CHAPTER THIRTEEN

The Chordin Morphogenetic Pathway

Edward M. De Robertis[1], **Yuki Moriyama**
Howard Hughes Medical Institute and Department of Biological Chemistry, University of California, Los Angeles, California, USA
[1]Corresponding author: e-mail address: ederobertis@mednet.ucla.edu

Contents

Abstract

The ancestral Chordin/bone morphogenetic protein (BMP) signaling pathway that establishes dorsal–ventral (D–V) patterning in animal development is one of the best understood morphogenetic gradients, and is established by multiple proteins that interact with each other in the extracellular space—including several BMPs, Chordin, Tolloid, Ont-1, Crossveinless-2, and Sizzled. The D–V gradient is adjusted redundantly by regulating the synthesis of its components, by direct protein–protein interactions between morphogens, and by long-range diffusion. The entire embryo participates in maintaining the D–V BMP gradient, so that for each action in the dorsal side there is a reaction in the ventral side. A gradient of Chordin is formed in the extracellular matrix that separates ectoderm from endomesoderm, called Brachet's cleft in *Xenopus*. The Chordin/BMP pathway is self-organizing and able to scale pattern in the dorsal half of bisected embryos or in Spemann dorsal lip transplantation experiments.

1. INTRODUCTION

A central question in developmental biology is how a perfectly patterned embryo consisting of many differentiated tissues is reliably generated time after time. A key experiment for understanding embryonic patterning

Current Topics in Developmental Biology, Volume 116
ISSN 0070-2153
http://dx.doi.org/10.1016/bs.ctdb.2015.10.003

was performed by Spemann's graduate student Hilde Mangold, who transplanted a small region of the amphibian gastrula called the dorsal lip of the blastopore into the ventral side of a host embryo (Spemann & Mangold, 1924). The blastopore is the region through which the cells that will form the endoderm and mesoderm layers of the embryo invaginate. The cells that remain on the outside form the third germ layer, the ectoderm. A small graft of this organizer region has a very potent effect on the recipient embryo, dividing the pattern of the whole into two Siamese twins. This experiment established that the dorsal side of the embryo had inductive abilities and that organizer tissue played a crucial role in self-regulation. The mechanism of this phenomenon would have to wait for many decades until gene cloning made molecular embryology a practical possibility. This chapter relates how a biochemical pathway that mediates embryonic induction was elucidated through the testing of a series of successive hypotheses suggested by experiments.

2. A GRADIENT OF GROWTH FACTOR SIGNALING

D–V patterning results from a gradient of activity of a family of secreted growth factors called bone morphogenetic proteins (BMPs). The first correlation between D–V tissue differentiation and BMPs came from the study of *Drosophila* mutations in a gene called decapentaplegic (*Dpp*), which is the homolog of vertebrate BMP2/4 (Ferguson & Anderson, 1992). In mammals, BMPs had a long history of being involved in bone differentiation. It was first noted that bone fragments transplanted subcutaneously or intramuscularly in rabbits could induce bone differentiation even after all cells had been killed with ethanol (Levander, 1938). Marshall Urist, an orthopedic surgeon working at UCLA, found that the proteinaceous bone extracellular matrix (ECM) (obtained by removing calcium from bones by soaking them in hydrochloric acid for several days), had potent ectopic bone morphogenetic activity after transplantation into rabbits or rats (Urist, 1965).The active proteins were purified and cloned by Genetics Institute and found to correspond to growth factors designated BMP2 to BMP7 (Wozney et al., 1988).

In zebrafish and *Xenopus* embryos, a gradient of BMP signaling activity spanning the entire embryo can be visualized indirectly, by staining embryos with an antibody specifically directed against the phosphorylated form of the Smad1 transcription factor. As shown in Fig. 1A and B, BMP signaling

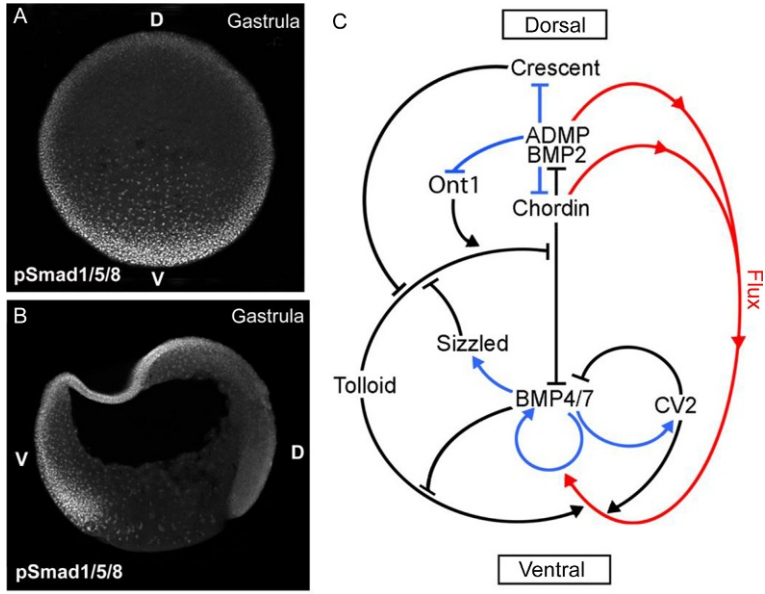

Figure 1 A gradient of nuclear phospho-Smad1 is established by a biochemical network of secreted proteins that interact with each other. (A) Horizontal optical section at gastrula (stage 11) showing a ventral to dorsal gradient of BMP activity using pSmad1/5/8 antibody as the readout. D, dorsal; V, ventral. (B) Gastrula (stage 11) embryo sectioned sagittally, showing higher BMP signaling in ventral nuclei. (C) The D–V morphogenetic signaling pathway results from a series of direct protein–protein interactions between Chordin and other partners (black arrows), transcriptional regulation (blue arrows), and protein flux (red arrows). The entire embryo participates in forming the BMP gradient, which results from the dueling activities of the dorsal and ventral signaling centers. *Images from Plouhinec, Zakin, Moriyama, and De Robertis (2013).*

activity is lowest on the dorsal side and gradually increases toward the ventral side, in which cell nuclei accumulate higher amounts of phospho-Smad1.

3. THE CLONING OF CHORDIN

When molecular biology, the great equalizer of modern biology, became practical, the search for the signals produced by the dorsal organizer tissue that mediate embryonic induction was on. In our laboratory, a gene library from manually dissected dorsal blastopore lips from *Xenopus* was prepared. We succeeded in isolating a homeobox gene designated *goosecoid* (Cho, Blumberg, Steinbeisser, & De Robertis, 1991). This gene allowed us, for the first time, to visualize Spemann's organizer using *in situ*

hybridizations to *goosecoid* mRNA. Previously, the existence of organizer tissue had to be deduced from its effects after transplantation experiments. Since Goosecoid is a transcription factor, we hypothesized that it might regulate a secreted target gene. In 1994, we isolated *chordin*, a gene activated by *goosecoid*, from our *Xenopus* organizer library (Sasai et al., 1994). Richard Harland had previously isolated *noggin* (Smith & Harland, 1992) and Douglas Melton *follistatin* (Hemmati-Brivanlou, Kelly, & Melton, 1994). We later found that all three gene products acted by antagonizing BMP signaling (Sasai, Lu, Steinbeisser, & De Robertis, 1995). The dorsal side of the gastrula embryo secretes BMP antagonists and the ventral side BMP4 and BMP7. Spemann's organizer proved a fertile fishing ground for novel molecules. Our initial hypothesis had been that dorsal tissue would be the source of novel growth factors but what was found instead was that it secreted a large number of secreted growth factor inhibitors (reviewed in De Robertis & Kuroda, 2004).

Intriguingly, BMP signaling levels are able to regulate cell differentiation in the ectoderm, mesoderm, and endoderm layers simultaneously. Explants of future ectoderm (cells from the animal cap of the embryo) differentiate into CNS at low BMP levels (e.g., in the presence of BMP antagonists such as Chordin, Noggin, or Follistatin) and into epidermis when BMP signaling levels are high. Similarly, in the mesoderm, low BMP gives rise to notochord, at slightly higher levels skeletal muscle (arranged in somites), then kidney (each embryonic segment develops a kidney tubule), then lateral plate (which gives rise to the body wall), and at the highest BMP levels blood islands. These histotypic differentiations represent the invariant body plan shared by all vertebrates. This raises the question of how the D–V development of the different germ layers is integrated. Is there a dedicated BMP gradient for each germ layer? How is each gradient regulated coordinately so that a perfectly harmonious embryo is formed each time? How is the BMP gradient regulated to ensure that each embryo is able to allocate these tissues perfectly?

While we isolated several growth factor antagonists secreted by Spemann's organizer cells, Chordin proved to be the most informative for the regulation of the D–V signaling gradient; it is at the heart of the organizer phenomenon. When Chordin mRNA is microinjected into a ventral cell, it recapitulates the organizer experiment, forming a second neural tube, somites, and even a second gut cavity (Sasai et al., 1994). Chordin can be depleted in *Xenopus* embryos using microinjected antisense morpholino oligos (MOs). Depletion of Chordin produced embryos that developed with

smaller heads and dorsal tissues and expanded ventral structures (Oelgeschläger, Kuroda, Reversade, & Robertis, 2003). However, an embryonic axis was still formed. Later, Harland found that the combined depletion of Chordin, Noggin, and Follistatin led to a catastrophic loss of all dorsal tissues (Khokha, Yeh, Grammer, & Harland, 2005). Similarly, in the mouse double knock-out of Chordin and Noggin, the forebrain, midbrain, and notochord are lost (Bachiller et al., 2000). Thus, the BMP antagonists secreted by organizer tissue are able to compensate for each other.

Chordin is essential for the activity of Spemann organizer grafts in *Xenopus*. When the organizer was depleted of Chordin, it remained as a patch of epidermis completely devoid of inducing activity (Oelgeschläger et al., 2003). Transplantation is a very powerful tool in biology. In this case, when dorsal cells were challenged by placing them in a new ventral surrounding their diminished biological capacity due to the loss of the Chordin gene product was strikingly revealed.

We realized very early on that Chordin had to be a very important molecule. A gene of similar sequence had been cloned in the fruit fly *Drosophila* called *short gastrulation* (*sog*) (Francois, Solloway, O'Neill, Emery, & Bier, 1994). We collaborated with F. Michael Hoffman and Edwin "Chip" Ferguson, who had independently cloned *sog*, to show that microinjected *chordin* and *sog* mRNA induced neural tissue both in *Drosophila* and frog embryos (Holley et al., 1995). This led to the realization that we had discovered an ancient D–V patterning molecule that had been conserved during evolution between fruit fly and amphibian embryos (reviewed in Bier & De Robertis, 2015; De Robertis, 2008). Importantly, the phenotypic effects of *sog* mutations were known to be enhanced when the dosage of *dpp*/BMP4 genes was increased in *Drosophila* (Ferguson & Anderson, 1992). This led us to investigate the fruitful working hypothesis that Chordin and BMPs worked on a common signaling pathway. Biochemical experiments showed that Chordin encodes a large protein containing four Cysteine-rich domains that serve as BMP-binding modules (Piccolo, Sasai, Lu, & De Robertis, 1996). Chordin has a cofactor called Twisted gastrulation (Tsg) that helps keep BMPs soluble and binds to both Chordin and BMPs (Little & Mullins, 2004; Oelgeschläger, Larraín, Geissert, & De Robertis, 2000). The ternary complex of Chordin, BMPs, and Tsg prevents binding of BMPs to BMP receptors on the cell surface, explaining why Chordin inhibits BMP signaling.

Chordin mRNA is expressed at high levels in dorsal cells in the exact region that has embryonic inducing activity after transplantation. We measured the amount of Chordin protein secreted by the frog embryo during

gastrulation and found that it is produced in prodigious amounts. If distributed uniformly in the extracellular space, Chordin protein would reach concentrations of 33 nM (Lee, Ambrosio, Reversade, & De Robertis, 2006). In the dorsal side, where it is produced, Chordin must reach much higher concentrations. BMP concentrations have not been measured in embryos, but in other tissues, they are active in the picomolar (pM) range. Thus, a vast excess of Chordin is present during early development.

Since Chordin is a BMP antagonist, in principle, its localized expression in the dorsal side could be sufficient to account for the BMP signaling gradient, even if BMP expression were uniform. Using systematic MO knockdown and transplantation experiments, we discovered that Chordin is part of a biochemical network of extracellular proteins that involves the entire embryo (Fig. 1C). It eventually became clear that the organizer effect is not due only to the action of the dorsal side but also to the reaction of ventral cells (Reversade & De Robertis, 2005). The embryo has a dorsal and a ventral center that communicate through secreted proteins that interact with each other. Dorsal genes are expressed when BMP levels are low, while ventral genes are expressed when BMP levels are high (Fig. 1C). The two signaling centers self-regulate after changes in signaling because all the components of the system are under opposing transcriptional control by BMP.

4. FUNCTION OF TOLLOID, SIZZLED, AND CROSSVEINLESS 2

Purified Chordin binds BMPs avidly, with an affinity (dissociation constant, K_D) in the low nM range (Piccolo et al., 1996). However, the inhibitory action of Chordin can be reversed by proteolysis of Chordin by metalloproteinases of the Tolloid family. Tolloid was identified in the classical *Drosophila* genetic screen (Nüsslein-Volhard & Wieschaus, 1980) as a gene that increased Dpp/BMP signaling. We hypothesized that Tolloid might act by inactivating the BMP antagonist Chordin/Sog and were able to show by biochemical experiments that the metalloproteinase Xolloid-related (Xlr) cleaved Chordin at two distinct sites (Piccolo et al., 1997). When this happened, the affinity of the cleaved Chordin for BMP decreased precipitously and the complex of BMP and Tsg was able to bind to BMP receptors, restoring signaling in *Xenopus* explants. Because Tolloid/Xlr is expressed in the ventral side, it serves as a ventral sink that degrades Chordin originating from Spemann's organizer, allowing the flux of BMPs from

more dorsal regions to the ventral side in which BMP signaling is maximal (Fig. 1C). Activity of the Tolloid protease is the rate-limiting step in D–V patterning and is subjected to stringent regulation.

Sizzled is a ventral center molecule that is expressed when BMP signaling levels are high (Collavin & Kirschner, 2003). In zebrafish, the *sizzled* mutation (called *ogon/mercedes*) had a phenotype intriguingly similar to that of Chordin mutants (Yabe et al., 2003). We microinjected *sizzled* mRNA into dorsal or ventral half-embryos in *Xenopus* and noted that it had strong dorsalizing effects on dorsal fragments (expansion of the CNS) but none at all in ventral fragments. Thus, *sizzled* function required a dorsal component, giving rise to the working hypothesis that it might inhibit the degradation of Chordin by Tolloid (Lee et al., 2006).

Sizzled encodes a secreted Frizzled-related protein (sFRP). This class of protein is normally involved in inhibiting Wnt signaling. However, in the case of Sizzled, the protein has evolved so that it is able to bind to the Tolloid proteolytic site, but is unable to be cleaved by this enzyme. In this way, Sizzled acts as a competitive inhibitor of Tolloid (Lee et al., 2006). Sizzled depletion by antisense morpholinos results in the same phenotype as Chordin loss-of-function, because in its absence the activity of Tolloid increases and Chordin is degraded. When overexpressed, Sizzled has anti-BMP effects because it inhibits the Tolloid proteinase, leading to the accumulation of Chordin and inhibition of BMP signaling. On the dorsal side of the embryo, another sFRP called Crescent also serves as a Tolloid inhibitor, but under the opposite transcriptional regulation (Ploper, Lee, & De Robertis, 2011; Fig. 1C).

Tolloid activity is also regulated by direct binding of BMP to protein domains located outside of its catalytic region. When BMP levels become high, BMPs bind to domains in Tolloid called CUB domains, inhibiting enzyme activity in a noncompetitive fashion (Lee, Mendes, Plouhinec, & De Robertis, 2009). This new negative feedback loop provided a molecular explanation for an old mystery in the field. Surprisingly, when the first peptide sequences were obtained from extracts with bone-inducing activity the first protein identified (Wozney et al., 1988), designated BMP1, had the sequence of a Tolloid enzyme containing three CUB domains. The reason for the copurification of BMP1 together with the BMP2 to 7 growth factors now had a simple explanation: Tolloids are BMP-binding proteins. This work showed that Tolloid activity is highly regulated and plays a key role in the communication between the dorsal and ventral sides of the embryo.

Ont-1, discovered in the Sasai laboratory, is a secreted protein member of the olfactomedin family that is expressed dorsally where BMP signaling levels are low (Inomata, Haraguchi, & Sasai, 2008). It serves as an adaptor that binds to Tolloid (via its coil–coil domain) and to Chordin (through its olfactomedin domain), facilitating Chordin proteolysis (Fig. 1C).

Another component of the Chordin/BMP pathway is Crossveinless 2 (CV2) which was first described in the *Drosophila* wing (Conley et al., 2000), where it helps reach the maximal BMP signaling required for formation of cross vein structures (Blair, 2007). CV2 has Chordin-like BMP-binding modules. CV2 expression is activated by high BMP signaling in the ventral side of *Xenopus* and is therefore a ventral center protein. When both Chordin and CV2 are depleted, ventral tissues are synergistically expanded (Ambrosio et al., 2008). Although CV2 is a secreted protein, it is unable to diffuse through the extracellular space because it remains anchored by glycosylated proteins (called glypicans) to the surface of the cells that secrete it (Serpe et al., 2008). Using biochemistry with purified proteins and embryo microinjection experiments, we found the mechanism of the CV2 pro-BMP activity. CV2 binds with considerable affinity (dissociation constant 1.4 nM) to Chordin and with even higher affinity to Chordin/BMP complexes (Ambrosio et al., 2008). Thus, in the frog embryo CV2 acts both as a BMP antagonist and as a molecular sink concentrating Chordin/BMP complexes in the ventral side (Fig. 1C). BMP signaling is boosted on the ventral side by the combined action of CV2 and Tolloid, which facilitate the flux of BMPs produced in more dorsal regions of the embryo and transported by Chordin (Ambrosio et al., 2008).

Extensive genetic screens in the zebrafish *Danio rerio* by the group of Christiane Nüsslein-Volhard provided strong support for the biochemical D–V pathway discovered in *Xenopus*. In a striking convergence, zebrafish loss-of-function mutations that specifically affected the allocation of dorsal–ventral tissues all involved genes in the Chordin/BMP pathway. Loss-of-function mutations that increased ventral tissues corresponded to *chordin* (called *chordino* in zebrafish) and *sizzled* (*ogon/mercedes*), while mutations increasing dorsal tissues corresponded to *bmp7* (*snailhouse*), *bmp2b* (*swirl*), the type I BMP receptor *alk8* (*lost-a-fin*), *smad5* (*somitabun*), and *tolloid* (*mini fin*) (Langdon & Mullins, 2011; Little & Mullins, 2006). These genetic experiments demonstrated that a common D–V patterning mechanism was utilized by vertebrate embryos.

Starting with the isolation of Chordin, we were able to identify, one at the time, multiple components that interact with each other in a

morphogenetic biochemical pathway that comprises the entire embryo. At the gastrula stage being studied, the embryo is composed of 10,000 cells, raising the question of how the information in the dorsal and ventral centers is transmitted long-range to produce a self-organizing morphogen gradient in different germ layers. We discuss this next.

5. LONG-RANGE DIFFUSION OF CHORDIN IN BRACHET'S CLEFT

We have recently been able to visualize endogenous Chordin protein by immunohistochemistry in the *Xenopus* gastrula (Plouhinec et al., 2013). *Chordin* mRNA is transcribed in an arc of 60° of the dorsal side. However, as shown in Fig. 2A, Chordin protein was present as a gradient extending much further than its mRNA expression domain, spanning from the dorsal side to the ventral-most part of the stage 12 gastrula. The staining was specific because it was eliminated by depletion of Chordin. Unexpectedly, Chordin did not diffuse randomly through the intercellular space but instead was greatly concentrated specifically in the extracellular space that separates the ectoderm from the endomesoderm. In amphibian embryos, this virtual cavity is called Brachet's cleft (in honor of Albert Brachet, the famous

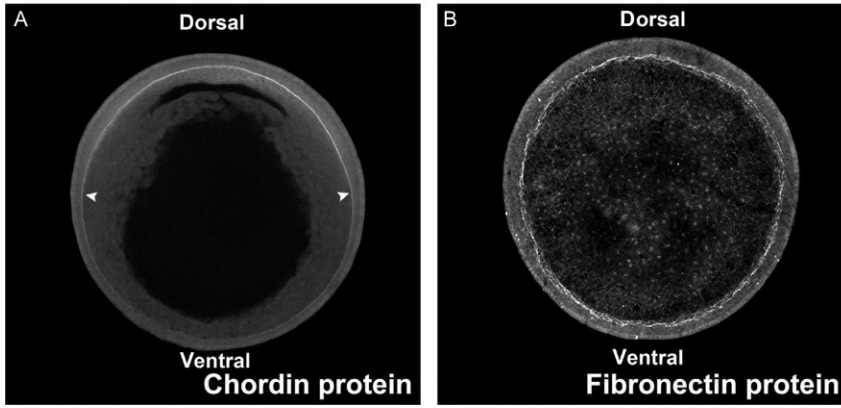

Figure 2 A gradient of endogenous Chordin protein in the extracellular matrix of Brachet's cleft. (A) Visualization of the dorsal to ventral gradient of endogenous Chordin protein in Brachet's cleft (arrowheads). The *Xenopus* embryo has a diameter of 1.3 mm and therefore the half-circumference of Brachet's cleft extends over a range of 2 mm. (B) Fibronectin is abundant in the Brachet's cleft ECM, but is uniformly distributed. *Panel A from Plouhinec et al. (2013). Panel B courtesy of Lise Zakin.*

Belgian embryologist). Brachet's cleft is not an amphibian-specific structure. All vertebrate embryos have an ECM-containing Fibronectin and other proteins separating ectoderm and endomesoderm. Chordin protein forms a D–V gradient in this signaling highway (Fig. 2A, arrowheads), while Fibronectin is uniformly distributed in this ECM (Fig. 2B).

It appears that the embryo may have a single gradient of Chordin that can pattern all three germ layers at the gastrula stage. This provides a simple solution to the question of how many BMP gradients exist: only one. From Brachet's cleft, Chordin/BMP can diffuse into both the ectoderm and the endomesoderm. As mentioned earlier, Chordin reaches high concentrations in the embryo. The fact that it diffuses through a narrow region suggests that Chordin, and perhaps the other components of the D–V patterning pathway, reach very high concentrations in the ECM between ectoderm and endomesoderm. During gastrulation, the germ layers undergo extensive morphogenetic movements; it is possible that cells read the Chordin/BMP gradient contained in this ECM as they move along its surface. Within this narrow cleft, Chordin behaves *in vivo* as predicted by our biochemical pathway in experiments using Tolloid and Sizzled antisense MOs (Plouhinec et al., 2013). We propose that the Chordin gradient is established and maintained by facilitated diffusion in the ECM and driven by the Tolloid protease. This study allowed the visualization, for the first time, of an endogenous gradient of a morphogen protein in a vertebrate embryo.

To demonstrate directly the long-range diffusion of the endogenous Chordin protein in the gastrula embryo, we performed Spemann transplants of lineage-traced dorsal lip tissue (Fig. 3A), which revealed a second gradient of Chordin protein originating from the graft and diffusing for considerable distances along Brachet's cleft (Fig. 3B, arrowheads). In an important precedent, it had been shown that overexpressed Nodal protein diffused along the ECM that separates the lateral plate mesoderm from ectoderm during left–right patterning in tailbud *Xenopus* embryos (Marjoram & Wright, 2011). It is possible that diffusion of morphogens along ECM separating cell layers may constitute a general phenomenon in long-range growth factor signaling.

The most intriguing property of the D–V patterning system is its ability to self-organize. After bisection in the D–V plane, the Chordin gradient in Brachet's cleft regenerated in dorsal halves, but not in the ventral ones (Plouhinec et al., 2013). We are currently investigating the regeneration of morphogenesis in half-embryos after sagittal bisections.

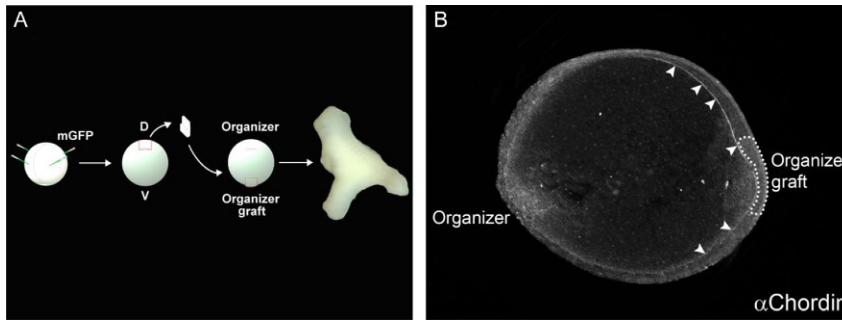

Figure 3 A long-range gradient of Chordin protein emanating from transplanted organizer tissue. (A) Diagram of the experimental procedure; membrane-targeted *mGFP* was injected in the donor embryo (four injections at the four-cell stage) to trace the lineage of grafted Spemann organizer tissue. (B) Embryo transplanted at stage 10 and fixed at stage 12 stained with anti-Chordin antibody shown in optical transverse section. A second gradient of Chordin was observed with Chordin protein staining in Brachet's cleft extending a considerable distance from the graft (arrowheads). The GFP fluorescence of the transplanted organizer cells is outlined by the dotted line. Chordin protein diffuses long-distance from the graft through Brachet's cleft ECM. *Data from Plouhinec et al. (2013)*.

6. THE ANCESTRAL CHORDIN/BMP MORPHOGENETIC PATHWAY

The Chordin/BMP pathway is ancestral and conserved in bilateral animals as diverse as *Drosophila*, spiders, amphioxus, hemichordates, sea urchins, and vertebrates (Bier & De Robertis, 2015). We proposed that the remarkable conservation of the Hox genes (Carrasco, McGinnis, Gehring, & De Robertis, 1984) and of the BMP/Dpp, and Chordin/Sog systems between *Drosophila* and *Xenopus* suggested that these patterning systems were present in *Urbilateria*, the common ancestor of all bilateral animals (De Robertis & Sasai, 1996). Recently, a remarkable study from the laboratory of Ulrich Technau has revealed that the Chordin morphogenetic network of genes is more ancient than previously thought, as it is present, although in modified form, in cnidarians (Fig. 4).

The sea anemone *Nematostella vectensis* is a diploblast with radial symmetry. During embryogenesis, however, it has traces of bilateral symmetry and a so-called directive axis in which nuclear pSmad1 forms a gradient of BMP signaling. *Chordin* is expressed where BMP signaling is low and coincides with the expression of *Nematostella Dpp* and *BMP5-8* (Fig. 4;

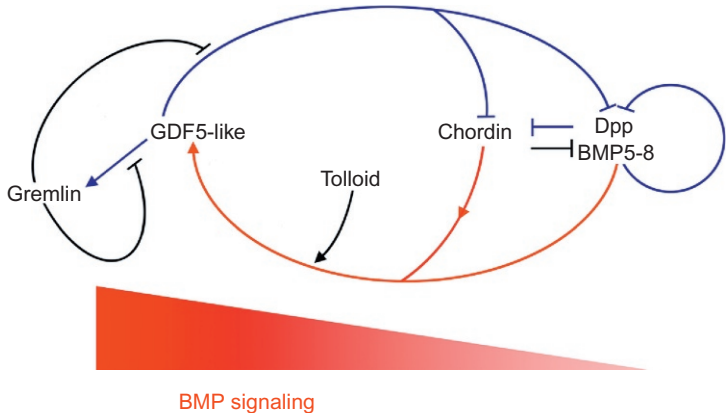

Figure 4 The directive axis of the cnidarian *Nematostella vectensis* is established by a Chordin/BMP gradient. As in *Xenopus*, BMP activity along the body axis results from a series of direct protein–protein interactions between Chordin and other protein partners (black arrows), transcriptional regulation (blue arrows), and protein flux (red arrows) in *Nematostella*. *Diagram based on Genikhovich et al. (2015).*

Genikhovich et al., 2015). Similarly, in *Xenopus*, the dorsal side expresses *Chordin*, *BMP2*, and *ADMP* (ADMP stands for the divergent BMP anti-dorsalizing morphogenetic protein; Moos, Wang, & Krinks, 1995). On the high BMP signaling side, the BMP *GDF5-like* (*growth and differentiation factor* 5) gene is expressed in *Nematostella*, while in *Xenopus* the ventral center expresses BMP4/7. One important difference is that the high BMP side expresses the BMP antagonist Gremlin (Fig. 4). Gremlin is a member of the Cerberus/DAN family of secreted growth factor antagonists first cloned by Harland in *Xenopus*, in which it is not known to play a role during gastrulation (Hsu, Economides, Wang, Eimon, & Harland, 1998). Sizzled, which plays a very prominent role in the *Xenopus* and zebrafish ventral gastrula centers, is not present in *Nematostella* or, to our knowledge, in any invertebrate. Knockdown experiments in sea anemone embryos showed that Chordin expression is the main determinant of where the low BMP side is formed, and mathematical modeling showed that Tolloid and Chordin are the rate-limiting elements in establishing the BMP gradient (Genikhovich et al., 2015). Different animals use variations of the ancestral BMP/Chordin/Tolloid D–V module. In the case of placental mammals, the *ADMP, crescent,* and *sizzled* genes have been lost, probably concomitantly with the loss of yolk in the egg, since these genes play a prominent role in the chick embryo (Ploper et al., 2011).

In conclusion, molecular studies on the Spemann organizer together with biochemical analyses and cell transplantation studies over a period of 20 years led to the formulation of successive working hypotheses that allowed the systematic discovery of a network of extracellular proteins that interact with each other in *Xenopus*. This biochemical pathway forms a morphogenetic field that encompasses the entire embryo and has been utilized since the time of our early animal ancestors to generate long-range gradients of positional information.

ACKNOWLEDGMENTS

We thank the many generations of postdocs and students that elucidated the Chordin pathway, in particular the late Yoshiki Sasai who first isolated Chordin. This work was supported by the Howard Hughes Medical Institute, of which Y.M. is an associate and E. D.R. is an investigator.

REFERENCES

Ambrosio, A. L., Taelman, V. F., Lee, H. X., Metzinger, C. A., Coffinier, C., & De Robertis, E. M. (2008). Crossveinless-2 is a BMP feedback inhibitor that binds Chordin/BMP to regulate *Xenopus* embryonic patterning. *Developmental Cell*, *15*, 248–260.

Bachiller, D., Klingensmith, J., Kemp, C., Belo, J. A., Anderson, R. M., May, S. R., et al. (2000). The organizer secreted factors Chordin and Noggin are required for forebrain development in the mouse. *Nature*, *403*, 658–661.

Bier, E., & De Robertis, E. M. (2015). BMP gradients: A paradigm for morphogen-mediated developmental patterning. *Science*, *348*, aaa5838.

Blair, S. (2007). Wing vein patterning in Drosophila and the analysis of intercellular signaling. *Annual Review of Cell and Developmental Biology*, *23*, 293–319.

Carrasco, A. E., McGinnis, W., Gehring, W. J., & De Robertis, E. M. (1984). Cloning of an X. laevis gene expressed during early embryogenesis coding for a peptide region homologous to Drosophila homeotic genes. *Cell*, *37*, 409–414.

Cho, K. W. Y., Blumberg, B., Steinbeisser, H., & De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: The role of the *Xenopus* homeobox gene *goosecoid*. *Cell*, *67*, 1111–1120.

Collavin, L., & Kirschner, M. W. (2003). The secreted Frizzled-related protein Sizzled functions as a negative feedback regulator of extreme ventral mesoderm. *Development*, *130*, 805–816.

Conley, C. A., Silburn, R., Singer, M. A., Ralston, A., Rohwer-Nutter, D., Olson, D. J., et al. (2000). Crossveinless 2 contains cysteine-rich domains and is required for high levels of BMP-like activity during the formation of the cross veins in Drosophila. *Development*, *127*, 3947–3959.

De Robertis, E. M. (2008). Evo-devo: Variations on ancestral themes. *Cell*, *132*, 185–195.

De Robertis, E. M., & Kuroda, H. (2004). Dorsal–ventral patterning and neural induction in *Xenopus* embryos. *Annual Review of Cell and Developmental Biology*, *20*, 285–308.

De Robertis, E. M., & Sasai, Y. (1996). A common plan for dorsoventral patterning in Bilateria. *Nature*, *380*, 37–40.

Ferguson, E. L., & Anderson, K. V. (1992). Localized enhancement and repression of the activity of the TGF-β family member, *decapentaplegic*, is necessary for dorsal–ventral pattern formation in the *Drosophila* embryo. *Development*, *114*, 583–597.

Francois, V., Solloway, M., O'Neill, J. W., Emery, J., & Bier, E. (1994). Dorsal–ventral patterning of the Drosophila embryo depends on a putative negative growth factor encoded by the short gastrulation gene. *Genes and Development*, *8*, 2602–2616.

Genikhovich, G., Fried, P., Prünster, M., Schinko, J., Gilles, A., Fredman, D., et al. (2015). Axis patterning by BMPs: Cnidarian network reveals evolutionary constraints. *Cell Reports*, *10*, 1646–1654.

Hemmati-Brivanlou, A., Kelly, O. G., & Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neutralizing activity. *Cell*, *77*, 283–295.

Holley, S. A., Jackson, P. D., Sasai, Y., Lu, B., De Robertis, E. M., Hoffman, F. M., et al. (1995). A conserved system for dorsal–ventral patterning in insects and vertebrates involving short gastrulation and chordin. *Nature*, *376*, 249–253.

Hsu, D., Economides, A., Wang, X., Eimon, P., & Harland, R. (1998). The Xenopus dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. *Molecular Cell*, *1*, 673–683.

Inomata, H., Haraguchi, T., & Sasai, Y. (2008). Robust stability of the embryonic axial pattern requires a secreted scaffold for chordin degradation. *Cell*, *134*, 854–865.

Khokha, M., Yeh, J., Grammer, T., & Harland, R. (2005). Depletion of three BMP antagonists from Spemann's organizer leads to a catastrophic loss of dorsal structures. *Developmental Cell*, *8*, 401–411.

Langdon, Y., & Mullins, M. (2011). Maternal and zygotic control of zebrafish dorsoventral axial patterning. *Annual Review of Genetics*, *45*, 357–377.

Lee, H. X., Ambrosio, A. L., Reversade, B., & De Robertis, E. M. (2006). Embryonic dorsal–ventral signaling: Secreted Frizzled-related proteins as inhibitors of Tolloid proteinases. *Cell*, *124*, 147–159.

Lee, H. X., Mendes, F. A., Plouhinec, J. L., & De Robertis, E. M. (2009). Enzymatic regulation of pattern: BMP4 binds CUB domains of Tolloids and inhibits proteinase activity. *Genes and Development*, *23*, 2551–2562.

Levander, G. (1938). A study of bone regeneration. *Surgery, Gynecology & Obstetrics*, *67*, 705–714.

Little, S., & Mullins, M. (2004). Twisted gastrulation promotes BMP signaling in zebrafish dorsal–ventral axial patterning. *Development*, *131*, 5825–5835.

Little, S. C., & Mullins, M. C. (2006). Extracellular modulation of BMP activity in patterning the dorsoventral axis. *Birth Defects Research. Part C, Embryo Today: Reviews*, *78*, 224–242.

Marjoram, L., & Wright, C. (2011). Rapid differential transport of Nodal and Lefty on sulfated proteoglycan-rich extracellular matrix regulates left-right asymmetry in Xenopus. *Development*, *138*, 475–485.

Moos, M., Jr., Wang, S., & Krinks, M. (1995). Anti-dorsalizing morphogenetic protein is a novel TGF-beta homolog expressed in the Spemann organizer. *Development*, *121*, 4293–4301.

Nüsslein-Volhard, C., & Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature*, *287*, 795–801.

Oelgeschläger, M., Kuroda, H., Reversade, B., & Robertis, E. M. (2003). Chordin is required for the Spemann organizer transplantation phenomenon in Xenopus embryos. *Developmental Cell*, *4*, 219–230.

Oelgeschläger, M., Larraín, J., Geissert, D., & De Robertis, E. M. (2000). The evolutionarily conserved BMP-binding protein Twisted gastrulation promotes BMP signalling. *Nature*, *405*, 757–763.

Piccolo, S., Agius, E., Lu, B., Goodman, S., Dale, L., & De Robertis, E. M. (1997). Cleavage of Chordin by the Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. *Cell*, *91*, 407–416.

Piccolo, S., Sasai, Y., Lu, B., & De Robertis, E. M. (1996). Dorsoventral patterning in Xenopus: Inhibition of ventral signals by direct binding of Chordin to BMP-4. *Cell*, *86*, 589–598.

Ploper, D., Lee, H. X., & De Robertis, E. M. (2011). Dorsal–ventral patterning: Crescent is a dorsally secreted Frizzled-related protein that competitively inhibits Tolloid proteases. *Developmental Biology*, *352*, 317–328.

Plouhinec, J.-L. L., Zakin, L., Moriyama, Y., & De Robertis, E. M. (2013). Chordin forms a self-organizing morphogen gradient in the extracellular space between ectoderm and mesoderm in the Xenopus embryo. *Proceedings of the National academy of Sciences of the United States of America*, *110*, 20372–20379.

Reversade, B., & De Robertis, E. M. (2005). Regulation of ADMP and BMP2/4/7 at opposite embryonic poles generates a self-regulating morphogen field. *Cell*, *123*, 1147–1160.

Sasai, Y., Lu, B., Steinbeisser, H., & De Robertis, E. M. (1995). Regulation of neural induction by the Chd and BMP-4 antagonistic patterning signals in *Xenopus*. *Nature*, *376*, 333–336.

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K., & De Robertis, E. M. (1994). Xenopus *chordin*: A novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell*, *79*, 779–790.

Serpe, M., Umulis, D., Ralston, A., Chen, J., Olson, D. J., Avanesov, A., et al. (2008). The BMP-binding protein Crossveinless 2 is a short-range, concentration-dependent, biphasic modulator of BMP signaling in Drosophila. *Developmental Cell*, *14*, 940–953.

Smith, W. C., & Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell*, *70*, 829–840.

Spemann, H., & Mangold, H. (1924). Induction of embryonic primordial by implantation of organizers from a different species. *Roux's Archives fur Mikroskopische Anatomie und Entwicklungsmechanik*, *100*, 599–638. (Reprinted and translated in *International Journal of Developmental Biology*. Special Issue, De Robertis, E.M., & Arechaga, J. (Eds.) *45*, 13–31 (2001)).

Urist, M. (1965). Bone: Formation by autoinduction. *Science*, *150*, 893–899.

Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., et al. (1988). Novel regulators of bone formation: Molecular clones and activities. *Science*, *242*, 1528–1534.

Yabe, T., Shimizu, T., Muraoka, O., Bae, Y. K., Hirata, T., Nojima, H., et al. (2003). Ogon/Secreted Frizzled functions as a negative feedback regulator of Bmp signaling. *Development*, *130*, 2705–2716.