsal restriction of the anterior marker. (b′) If AP and DV patterning were temporally independent, inhibiting BMP at an early gastrula stage would restrict the normal domain and the posteriorly expanded region would be restricted at a later gastrula stage. (c) BMP restricts posterior marker expression (green) at late gastrula stages. Embryo posteriorized by overexpressing FGF, Wnt, or RA (dashed black arrow: anterior expansion of posterior marker). (d) Inhibiting BMP signaling in a posteriorized embryo at a mid-blastula stage causes a compound posteriorized-dorsalized phenotype (dashed yellow arrow: ventral expansion of the posterior marker). Since AP and DV patterning are temporally coordinated, inhibiting BMP signaling only at a late gastrula stage (when the posterior marker is normally patterned) causes complete dorsal restriction of the posterior marker. (d′) If AP and DV patterning were temporally independent, inhibiting BMP at an early gastrula stage would still restrict the anteriorly expanded domain, but not the normal domain of the posterior marker.

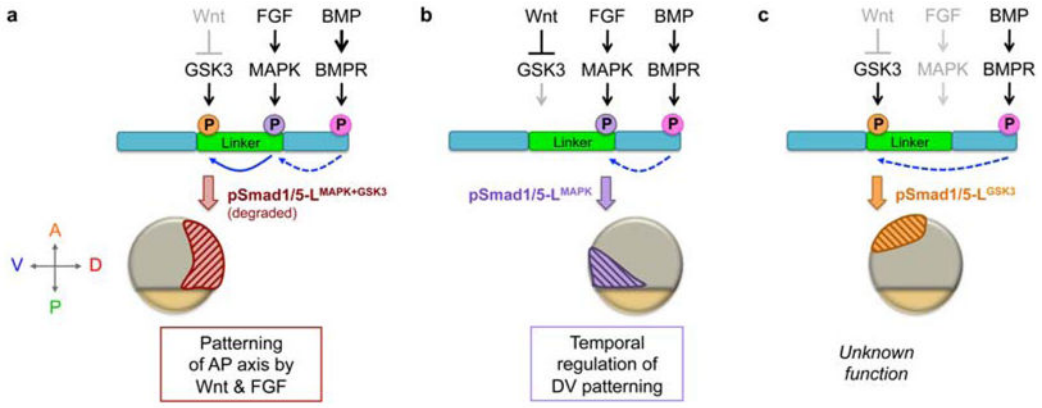

Fig. 5.

Model of spatially restricted functions of distinct phosphorylated Smad1/5 linker forms, depicted in mid-gastrula stage embryos. The N- and C-termini of Smad1/5 are shown in teal and the linker region is in green. C-terminal phosphorylation of Smad1/5 (Ct-pSmad1/5) a prerequisite to linker phosphorylation (dashed blue arrow). (a) In dorsal regions, MAPK and GSK3 sequentially phosphorylate the Smad1/5 linker (solid blue arrow), leading to pSmad1/5-$L^{MAPK+GSK3}$ degradation and blockage of BMP signaling. This mechanism may be most important during blastula stages when BMP signaling is more widespread and present dorsally (Section 3.1). (b) In ventral-posterior regions both FGF and Wnt are present, activating MAPK and inhibiting GSK3, respectively, to generate pSmad1/5-L^{MAPK}. pSmad1/5-L^{MAPK} may have reduced activity, which regulates timing of DV patterning. During epiboly, pSmad1/5-L^{MAPK} localizes progressively more posteriorly with the margin, patterning anterior DV tissues in its wake (not shown). (c) In animal regions GSK3 is uninhibited by Wnt, resulting in pSmad1/5-L^{GSK3}, though its significance remains unknown. Since pSmad1/5-L^{GSK3} does not overlap with pSmad1/5-L^{MAPK}, it is possible that in the zebrafish embryo (versus *in vitro* studies) either GSK3 does not require MAPK to prime the linker or those residues may be rapidly de-phosphorylated.

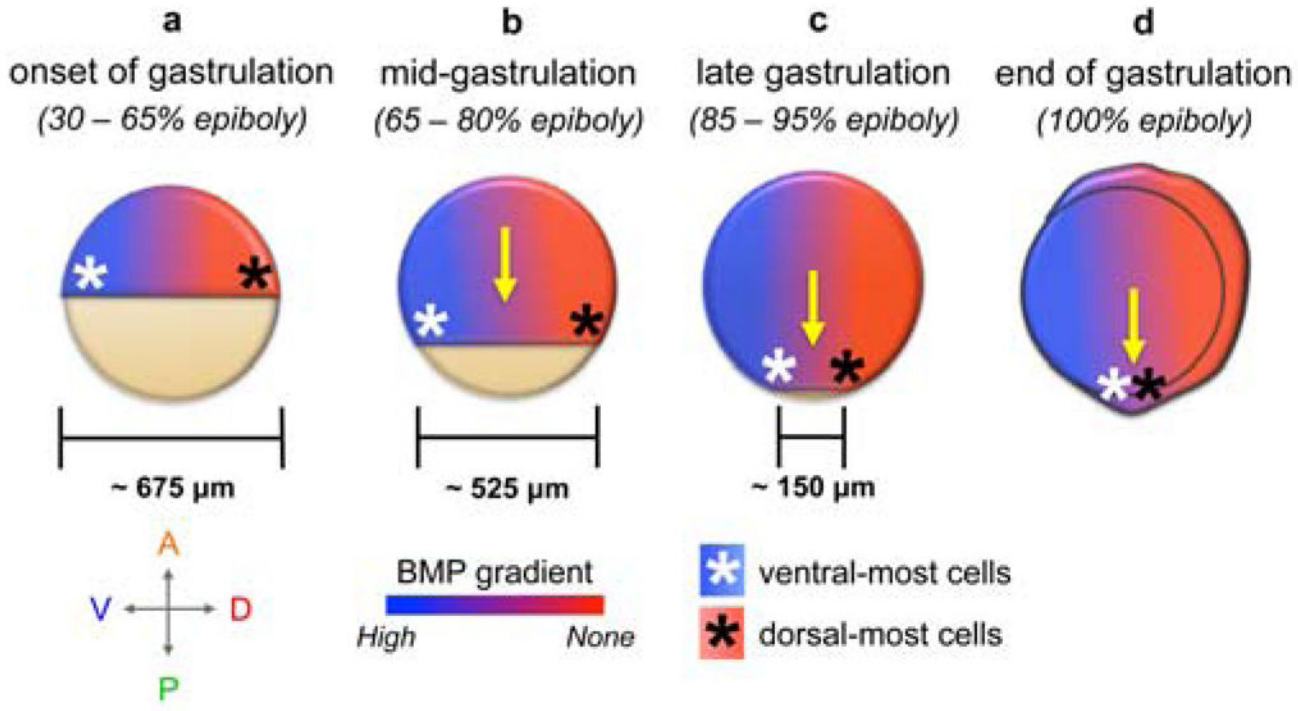

Fig. 6.

During gastrulation, there is a dramatic decrease in distance between the ventral- and dorsal-most cells. (a) From late blastula to early gastrula stages (4 – 6 hpf), the ventral-most cells (white asterisk), which have the highest levels of BMP signaling, are farthest (approximately 675 µm) from the dorsal-most cells (black asterisk), which have the lowest levels of BMP signaling. (b – d) As gastrulation proceeds (6 – 10hpf), epiboly movements advance the margin posteriorly (yellow arrow) and the distance between the ventral- and dorsal-most cells decreases rapidly until, by the end of epiboly, they are in direct contact.