Systems control of BMP morphogen flow in vertebrate embryos

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Embryonic morphogenetic programs coordinate cell behavior to ensure robust pattern formation. Having identified components of those programs by molecular genetics, developmental biology is now borrowing concepts and tools from systems biology to decode their regulatory logic. Dorsal–ventral (D–V) patterning of the frog gastrula by Bone Morphogenetic Proteins (BMPs) is one of the best studied examples of a self-regulating embryonic patterning system. Embryological analyses and mathematical modeling are revealing that the BMP activity gradient is maintained by a directed flow of BMP ligands towards the ventral side. Pattern robustness is ensured through feedback control of the levels of extracellular BMP pathway modulators that adjust the flow to the dimensions of the embryonic field.

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Introduction

Developmental biology aims at reverse engineering the genetic program that converts a single cell into a fully functional adult animal. Cell–cell communication over long distances is an essential component of the program that organizes embryonic tissues. The BMP pathway is the main signaling pathway involved in the D–V patterning of the gastrula embryonic field, which consists of about 10,000 cells in embryos of the frog *Xenopus*. Most of its molecular components have been conserved throughout the evolution of metazoans, but despite years of genetic and biochemical studies, we are only now starting to unravel the built-in complexity of the pathway. The main focus of this review is the extracellular regulation of D–V patterning, which has emerged as a paradigm in vertebrates for the study of positional information

and patterning. Its role is to reliably provide positional information along the D–V axis to instruct the histotypic differentiation of the three germ layers. For example in the ectoderm, low levels of BMP signaling give rise to central nervous system, intermediate levels to neural crest, and high levels to epidermal differentiation, while in the mesoderm the BMP gradient induces notochord, somite, lateral plate and blood island differentiation. We will first briefly review the biochemistry of the system (detailed reviews are available in [1,2•,3]). Second, we address the current understanding of the organization of the genetic network that controls D–V patterning. Finally, we examine how systems biology modeling helps understand its logic.

Components of the BMP pathway

BMP proteins are extracellular ligands of the TGFβ superfamily of growth factors. Several BMPs are expressed during *Xenopus* gastrulation, including BMP2/4/7 and ADMP (Anti Dorsalizing Morphogenetic Protein). BMP signaling is triggered by the binding of secreted BMP homodimers or heterodimers to two type I and type II BMP receptors. The activated receptor complex phosphorylates two serines in the C-terminus of transcription factors of the Smad family (Figure 1), resulting in their accumulation in the nucleus and activation of target genes [3]. At the extracellular level, multiple evolutionarily conserved nodes of regulation allow embryos to self-regulate, adjusting to changes in BMP gradient. Regulation occurs at three important steps: binding of BMPs to their receptors, cleavage of the BMP antagonist Chordin (Chd), and diffusion of BMP ligands in the extracellular space.

Binding of BMPs to their type I and II heterotetrameric plasma membrane receptors is highly regulated by diffusible and cell-surface bound antagonists (Figure 1). Diffusible antagonists include Chordin, Noggin, Follistatin and Chordin-like proteins [4–9]. They were originally identified as proteins secreted by the dorsal Spemann organizer able to induce twinned axes when expressed on the ventral side of *Xenopus* embryos. Chordin [10,11] and Noggin [12] physically prevent BMPs from binding to their cognate receptors (step 1 in Figure 1). In the case of Chordin, the stability of the Chd/BMP complex is greatly increased by a third protein, Twisted Gastrulation (Tsg) [13–15] (step 2 in Figure 1). The transmembrane antagonist BAMBI (BMP and Activin Membrane-bound Inhibitor) is a type I BMP truncated pseudo-receptor that associates with type II receptors and BMP ligands to form inactive signaling complexes [16] (step 8 in Figure 1).

Figure 1
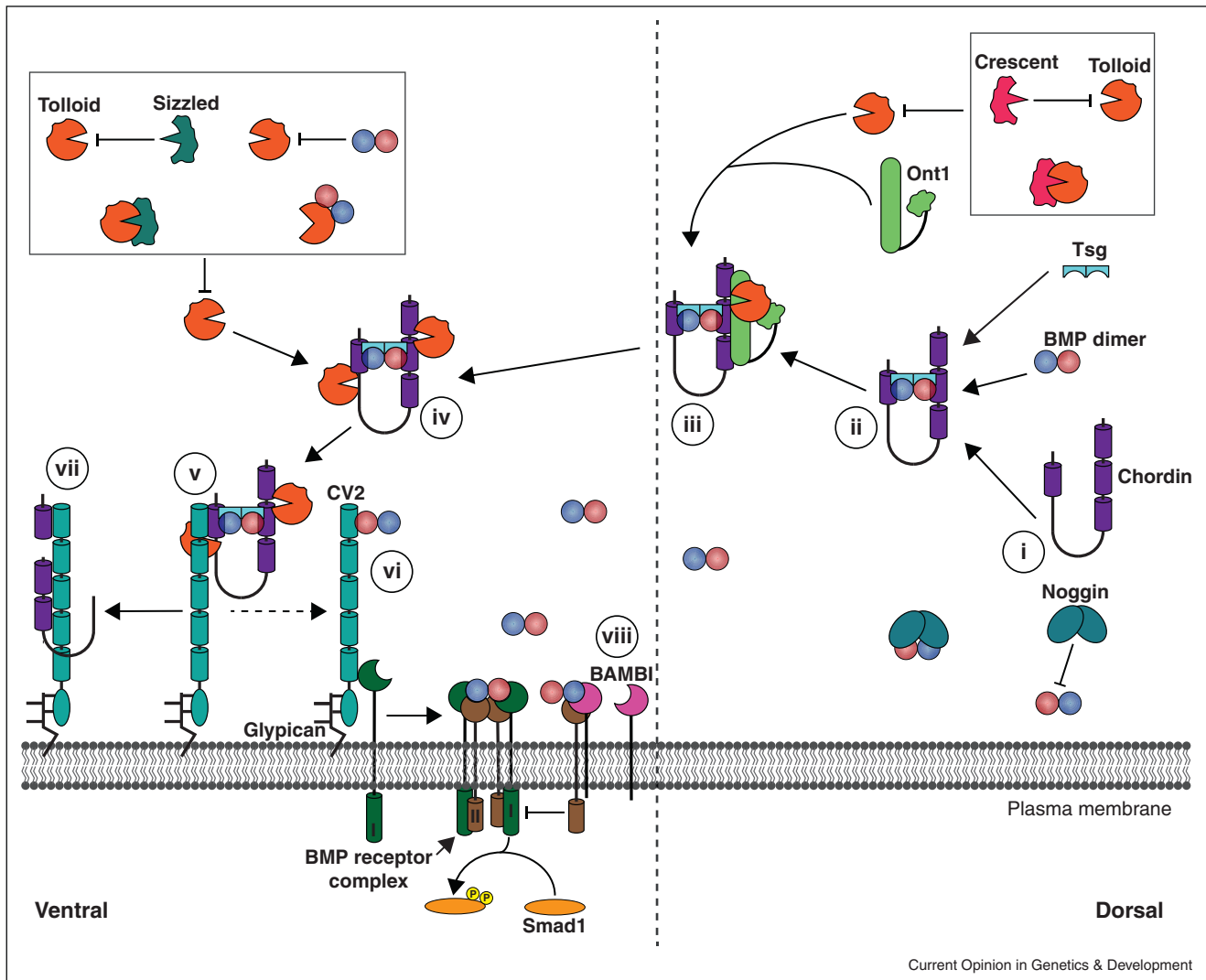
Schematic representation of the biochemical pathway controlling *Xenopus* dorsal–ventral differentiation. Components of the extracellular space are depicted according to their dorsal or ventral localization within the embryo. **(i)** Chordin and Noggin are BMP antagonists secreted by the dorsal center [10–12]. **(ii)** Chordin forms together with Tsg and BMP a trimolecular complex [13]. **(iii)** Ont1 enhances the binding between the substrate Chordin and its proteolytic enzyme Tolloid [26**]. Tolloid activity is competitively inhibited by Sizzled, Crescent, and non-competitively by BMPs (see boxes) [27–29,30**]. **(iv)** The Chd/BMP/Tsg complex flows towards the ventral side, **(v)** where it binds to CV2 that acts as a Chd/BMP sink [21**]. Upon digestion of Chordin by Tolloid [23], **(vi)** CV2-bound BMPs are transferred to the receptor complex that phosphorylates Smad1/5/8 transcription factors. Smad1/5/8 transduce the BMP signal, bind to a co-Smad (Smad4), and translocate to the nucleus to activate transcription [65]. **(vii)** Proteolytic fragments of Chordin also bind CV2 [21**], which presumably clears them by endocytosis [20*]. **(viii)** The pseudo-receptor BAMBI binds BMPs and inhibits formation of the BMP receptor complex [16].

An interesting case is that of the secreted BMP modulator Crossveinless 2 (CV2). CV2 contains Chordin-like Cysteine-Rich (CR) domains that serve as BMP-binding modules [17,18*,19*] (steps 3 and 6 in Figure 1). Although it is a secreted protein, the diffusion of CV2 is restricted to the cells that produce it and a few additional nearby cells, because it binds tightly to cell-surface Heparan Sulfate Proteoglycans (HSPG) such as glypicans [18*] (Figure 1). CV2 displays both pro-BMP and anti-BMP activities depending on its concentration [18*]. At low

concentrations, it can form a transient ternary complex with type I BMP receptors and BMP ligands that promotes the formation of signaling receptor complexes (step 6 in Figure 1). At high concentrations, CV2 sequesters BMP ligands and drives their endocytosis and degradation [20*]. Remarkably, CV2 in the ventral side of the embryo is able to bind with high affinity to Chd/BMP complexes diffusing from the dorsal side [21**]. By acting as a sink concentrating Chd/BMP, CV2 promotes peak BMP signaling on the ventral side of the embryo by

facilitating the release of BMPs from Chordin [21••,22] (steps 5 and 7 in Figure 1).

The second key node of regulation is the cleavage of Chordin by Tolloid Zinc metalloproteinases, which triggers the release of active BMPs from the Chd/BMP complex [23,24] (steps 4 and 5 in Figure 1). Thus, inhibition of BMP by Chordin is reversed by a proteolytic step that constitutes the rate-limiting reaction in D–V patterning [25•]. Consequently, the activity of this protease is exquisitely regulated. Chordin cleavage is enhanced by Ont1, a dorsal secreted protein of the Olfactomedin family, which provides a scaffold that brings the Chordin substrate and the Tolloid enzyme together, facilitating proteolysis [26••] (step 3 in Figure 1). There are three Tolloid enzymes in *Xenopus*, and their activity is negatively regulated by the secreted Frizzled-related proteins Sizzled and Crescent that act as competitive inhibitors of Chordin cleavage [27–29], and by BMPs through a non-competitive inhibition mechanism [30••] (see boxes in Figure 1).

Finally, regulation of BMP transport is crucial to its role as a morphogen. Different modes of morphogen transport have been proposed, including diffusion, transcytosis and cytonemes [31–33]. So far, only diffusion has been shown to occur in vertebrates [34]. In *Drosophila*, where this question has been more thoroughly investigated, extracellular HSPGs [34,35], Collagen IV [36••], and Pentagone/Smoc [37] have been implicated in the regulation of BMP transport. Collagen IV, a component of the basement membrane, offers a particularly interesting example of the molecular control of directional diffusion. Collagen IV binds to the *Drosophila* BMP4 and Chordin homologs, sequestering them in the extracellular matrix. Twisted gastrulation (a protein that binds both to BMP and Chordin/Short gastrulation [13]) serves as a solubilizing agent that releases Chd/BMP complexes from the collagen IV matrix of the perivitelline space, allowing their diffusion [36••]. In *Xenopus*, it has been found that BMP4 has a shorter range of diffusion than that of other TGFβ ligands [38], because the N-terminus of the mature peptide binds heparan sulfate in HSPGs via basic residues [39]. HSPGs are important for the transport of another TGFβ morphogen, Nodal [40•], and regulate BMP signaling during D–V patterning [41–43]. Finally, HSPGs and Collagen IV have also been shown to enhance the binding of BMPs to their plasma membrane receptors [36••,44].

Organizing a self-regulating gradient

Experimental studies on genes regulating the BMP pathway in *Xenopus* have uncovered a biochemical pathway consisting of extracellular proteins, which explains how gradients of BMP activity arise and are robustly maintained in embryonic tissues. In a pleasing convergence of independent lines of evidence, extensive genetic screens

in the zebrafish *Danio rerio* uncovered most of the same genes as mutations specifically affecting embryonic D–V patterning [41,43]. Early work in *Xenopus* showed that BMP4 and BMP7 were expressed on the ventral side of the gastrula (the ventral center) [4], while the BMP antagonists Chordin and Noggin were expressed in the dorsal center and behaved as morphogens that could act over long distances to pattern the embryo [10,45]. These findings suggested that a D–V gradient of BMP activity could be generated simply by these two antagonistic activities generated at opposite poles of the embryo.

As shown in Figure 2, these factors proved to provide only the core of the D–V biochemical network, as many other genes co-expressed in both centers were subsequently uncovered [46]. The two centers are under opposite transcriptional control [47,48], as ventral genes are transcribed

Figure 2

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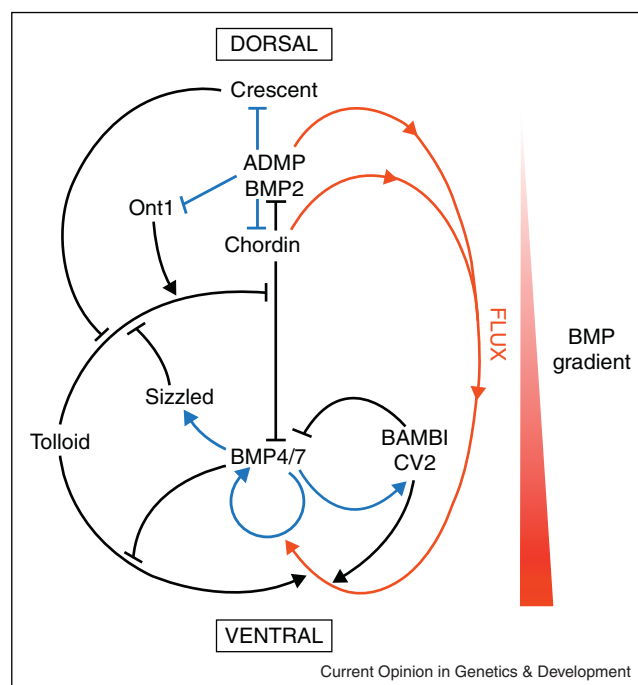
Extracellular network of proteins involved in the self-regulation of *Xenopus* dorsal–ventral patterning. Black arrows indicate protein-protein interactions, blue arrows transcriptional regulations, and red arrows the direction of the flow of Chordin–BMP complexes from the dorsal to the ventral side of the embryo. The resulting BMP activity gradient is indicated on the right. The rate-limiting reaction in this network is that of Tolloid, a Zinc metalloproteinase that cleaves Chordin, releasing BMPs from its inactive complexes [23]. Tolloid activity is regulated by the competitive inhibitors emanating from the dorsal (Crescent [29]) and ventral (Sizzled [27]) centers, as well as by BMP4 that acts as a non-competitive inhibitor of Tolloid on the ventral side [30••]. The behavior of the entire biochemical pathway can be modeled using reaction-diffusion equations. Mathematical analyses have shown that the flow of BMPs from dorsal towards ventral regions provides robustness to the system [56••], and that the Chordin substrate, which is produced in very large amounts, is a major inhibitor of Tolloid activity on the dorsal side [30••].

when BMP signaling is high and dorsal genes when it is low (indicated by blue arrows in Figure 2). Unexpectedly, several of these proteins proved to be feedback inhibitors of the activity of the center they were expressed in, promoting the activity of the opposite center (Figure 2). For example, low BMP signals cause Ont1 expression in the dorsal center, enhancing Chordin degradation [26••], as well as transcriptional activation of two BMP proteins, BMP2 and ADMP. This dampens excessive activation of dorsal center genes, and promotes the expression of ventral center genes at a distance by increasing BMP signaling [47,48]. The ventral center expresses two anti-BMPs, BAMBI and CV2, as well as a competitive inhibitor of Chordin cleavage, Sizzled (Figure 2). For each action of the dorsal side there is a reaction in the ventral center. These negative feedback loops and positive cross-regulations are required for the precise adjustment of the BMP activity gradient, balancing the strength of each center [27,47]. This ensures that each embryo achieves a perfect allocation of tissue types, adjusting to environmental or experimental perturbations [46,47]. One of the best examples is when experimental bisection of a *Xenopus* blastula results in identical twins of perfect proportions; this requires extensive scaling of the size of the self-regulating morphogenetic field [48].

Communication between the two opposing embryonic centers is enhanced by a 'sink' mechanism. Early evidence in *Drosophila* demonstrated that a Tolloid-generated Chordin gradient could produce a directional flow of BMP ligands towards higher BMP concentrations to generate peak levels of signaling [49,50•,51]. It was later found in the frog gastrula that the ventrally expressed protein CV2 could enhance this flow by binding to Chd/BMP complexes with high affinity [21••], inducing release of active BMPs from the complex after cleavage by Tolloid [23]. This generates a molecular sink driving Chd/BMP flow towards the ventral center. The essential role of CV2 in regulating the flow of Chordin over long distances was demonstrated genetically in the mouse vertebral morphogenetic field [52•]. CV2 is normally expressed in the developing vertebral body, in which Chordin protein secreted by the intervertebral disc also accumulates. However, in CV2 mutant mice the relocalization of Chordin to vertebral bodies cannot take place, as shown in Figure 3.

Experimental studies in *Xenopus* have provided estimates of the *in vivo* concentrations of many extracellular factors of the BMP pathway and the chemical reaction rates of their interactions [27,30••]. Together with functional

Figure 3
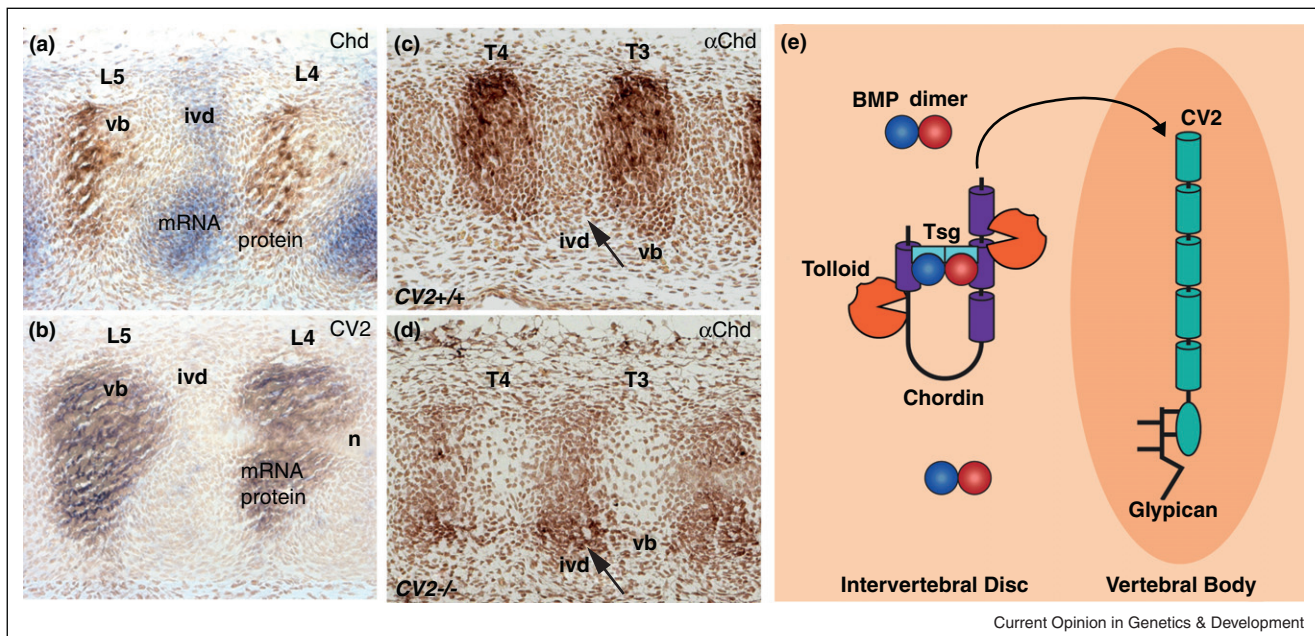
Chordin relocalization in the vertebral field of the mouse embryo requires CV2. **(a)** At 12 days post coitum, *Chd* mRNA is transcribed (blue staining) in the intervertebral disc (ivd) but its protein (brown staining) accumulates in the vertebral body (vb). This indicates a relocalization of Chd protein, whereas **(b)** CV2 transcripts and protein co-localize in the vertebral body (histological sections at the lumbar L4 and L5 level). **(c, d)** Chd accumulation in the vertebral body of wild-type embryos is lost in CV2$^{-/-}$ mutant embryos, in which Chordin is found instead in the intervertebral disc (arrow). This shows that CV2 is required for the relocalization of Chd protein from the intervertebral disc, where it is transcribed, to the vertebral body (sections at the thoracic T3 and T4 level). **(e)** Schematic model of the developing vertebral field. CV2 is retained in the vertebral body through his interaction with Heparan Sulfate Proteoglycans (such as Glypican) and acts as a sink for Chd/BMP complexes produced in the intervertebral disc. Upon cleavage of Chd by Tolloid metalloproteinases, BMPs are released in the vertebral body producing the high-BMP signals required for cartilage differentiation. Adapted from [52•], with permission.

analyses of the expression domains and transcriptional regulation of these BMP regulators, this has paved the way for computational modeling of the D–V patterning network.

Insights from modeling the BMP gradient

Mathematical and computational modeling has provided insights into the function of each component and the global behavior of the system. Intuition alone is insufficient to directly understand how such a complex network with its many negative and positive feedback loops (Figure 2) generates robust and reproducible pattern. Mathematical modeling of biological gradients was pioneered by Turing [53••], who coined the term 'Morphogen' in 1952, and extended by Meinhardt [54,55]. They introduced partial differential equations as mathematical tools to model systems of diffusing morphogens through elaborations of the following basic equation:

$$\frac{\partial C}{\partial t} = D \cdot \nabla^2 C + \text{reactions}\,(C)$$

Variations in morphogen concentration in the embryo over time ($\partial C/\partial t$, first derivative with respect to time) are driven by Fick's law of diffusion ($D \cdot \nabla^2 C$, where D indicates diffusion rate and $\nabla^2 C$ the second derivative of morphogen concentration with respect to space) and the chemical 'reactions' that lead to the formation/degradation of the morphogen species (C). These reaction-diffusion equations have shown that an activator and inhibitor pair (such as ADMP and Chordin) diffusing from the same source can generate stable patterns, provided that the inhibitor diffuses faster [54,55].

With knowledge of the biochemical affinities of the system, it has become possible to use these equations to conduct extensive quantitative simulations. Modeling predicted that a requirement for robust D–V patterning was the existence of a Chordin-driven flow of BMPs towards high concentrations of that same morphogen, both in *Drosophila* [49] and in *Xenopus* [56••]. The flow of BMP ligands towards the ventral side can be visualized indirectly in BMP-depleted *Xenopus* embryos, in which a wild-type organizer graft expressing BMP ligands (*ADMP and BMP2*) can restore BMP-dependent epidermal differentiation, but only at a long distance from the BMP source where they are released from inactive complexes by the action of Tolloid, as shown in Figure 4. The flow of dorsal BMPs towards higher concentrations seems to contradict Fick's law of diffusion, but the maintenance of a steady Chordin concentration gradient through its degradation by the protease Tolloid provides the chemical work that drives this facilitated diffusion. These mathematical predictions were verified experimentally in the *Drosophila* and *Xenopus* systems [49,56••].

Computational analyses have also confirmed the importance of feedback and cross-regulatory loops in ensuring

Figure 4
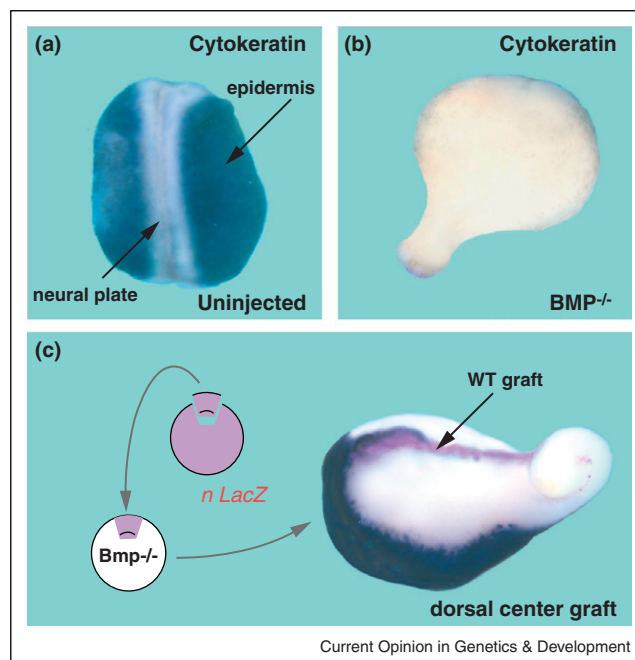
The frog dorsal center can induce epidermal differentiation at a distance. **(a)** *Cytokeratin* mRNA marks the epidermis, a region of high BMP signaling in the *Xenopus* embryo at neural plate stage. **(b)** When four BMPs are depleted by knocking down simultaneously BMP2/4/7 and ADMP with antisense Morpholino oligonucleotides, the entire ectoderm is converted to central nervous system tissue as indicated by the absence of *Cytokeratin* mRNA staining. **(c)** Transplantation of a wild-type (WT) dorsal center into an embryo depleted of BMPs restores dorsal–ventral patterning and epidermal differentiation at a distance. The transplanted wild-type tissue, which was labeled with the lineage tracer nuclear-LacZ, gives rise to notochord. These experiments show that the dorsal center is a source of BMP ligands that are transported and induce BMP signaling at a distance.
Adapted from [47], with permission.

the robustness of the gradient [55–57]. The study by Ben-Zvi *et al.* [56••] showed that the function of ADMP is to maintain a low but steady level of BMP signaling in the dorsal side and to promote at a distance peak levels of BMP signaling in the ventral center, in agreement with experimental embryology studies [47]. In addition, the ventral feedback regulator BAMBI helps cells to reliably sense a broad range of BMP concentration by linearizing the response to BMP signaling, avoiding immediate saturation and reducing variability in D–V patterning [58••]. Computational modeling also revealed that while the competitive inhibitor Sizzled and the non-competitive inhibitor BMP are feedback inhibitors of the activity of Tolloid in the ventral center, a main inhibitor of activity of this protease on the dorsal side is its own substrate, Chordin, which is produced very abundantly [30••].

Embryos are increasingly recognized as systems that need to meet multiple and often contradictory performance objectives in order to develop into functional and

well-proportioned animals, bearing parallels with arti-facts designed by engineers [59]. Therefore, concepts developed for the control of engineered systems are becoming increasingly useful in understanding the mechanics of developmental networks. Indeed, Ben-Zvi and Barkai [60] have shown that the D–V network is topologically analogous to an error-correcting integral-feedback controller that adjusts morphogen activity to match the size of the embryo.

Perspectives

We have come a long way since the discovery of the embryonic dorsal organizer by Hans Spemann and Hilde Mangold in 1924 [61]. Deciphering the molecular blue-print of self-regulating morphogenetic fields is now possible. Studies in *Xenopus* have revealed the biochemical interactions of many components of a novel extracellular pathway of BMP signaling regulation and discovered the principles that explain how they generate a self-regulating D–V morphogen gradient. An area still underexplored in the vertebrates is the role of proteoglycans and the extra-cellular matrix in the control of morphogen diffusion, which has already been more extensively studied in *Dro-sophila* [34–37]. Moreover, to gain further understanding of the vertebrate D–V pathway we need to go beyond bio-chemistry and genetics and measure the localization of its proteins as has been done in *Drosophila*, where it has enabled the elaboration of detailed and testable quanti-tative models of patterning [62,63].

Finally, our understanding of how the Chordin/BMP network is integrated both at the intracellular and extra-cellular level with other signaling pathways, transcrip-tional programs, and morphogenetic movements that specify positional information in the embryo is still a very active field of investigation [64]. Beyond D–V cell differ-entiation, the BMP pathway also plays essential roles in the regulation of many other organ-forming fields [48,52•]. It remains to be tested how the knowledge acquired from *Xenopus* dorso-ventral patterning translates to organogenesis.

Acknowledgements

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- •• of outstanding interest

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