

DORSAL-VENTRAL PATTERNING AND NEURAL INDUCTION IN *XENOPUS* EMBRYOS

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■ **Abstract** We review the current status of research in dorsal-ventral (D-V) patterning in vertebrates. Emphasis is placed on recent work on *Xenopus*, which provides a paradigm for vertebrate development based on a rich heritage of experimental embryology. D-V patterning starts much earlier than previously thought, under the influence of a dorsal nuclear β -Catenin signal. At mid-blastula two signaling centers are present on the dorsal side: The prospective neuroectoderm expresses bone morphogenetic protein (BMP) antagonists, and the future dorsal endoderm secretes Nodal-related mesoderm-inducing factors. When dorsal mesoderm is formed at gastrula, a cocktail of growth factor antagonists is secreted by the Spemann organizer and further patterns the embryo. A ventral gastrula signaling center opposes the actions of the dorsal organizer, and another set of secreted antagonists is produced ventrally under the control of BMP4. The early dorsal β -Catenin signal inhibits BMP expression at the transcriptional level and promotes expression of secreted BMP antagonists in the prospective central nervous system (CNS). In the absence of mesoderm, expression of Chordin and Noggin in ectoderm is required for anterior CNS formation. FGF (fibroblast growth factor) and IGF (insulin-like growth factor) signals are also potent neural inducers. Neural induction by anti-BMPs such as Chordin requires mitogen-activated protein kinase (MAPK) activation mediated by FGF and IGF. These multiple signals can be integrated at the level of Smad1. Phosphorylation by BMP receptor stimulates Smad1 transcriptional activity, whereas phosphorylation by MAPK has the opposite effect. Neural tissue is formed only at very low levels of activity of BMP-transducing Smads, which require the combination of both low BMP levels and high MAPK signals. Many of the molecular players that regulate D-V patterning via regulation of BMP signaling have been conserved between *Drosophila* and the vertebrates.

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INTRODUCTION

All vertebrates share a conserved body plan. In the anterior-posterior (A-P) axis they form head, trunk, and tail, and in the dorsal-ventral (D-V) axis the backbone and belly of the animal. During development, dorsal ectoderm gives rise to neural plate and eventually to the entire central nervous system (CNS), and ventral ectoderm gives rise to epidermis and its derivatives. The mesoderm differentiates—from dorsal to ventral—into prechordal plate and notochord, somite (which forms skeletal muscle, vertebral column, and dermis), kidney, lateral plate mesoderm, and ventral blood islands. The molecular nature of the signals that ensure these stereotypical choices of cell and tissue differentiations in the embryo is beginning to emerge.

Inductive Properties of Dorsal Mesoderm

The starting point was provided by an experiment carried out by Hans Spemann and Hilde Mangold in 1924 in which they transplanted a small fragment of dorsal mesoderm into ventral mesoderm of the salamander gastrula (Spemann & Mangold 1924) and found it could induce a twinned embryo. They grafted a mesoderm fragment of different pigmentation, as in the experimental reproduction shown in Figure 1. The transplanted cells changed the differentiation of neighboring ventral cells in the host, inducing CNS, somite, and other axial components. In other words, both the ectoderm and the mesoderm became dorsalized. Research stimulated by this experiment has continued to this day, in particular with the molecular

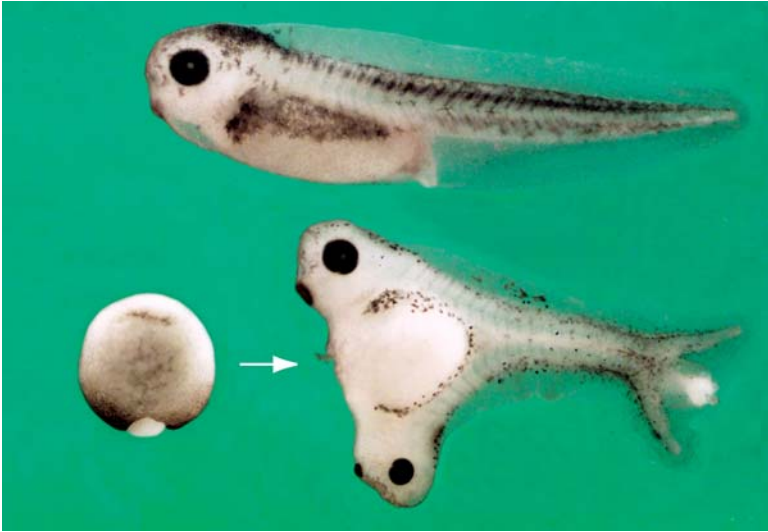


Figure 1 The Spemann-Mangold organizer experiment repeated in *Xenopus laevis*. (Top) control swimming tadpole; (bottom-right) Spemann organizer graft at the same stage. In the bottom-left embryo the size of the graft can be visualized as a white patch (Spemann organizer from an albino donor embryo) at early gastrula (vegetal view). The dorsal lip of the blastopore may be seen as a thin crescent opposite the graft.

exploration of Spemann's organizer phenomenon (De Robertis & Aréchaga 2001, Grunz 2004, Stern 2004).

D-V Patterning Mutations Affect the BMP Pathway

Numerous mutations that affect D-V embryonic pattern have been isolated in zebrafish and *Drosophila*. Most, if not all, of these mutations affect the bone morphogenetic protein (BMP) signaling pathway. In zebrafish, seven genes affecting D-V patterning were isolated in large-scale mutant screens (Hammerschmidt & Mullins 2002). *swirl* encodes BMP2b, and *snailhouse* BMP7. *chordino* encodes the homologue of *Xenopus* Chordin, a BMP antagonist secreted by Spemann's organizer. *mini fin* encodes Tolloid, an extracellular zinc metalloprotease that cleaves and inactivates Chordin. *lost-a-fin* encodes a BMP receptor. *somitabun* and *piggyltail* are mutations of the BMP-regulated transcription factor Smad5. Finally, *ogonlmercedes* is caused by mutations in Sizzled, a secreted Frizzled-related protein that displays anti-BMP phenotypic effects (Yabe et al. 2003, Martyn & Schulte-Merker 2003). In *Drosophila*, seven genes that affect D-V patterning were isolated in the classical zygotic mutant screens of Nüsslein-Volhard and Wieschaus. These were: *Decapentaplegic* and *screw* (Dpp and Scw, which are BMP growth factors), *short gastrulation* (Sog, a BMP antagonist homologous to vertebrate Chordin), *twisted gastrulation* (dTsg, a BMP-binding protein that functions as a cofactor of

Sog), *tolloid* (Tld, a zinc metalloprotease that cleaves and inactivates Sog), *zen* (a homeobox gene activated by BMP), and *shrew* (which has not yet been identified molecularly) (De Robertis & Sasai 1996, Lall & Patel 2001). From these genetic studies one can conclude that the BMP signaling pathway plays a major role in D-V axis formation in animal development.

In this review, we examine the current status of research on D-V patterning and the induction of neural tissue. We place emphasis on recent work on *Xenopus* embryos because this organism provides a paradigm for vertebrate development. We build on previous reviews on this topic (Harland & Gerhart 1997, Weinstein & Hemmati-Brivanlou 1999, De Robertis et al. 2000, Harland 2000).

THE EARLY β -CATENIN SIGNAL

The Cortical Rotation

Sperm entry initiates a rotation of the cortex of the egg with respect to the internal, more yolky, cytoplasm. The rotation is driven by parallel arrays of microtubules that are nucleated by the centriole, which in all animals is contributed by the sperm (reviewed by De Robertis et al. 2000). These egg cortical microtubules may correspond to the astral cortical microtubules present in most mitotic somatic cells. Organelles that can be labeled with hydrophobic membrane dyes are transported from the vegetal pole toward the dorsal side along these microtubule tracks. The dorsal lip is formed later on, at gastrula, opposite to the sperm entry point. Some of the molecular components of the dorsal determinants that move from the vegetal pole to the dorsal side are beginning to emerge and include Dishevelled and the GSK3-binding protein GBP (Miller et al. 1999, Weaver et al. 2003). Transport of dorsal determinants results in the stabilization and nuclear translocation of β -Catenin protein on the dorsal side of the *Xenopus* blastula (Schneider et al. 1996, Schohl & Fagotto 2002), providing the earliest molecular D-V asymmetry.

Two Dorsal Signaling Centers at Blastula

The nuclear localization of β -Catenin on the dorsal side extends from the bottom (vegetal) to the top (animal) pole of the blastula. The egg cytoplasm is heterogeneous, and when zygotic gene transcription starts, after the midblastula transition, the Nieuwkoop center is formed in dorsal-vegetal cells (Figure 2). Nieuwkoop center cells express *Xenopus* Nodal-related factors (Xnr1, 2, 4, 5, and 6) that are potent mesoderm inducers (Agius et al. 2000, Takahashi et al. 2000). High levels of nodals induce dorsal mesoderm (Spemann organizer) in overlying cells, whereas the Nieuwkoop center cells themselves go on to form anterior endoderm. In cells located above the Nieuwkoop center, in the dorsal animal cap and marginal zone, the β -Catenin signal induces the expression of BMP antagonists such as Chordin and Noggin (Wessely et al. 2001). This region of the animal cap was initially

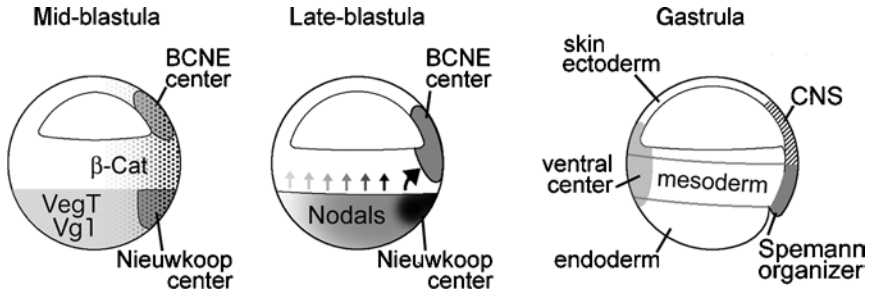


Figure 2 Organizer formation in *Xenopus laevis*. At blastula stages, two signaling centers, the BCNE center in the animal region and the Nieuwkoop center in the vegetal region, pattern the embryo. Both are dependent on nuclear localization of β -Catenin on the dorsal side of the embryo. The Nieuwkoop center is formed in vegetal cells at the intersection of the *VegT*, *Vg1*, and β -Catenin gene products. The BCNE center is involved in the formation of anterior neural tissue and expresses *chordin*, *noggin*, and *Xnr3*. The Nieuwkoop center releases Nodal-related signals that induce Spemann's organizer in dorsal mesoderm at gastrula.

designated as the preorganizer region and has more recently been renamed the BCNE center, for blastula Chordin and Noggin expression center (Kuroda et al. 2004). Molecular studies show that there is limited overlap between the Nieuwkoop and BCNE centers. Additional genes are expressed in a localized fashion in the blastula: The Nieuwkoop center expresses the secreted antagonist *cerberus*, and the BCNE center expresses the homeobox gene *Siamois*, the winged-helix gene *pintallavis/FoxA4a/HNF3 β* , and *Xnr3* (Kuroda et al. 2004, Wessely et al. 2004). Both blastula centers are formed simultaneously, as soon as zygotic transcription starts. Both require the β -Catenin signal on the dorsal side of the embryo, but the Nieuwkoop center also requires mRNAs located in the vegetal pole of the unfertilized egg (Figure 2).

In later development, BCNE cells give rise to all of the forebrain, most of mid- and hind-brain, floor plate, and notochord (Kuroda et al. 2004). Thus *chordin* and *noggin* are transiently expressed in prospective neuroectoderm at blastula. Later on, at gastrula stages, the same genes are expressed in the Spemann organizer mesoderm under the control of Nodal-related signals (Agius et al. 2000, Wessely et al. 2001). When BCNE explants are cultured in saline they form CNS tissue, indicating that neural specification takes place very early, at the blastula stage, in *Xenopus* ectoderm. When the BCNE center is excised, brain formation fails in the resulting embryos. This requirement of BCNE cells for brain formation can be rescued by transplantation of dorsal, but not ventral, animal cap tissue (Kuroda et al. 2004). Thus the early β -Catenin signal triggers the formation of two signaling centers at blastula, one involved in dorsal endoderm development (Nieuwkoop center) and the other in neural specification (BCNE center).

THE DORSAL GASTRULA CENTER

The Spemann Organizer Is a Source of Secreted Antagonists

When cDNA cloning became practical, researchers focused on the identification of the molecules that execute Spemann's organizer phenomenon. Starting with the cloning of the homeobox gene *gooseoid* (Cho et al. 1991), the organizer proved a very productive fishing ground for new genes. The success of these efforts may be compared with the large genetic screens of *Drosophila* and zebrafish, except that molecular methods were used and several laboratories were involved. The most surprising finding was that the Spemann organizer secretes a cocktail of growth factor antagonists (Figure 3).

Chordin, Noggin, and Xnr3

Chordin and *Noggin* are expressed in the Spemann organizer at gastrula and in notochord and prechordal plate at later stages. They encode BMP antagonists that bind to BMPs in the extracellular space, blocking binding to BMP receptors (Piccolo et al. 1996, Zimmerman et al. 1996). Work on the *Noggin* and *Chordin* secreted proteins has provided a molecular framework for understanding the function of many

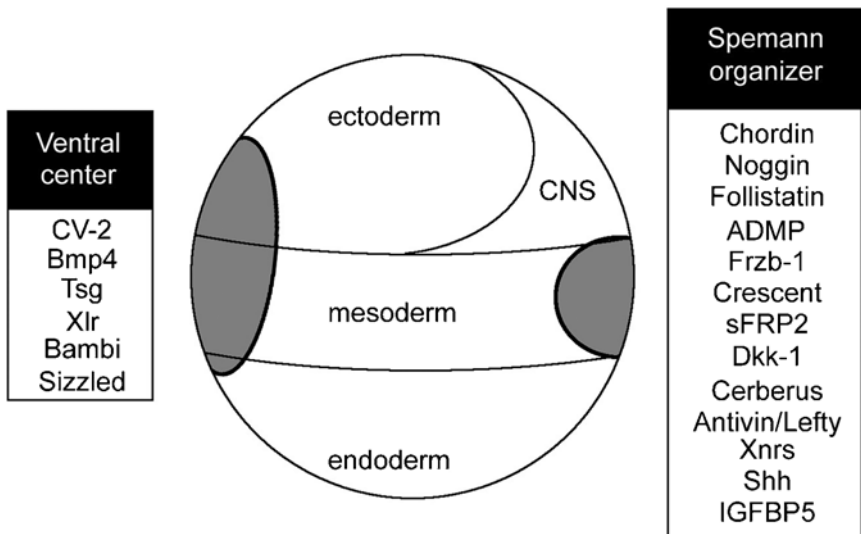


Figure 3 Proteins secreted by dorsal (Spemann organizer) or ventral gastrula signaling centers. Many are growth factor antagonists required for D-V patterning and are described in the text. ADMP (anti-dorsalizing morphogenetic protein) is a BMP family member that, paradoxically, is expressed in the dorsal center. Shh is sonic hedgehog and IGFBP5 is an insulin-like growth factor-binding protein that enhances IGF activity.

extracellular proteins. The three-dimensional structure of Noggin bound to BMP7 revealed that this BMP antagonist contains a cystine knot (Groppe et al. 2002). The cystine knot structural motif is found in many extracellular proteins, such as the TGF- β superfamily, luteinizing hormone (LH), follicle stimulating hormone (FSH), platelet-derived growth factor (PDGF), nerve growth factor (NGF), and others (Avsian-Kretchmer & Hsueh 2004). Because BMP and Noggin share cystine knots and conserved protein folds, it has been proposed that the ligand and its antagonist may have evolved from ancestrally related proteins (Groppe et al. 2002). Chordin is a large protein of about 1000 amino acids containing four cysteine-rich domains of about 70 amino acids each (called CR1 to CR4), which constitute BMP binding modules (Larraín et al. 2000). The structures of Chordin or CR domains have not been solved, but some predictions indicate a possible cystine knot (Avsian-Kretchmer & Hsueh 2004). CR repeats, also called von Willebrand factor C domains (vWF-C), are present in a large number of extracellular proteins, many of which have been found to regulate BMP or TGF- β signaling. Proteins containing CR domains include CTGF (connective tissue growth factor), Procollagens, Amnionless, Chordin-like/Ventropin/Neuralin-1 and -2, CRIM-1 (cysteine-rich motor neuron protein), Nel (neural tissue protein containing Egf-Like domains), Nel-like 1 and 2, Keilin, and Crossveinless-2 (Garcia-Abreu et al. 2002).

On the dorsal side of the *Xenopus* embryo, Chordin protein is present at concentrations of 6 to 12 nM in the extracellular space (Piccolo et al. 1996). Because BMPs are expressed in the picomolar range in the embryo, Chordin alone should suffice to block BMP signaling on the dorsal side. However, the knockout of the Chordin gene in mouse causes only a small percentage of embryos to become ventralized at the gastrula stage (Bachiller et al. 2003). These infrequent embryos have a small neural plate and embryonic region, and an enlarged allantois (ventral mesoderm). Most *chd*^{-/-} embryos have a normal CNS and die at birth, mimicking a human malformation called DiGeorge syndrome, which is caused by the lack of Chordin in pharyngeal endoderm at a later developmental stage (Bachiller et al. 2003). The mouse Noggin knockout has normal gastrulation and neural plate formation, and a strong skeletal phenotype later on (McMahon et al. 1998). Mouse embryos mutant for HNF3 β , in which Hensen's node—the equivalent of Spemann's organizer—does not develop, do not express Chordin or Noggin at gastrula but still form a neural plate (Klingensmith et al. 1999). Because these two BMP antagonists are required for neural plate development, they must also function at earlier stages before HNF3 β is expressed. Indeed, *chordin;noggin* double mutant embryos display a loss of the prosencephalic vesicle, lack of anterior notochord (ventralization of the mesoderm), and randomization of heart left-right asymmetry (Bachiller et al. 2000). Thus the Chordin and Noggin BMP antagonists have redundant functions and are required for the patterning of the three embryonic axes of the mouse.

In *Xenopus*, knockdown of Chordin expression is achieved using *chordin* anti-sense morpholino oligos (Chd-MO) (Oelgeschläger et al. 2003a). The phenotype obtained is very similar to that of the *chordino* zebrafish mutant (Schulte-Merker et al. 1997), displaying a reduction of the size of the neural plate and eventually

CNS tissue, and an expansion of ventral mesoderm. When the *Xenopus* embryo is experimentally manipulated, strong requirements for Chordin are observed. For example, the dorsalizing effects of lithium chloride (LiCl), a treatment that stabilizes β -Catenin, can be completely blocked by Chd-MO (Oelgeschlager et al. 2003a). When the Spemann organizer transplantation experiment is repeated using dorsal lip explants injected with Chd-MO, the grafts completely lose their inducing activity (Oelgeschlager et al. 2003a). Organizer grafts probably require a full complement of BMP antagonists, and even the loss of a single one, Chordin, has profound effects.

Xnr-3 encodes a Nodal-related protein that lacks mesoderm-inducing activity, presumably because it is mutated in a critical cysteine residue of the cystine knot. Xnr3 is able to induce neural differentiation when overexpressed in animal caps (Hansen et al. 1997) and is able to antagonize BMP signaling through its amino-terminal proregion (Haramoto et al. 2004). Xnr3 homologues have not been found in any other vertebrates, but in *Xenopus* it is, after Chordin, the gene most strongly induced by the early β -Catenin signal in genome-wide studies (Wessely et al. 2004).

The Wnt Antagonists: Frzb-1, Crescent, sFRP-2, and Dickkopf

Secreted Frizzled-related proteins (sFRPs) constitute a large family of Wnt antagonists that encode secreted forms of the amino-terminal, cysteine-rich domain of the Wnt receptor Frizzled (Kawano & Kypta 2003). They bind Wnt proteins in the extracellular space and prevent them from signaling (Leyns et al. 1997). The *Xenopus* gastrula expresses high levels of sFRPs; a screen for cDNAs encoding secreted proteins resulted in a surprising 24% of isolates encoding sFRPs (Pera & De Robertis 2000). The Spemann organizer expresses Frzb-1/sFRP3, Crescent and sFRP2 (Figure 3).

Xenopus Dickkopf-1 (Dkk-1) is a secreted inhibitor of Wnt signaling that functions through a different and interesting molecular mechanism. It encodes a cysteine-rich secreted protein expressed in dorsal endomesoderm that defines a new protein family (Glinka et al. 1998). Dkk-1 binds to a Wnt coreceptor called LDL receptor-related protein-5/6 (LRP5/6) (Mao et al. 2001). Wnt binds to both Frizzled and LRP6, forming a ternary receptor complex on the cell surface (Tamai et al. 2000). This triggers phosphorylation of the intracellular domain of LRP5/6 at conserved PPPSP sites, causing the recruitment of the β -Catenin destruction complex protein Axin to the cell membrane and inhibition of β -Catenin degradation (Tamai et al. 2004). Thus LRP5/6 specifically links Wnt signaling to β -Catenin stabilization. Dkk-1 binds not only to LRP5/6 but also to a second transmembrane protein called Kremen. The resulting trimolecular complex of LRP5/6, Dkk, and Kremen is endocytosed, resulting in the depletion of LRP5/6 coreceptor from the plasma membrane (Mao et al. 2002). This provides an elegant molecular explanation for how Dkk selectively inhibits the action of Wnt on the canonical β -Catenin Wnt pathway without affecting other aspects of Wnt signaling. In *Xenopus*, Dkk1

neutralizing antibodies inhibit head and prechordal plate formation (Glinka et al. 1998, Kazanskaya et al. 2000). In the mouse, *Dkk-1* homozygous mutants lack CNS structures anterior to the midbrain (Mukhopadhyay et al. 2001), and heterozygotes show strong cooperation with *noggin* in head formation (del Barco et al. 2003).

Cerberus

Cerberus, a secreted protein expressed at high levels in the anterior dorsal endoderm of gastrula, has the remarkable property of inducing ectopic head structures in the absence of trunk formation (Bouwmeester et al. 1996). Its discovery identified the first head-inductive signal from endoderm, a finding that was later supported by studies in the mouse on the role of the anterior visceral endoderm (AVE) in head development (Beddington & Robertson 1999). Cerberus protein binds to, and prevents signaling by, Nodal, BMP, and Wnt-8 (Piccolo et al. 1999). A fragment of Cerberus consisting of its carboxy-terminal cystine knot has only the Nodal-inhibiting activity. This artificial construct, called Cerberus-short (Cer-S), provides a useful reagent to block Nodal signaling in embryos. For example, the use of Cer-S allowed Agius et al. (2000) to demonstrate that the induction of both dorsal and ventral mesoderm is mediated by a gradient of Nodal-related signals emanating from endoderm at the late-blastula stage (Figure 2).

In *Xenopus*, Cerberus is required for head induction; a Cer-MO inhibits head but not trunk-tail development (Kuroda et al. 2004). In the mouse, knockout of a *cerberus-like* gene expressed in the AVE lacks gastrulation phenotypes. Mutations in another nodal antagonist expressed in AVE cells, *Lefty-1*, also lacks gastrulation phenotypes. However, when *cer-1^{-/-}*; *lefty-1^{-/-}* double mutants are generated, development of the anterior embryo is greatly impaired owing to excessive Nodal signaling in the anterior region of the embryo (Perea-Gomez et al. 2002, Yamamoto et al. 2004). In chick, a *cerberus* homologue expressed in the hypoblast (the equivalent of mouse AVE) prevents formation of trunk mesoderm in prospective head neuroectoderm via its anti-Nodal activity (Bertocchini & Stern 2002). In summary, these studies in vertebrate embryos support the view that secreted antagonists of Nodal, BMP, and Wnt signals play a fundamental role in promoting head development and repressing trunk-tail development (Piccolo et al. 1999, Niehrs 2001, Agathon et al. 2003).

THE VENTRAL GASTRULA CENTER

The BMP4 Synexpression Group

Evidence indicating that a ventral signaling center exists in the gastrula has been steadily accumulating. Several genes encoding secreted or cell surface proteins are expressed in ventral mesoderm and ectoderm 180° from Spemann's organizer (Figure 3). Their expression patterns indicate they are members of the BMP4

synexpression group. Synexpression groups consist of genes that are coordinately expressed in the embryo and frequently function in a common signaling pathway (Niehrs & Pollet 1999). Early indications of the existence of a ventral center came from studies on the expression of homeobox genes such as *Vent/Vox/Vega* and *Eve1* that are induced by BMP4 in the ventral region of the gastrula (Gawantka et al. 1995, Kawahara et al. 2000, Joly et al. 1993). Interestingly, many of the proteins secreted by the ventral gastrula center have biochemical activities similar to those of the Spemann organizer but are under the opposite transcriptional regulation (see below).

Crossveinless-2, Twisted Gastrulation, Xolloid-Related, and Bambi

Drosophila Crossveinless-2 (CV-2) is a gene required for the formation of the wing crossveins, structures that require Dpp signaling during development. *Drosophila* and vertebrate CV-2 contain five CR domains of the types present in the BMP-binding modules of Chordin, as well as a von Willebrand factor-D domain (Figure 4A) (Conley et al. 2000, Coffinier et al. 2002). Mouse and *Xenopus* CV-2 are expressed in ventral mesoderm and ectoderm and when overexpressed behave as BMP antagonists (Coffinier et al. 2002, Moser et al. 2003, Binnerts et al. 2004; C. Coffinier & E.M. De Robertis, unpublished observations). Whereas Chordin expression is repressed by BMP4, CV-2 expression is upregulated by BMP4.

Twisted gastrulation (Tsg) encodes a protein that binds to both BMP and Chordin (Oelgeschläger et al. 2000, Chang et al. 2001, Scott et al. 2001, Ross et al. 2001, Blitz et al. 2003). Chordin, BMP, and Tsg form ternary complexes that prevent binding of BMP to its receptor (Figure 4B). Chordin is cleaved by the Tolloid/Xolloid protease at two specific cleavage sites. The resulting proteolytic fragments have lower affinity, and BMP signaling through its receptors is restored (Figure 4B). Tsg facilitates this proteolytic cleavage and promotes BMP signals (Larraín et al. 2001, Oelgeschläger et al. 2003b). The key switch is provided by the levels of Xolloid protease. The effects of Tsg overexpression depend on the amount of Tolloid present; at high Xolloid levels Tsg promotes BMP signaling and at low levels it inhibits it (Larraín et al. 2001, Oelgeschläger et al. 2003b). In mouse knockouts, Tsg mutants have phenotypes and genetic interactions that reflect both its anti- and pro-BMP4 activities (Nosaka et al. 2003, Petryk et al. 2004, Zakin & De Robertis 2004). In *Drosophila*, *dTsg* is required for maximal Dpp signaling levels in the dorsal-most region (called the amnioserosa) and for the diffusion of Dpp in the early embryo (Eldar et al. 2002). In vertebrates, it appears likely that Tsg may interact with other CR-containing proteins in addition to Chordin (Oelgeschläger et al. 2003b).

Xolloid-related (Xlr) is a recently identified Tolloid metalloprotease that is specifically expressed in the ventral gastrula center of *Xenopus* (Dale et al. 2002). There are multiple Tolloid-like genes in vertebrates, and *Xlr* is most similar to zebrafish *mini-fin/Tolloid* (Connors et al. 1999) and mouse *Tolloid-like-1* (Scott

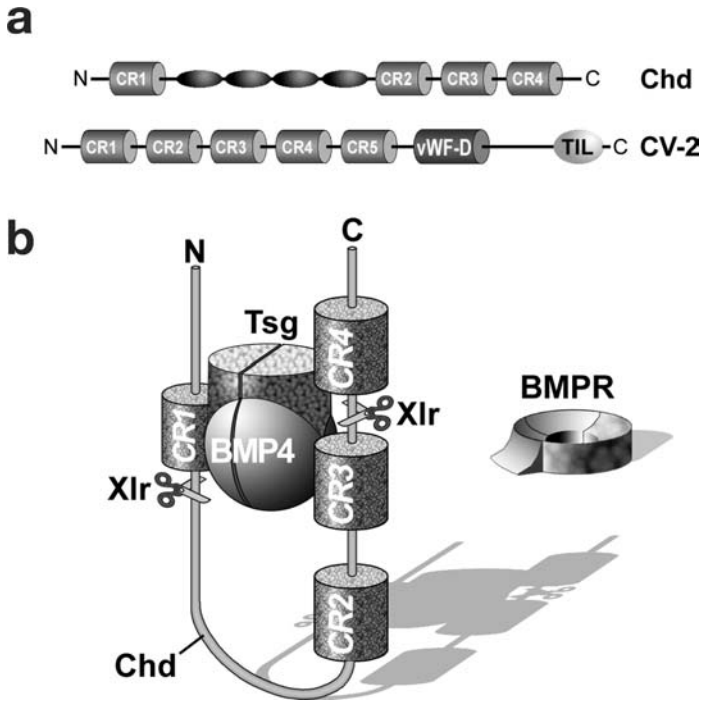


Figure 4 Cysteine-rich (CR) BMP-binding modules in Chordin and Crossveinless-2. (a) Chordin contains four CR modules; Crossveinless-2 (CV-2) has five. Chordin contains four additional repeats (*ovals*) in the region between CR1 and CR2 designated CHRD domains. These immunoglobulin-like beta-barrel domains are also present in Short-gastrulation and some secreted bacterial proteins (Hyvönen 2003). CV-2 contains a von Willebrand factor-D domain (vWF-D) and a trypsin-like cysteine-rich domain (TIL) at its carboxy-terminal end. (b) A molecular complex involving BMP, Chordin, and Tsg regulates the D-V activity gradient of BMP4 in *Xenopus*. Chordin binds BMP4 through cysteine-rich domains CR1 and CR3. After cleavage of Chordin by the Xolloid-related (Xlr) zinc metalloprotease, the affinity between CR modules and BMP4 is greatly reduced, and BMP4 protein is able to bind to and activate BMP receptor (BMPR) on the cell surface.

et al. 1999). *Xlr* expression is upregulated by BMP4 (Dale et al. 2002), and this gene is likely to play a critical role in inactivating Chordin during D-V patterning in the gastrula.

Bambi, an acronym for BMP and Actinmembrane-bound inhibitor, encodes a transmembrane protein related to BMP receptor type I. It lacks the serine-threonine kinase intracellular domain present in BMPR and functions as a cell surface inhibitor of BMP and Activin signaling that is expressed as an integral part of the BMP4 synexpression group (Onichtchouk et al. 1999).

Sizzled

Sizzled is a ventral center gene of particular interest. It encodes an sFRP-like molecule (Salic et al. 1997). Microinjection of *sizzled* antisense morpholino oligos causes ventralization of the *Xenopus* embryo, including an increase of ventral mesoderm and reduction of the neural plate (Collavin & Kirshner 2003). The most surprising aspect of this phenotype is that it is indistinguishable from that of the loss-of-function of Chordin in *Xenopus* (Oelgeschlager et al. 2003a). Knockdown of Chordin expands the expression of *sizzled* in the ventral gastrula center, presumably through increased BMP4 signaling on the ventral side. Understanding how these two secreted proteins expressed at opposite poles of the embryo can generate similar phenotypes when inhibited may hold the key to understanding D-V regulation in *Xenopus*.

In zebrafish, only two ventralized mutants have been isolated, *chordino* and *ogon/mercedes*. Both result in reduced CNS and expanded ventral tail structures consistent with increased BMP signaling. The *ogon* (meaning tail in Polish) gene encodes *sizzled* (Yabe et al. 2003, Martyn & Schulte-Merker 2003). This gene, which has the molecular structure of a Wnt inhibitor, must have important interactions with the BMP pathway because *ogon/sizzled* mRNA injection dorsalizes the wild-type zebrafish embryo. Interestingly, microinjected *sizzled* mRNA is inactive in *chordino* mutants (Yabe et al. 2003). Thus Sizzled must work through Chordin. The molecular mechanism of Sizzled action remains a puzzle. An initial study indicated that it functioned as an xWnt-8 antagonist (Salic et al. 1997), but subsequent studies suggested that Sizzled behaved as a BMP4 antagonist (Collavin & Kirschner 2003, Yabe et al. 2003). One of the challenges for the near future is determining whether the Sizzled protein regulates the activity of a Wnt signal, which in turn affects BMP expression, or whether it acts directly on BMP or Chordin activity. Sizzled is most similar to Crescent, an sFRP that functions as a Wnt antagonist on the dorsal side of the embryo (Pera & De Robertis 2000, Bradley et al. 2000). Thus Chordin and Crescent are expressed in the Spemann organizer, and two other genes of related structures, Crossveinless-2 and Sizzled, respectively, are expressed in the ventral center. Dorsal and ventral center genes have similar activities when overexpressed in embryos, but whereas the expression of organizer genes is inhibited by BMPs, expression of Crossveinless-2 and Sizzled/Ogon is increased by BMP4 signaling. The importance of the ventral signaling center was unrecognized for a long time, perhaps because it lacks inductive activity upon transplantation. With the availability of morpholino loss-of-function reagents, the gastrula ventral center is likely to become the focus of much research.

THE ROLE OF NEUROECTODERM IN CNS FORMATION

Traditionally, research in amphibian neural induction centered on the role of the mesodermal Spemann organizer signals at gastrula, also known as the primary embryonic induction (Spemann 1938). A possible role for the prospective

neuroectoderm itself in CNS formation has long been debated (Goerttler 1925, Holtfreter 1933, Spemann 1938). In the very influential exogastrulation experiment, Holtfreter removed the egg membranes and placed axolotl embryos in hypertonic saline, preventing the involution of endomesoderm. In the absence of its mesodermal substratum, the entire ectodermal layer became epidermis, and no CNS developed (Holtfreter 1933). Since then, traditional embryological thinking has been that the Spemann organizer mesoderm secretes the neural inducers and that the ectoderm itself has no role (Spemann 1938). One way of understanding Holtfreter's intriguing result in modern terms is that perhaps in exogastrulated ectoderm BMPs reach very high levels, reversing any labile bias present in the ectoderm toward neural formation. Below, we discuss recent evidence indicating that the ectoderm of the BCNE center does indeed play a crucial role in neural induction.

The Neural Induction Default Model

Early efforts to identify neural inducers used ectodermal explants exposed to many substances, such as dead organizers, methylene blue, sterols, fatty acids, and even sand particles. All were found to neuralize embryonic ectoderm. Gradually, the search for the Spemann organizer neural inducer became a funeral march for the field (reviewed by Holtfreter & Hamburger 1955). Eventually it was realized that neuralization of axolotl ectoderm could be obtained in the complete absence of any inducer simply by culturing animal caps in an inadequate saline solution (Barth 1941). This effect could be mimicked in other amphibians by partial cell dissociation with citrate, oxalate, or low pH treatments that received the unfortunate name of "sublethal cytolysis" (Holtfreter & Hamburger 1955). *Xenopus* ectoderm is relatively resistant to neuralization, but neural differentiation can be elicited by cell dissociation and culture for several hours (reviewed by Weinstein & Hemmati-Brivanlou 1999, Munoz-Sanjuan & Brivanlou 2002). This neuralization can be reversed by adding BMP4 to the culture medium, which led to the proposal that during dissociation BMP4 protein is diluted by diffusion into the culture medium (Wilson & Hemmati-Brivanlou 1995). BMP acts within the ectoderm to induce epidermis, and it was proposed that when it diffuses away in dissociated cells, neural differentiation by a default pathway would ensue. However, given the recent realization that activation of MAPK signaling can downregulate the BMP signaling pathway at the level of Smad1 phosphorylation (see below), alternative interpretations for why cell dissociation and abnormal substances have neural-inducing activities in ectodermal cells will have to be explored.

Although the BMP default neural induction model has generally received support in *Xenopus* (Harland 2000), work in other model systems has highlighted the role of FGF and Wnt signals in neural induction and de-emphasized a role for BMP (reviewed in Wilson & Edlund 2001, Streit & Stern 1999, Stern 2002, Lemaire et al. 2002). In chick, FGF can initiate ectopic expression of neural markers but Chordin and Noggin cannot. However, Chordin can stabilize expression of transiently induced neural markers, expand an already formed neural plate, and

induce ectopic primitive streaks (Streit & Stern 1999). Explants of the medial chick epiblast differentiate into neural tissue, but when BMP4 is added develop into epidermis instead. Lateral chick epiblast explants develop into epidermis and do not respond to FGF or BMP antagonists; however, if Wnt signaling levels are lowered by treatment with Wnt antagonists, then both FGFs and BMP antagonists can induce neural differentiation in lateral epiblast cells (Wilson & Edlund 2001). Additional lines of evidence indicate that Wnt signals are involved in the choice between epidermal and neural differentiation. A functional screening of cDNA for genes able to cause differentiation of mouse embryonic stem cells into neurons resulted in the isolation of the sFRP-2 Wnt antagonist (Aubert et al. 2002). In *Xenopus* animal cap explants, Wnt antagonists are able to transiently induce neural markers (Glinka et al. 1997), and FGFs function as neural inducers (Harland 2000). Taken together, the available evidence suggests that multiple signaling pathways are involved in neural induction in all vertebrates. Rather than emphasizing the differences between organisms, the field now needs ways of integrating these diverse neural-inducing signaling pathways. One such integration of cell-cell signals may occur at the level of Smad1 phosphorylation.

Integrating Signals at the Level of Smads

At any given time, cells are exposed to a multitude of cell-cell signals. Understanding how multiple signaling pathways are integrated is a major challenge in cell biology. The differentiation of the ectoderm provides a good material because at the gastrula stage cells must choose between two different fates, epidermis or neural tissue, for which excellent molecular markers exist. Smads 1/5/8 transduce signals of the BMP serine/threonine kinase receptors through phosphorylation of carboxy-terminal SXS sequences, thus triggering nuclear translocation (Shi & Massagué 2003). BMP antagonists, such as Chordin and Noggin, inhibit this carboxy-terminal phosphorylation and promote neural gene expression by decreasing Smad1 activity (Figure 5). Positively acting neural inducers such as FGF8 (Hardcastle et al. 2000) and IGF (Pera et al. 2001, Richard-Parpaillon 2002) signal through tyrosine kinase (RTK) transmembrane receptors. It has recently been shown that FGF and IGF induce, via mitogen-activated protein kinase (MAPK), phosphorylation in the linker (middle) region of Smad1 at four conserved PXSP sites (Pera et al. 2003). Linker phosphorylation prevents nuclear translocation and has an inhibitory effect on Smad activity (Figure 5). This effect had been initially observed in cultured cells treated with EGF (epidermal growth factor) or HGF (hepatocyte growth factor), but its physiological significance had remained unclear (Kretzschmar et al. 1997, Massagué 2003). Overexpression of Smad1 in *Xenopus* has little ventralizing (pro-BMP) effect except in the case of mutant proteins that cannot be phosphorylated by MAPK (Pera et al. 2003, Sater et al. 2003). This indicates that MAPK signals are active in the developing embryo and inhibit Smad activity. The emerging picture indicates that neural genes are expressed

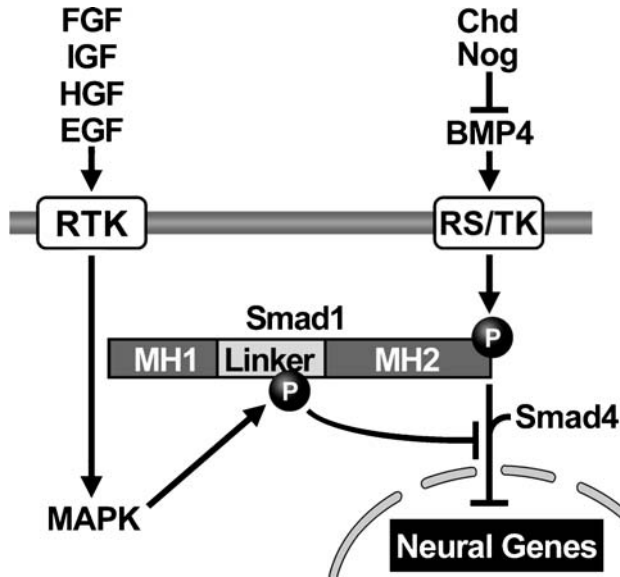


Figure 5 Integration of multiple signaling pathways at the level of Smad1 phosphorylation during neural induction. Neural induction requires extremely low levels of Smad1 activity that are reached through the combination of two signaling systems. One is inhibition of binding between BMP4 and its serine/threonine kinase (RS/TK) receptor by anti-BMP molecules such as Chordin and Noggin. The other is inhibitory phosphorylation of Smad1 in the linker region by receptor tyrosine kinase (RTK) signals such as FGF, IGF, HGF, and EGF mediated by activation of MAPK. MH1 and MH2 are evolutionarily conserved globular Mad-homology domains; MH1 contains the DNA-binding domain and MH2 multiple protein interaction sites.

only at very low levels of Smad1 activity, which requires both low BMP levels and high MAPK signals (Figure 5). In agreement with this view, neural induction by Chordin can be blocked by agents that inhibit FGF or IGF signaling (Pera et al. 2003).

In the mouse, knock-in of Smad1 forms that are insensitive to MAPK phosphorylation in the linker region exhibit phenotypes in gastrointestinal epithelium and germ cells (Aubin et al. 2004). These animals express normal levels of mutant Smad1 (as well as of wild-type Smad5 and Smad8), supporting the view that integrating BMP and MAPK signals at the level of Smad1 is required *in vivo*.

The finding that FGF signaling can cause inhibition of signaling by BMP Smads via the hard-wired mechanism shown in Figure 5 may help explain other situations in which FGF and BMP signals oppose each other during development. A classical example is the antagonism between FGF4 and BMP2 in limb development (Niswander & Martin 1993). Similarly, opposing effects of FGFs and BMPs have

been reported in lung morphogenesis, cranial suture fusion, and tooth development (Weaver et al. 2000, Warren et al. 2003, Thesleff & Mikkola 2002). In future, one aspect of the neural induction default model that should be reinvestigated is whether animal cap dissociation, in addition to lowering BMP levels, causes the activation of other signaling pathways such as MAPK.

β -Catenin, Chordin, and Noggin Are Required in Blastula Ectoderm

It has long been known that the *Xenopus* gastrula dorsal animal cap has a predisposition for neural induction by dorsal endomesoderm (Sharpe et al. 1987, London et al. 1988) and that it has lower levels of BMP4 expression (Fainsod et al. 1994). In the course of a functional cDNA screen, Baker et al. (1999) made the important discovery that an activated form of mouse β -Catenin was able to induce neural tissue in animal caps. This neural induction was accompanied by the extinction of BMP4 expression in animal cap explants and could be inhibited by constitutively activated BMP receptor (Baker et al. 1999). In zebrafish, a homeobox gene called *bozozok/dahrma* is expressed on the dorsal side in response to the early β -Catenin signal and directly represses *BMP2b* gene transcription (Leung et al. 2003). Because *bozozok* and *chordino* double mutants show synergistic losses of neural tissue and dorsal structures (Gonzalez et al. 2000), it appears that during development dorsal BMP levels are inhibited both by transcriptional regulators and by BMP antagonists.

In *Xenopus*, the early β -Catenin signal induces the early expression of Chordin and Noggin in the BCNE center (Wessely et al. 2001, Kuroda et al. 2004). In later development, the BCNE becomes the brain and floor plate. When mesoderm formation is blocked (by inhibiting Nodal signals with Cer-S), brain structures still develop, even though these embryos lack expression of *chordin*, *noggin*, and *follistatin* at gastrula. However, transcription of BMP antagonists can be transiently detected at blastula in the BCNE center that forms under the influence of the β -Catenin signal (Wessely et al. 2001). Anterior CNS differentiation in the absence of mesoderm is entirely dependent on the early β -Catenin signal and can be blocked by Chd-MO or Noggin-MO; posterior neural markers are not affected and require FGF signals (Kuroda et al. 2004). The labile neural determination of the BCNE region is reinforced by signals from the underlying endomesoderm. The Nieuwkoop center, which expresses *cerberus*, involutes and comes into intimate contact with the future brain (Figure 6), providing a “double assurance” mechanism for brain formation. The requirement for signals from two different cell layers during anterior CNS formation can be revealed by injecting Chd-MO into prospective neuroectoderm and Cer-MO into the future endomesoderm (Kuroda et al. 2004). A crucial role is played in the ectodermal layer itself by the early dorsal β -Catenin signal that activates expression of the BMP antagonists Chordin and Noggin in the future CNS of the embryo at the blastula stage.

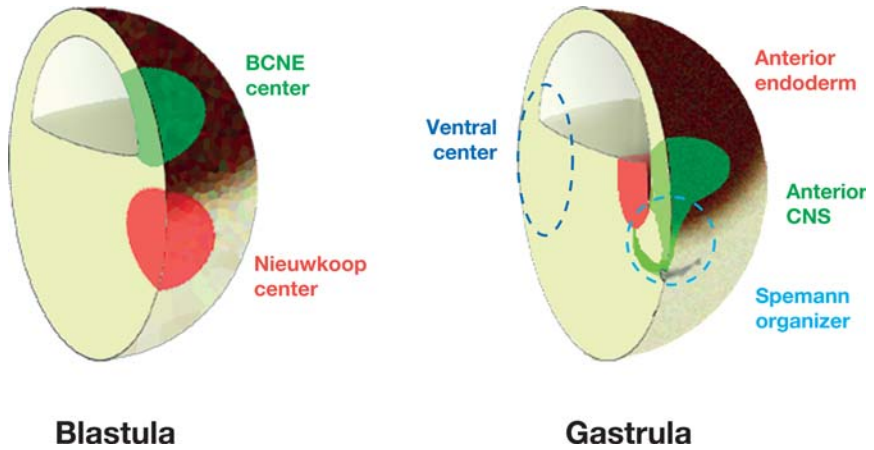


Figure 6 Signaling centers at blastula and gastrula that have critical roles for body plan formation in *Xenopus*. The BCNE center is located in the dorsal animal cap region (*left*) and gives rise to prospective brain and floor plate, as well as the notochord region of the Spemann organizer at gastrula (*right*). Nieuwkoop center cells become anterior endoderm at gastrula, coming into close apposition with prospective anterior CNS. Both signaling centers are required for brain formation. At gastrula, a ventral signaling is formed opposite the organizer.

CONSERVED MOLECULAR MECHANISMS OF BMP REGULATION

There is general agreement that Urbilateria, the last common ancestor of the vertebrate and invertebrate lineages, had a conserved A-P patterning system regulated by Hox genes. Many D-V patterning genes have also been conserved between *Xenopus* and *Drosophila*, except that their expression patterns have been inverted with respect to each other (De Robertis & Sasai 1996, Carroll et al. 2001).

Inversion of the D-V Axis in Evolution

As shown in Figure 7, Sog is expressed ventrally in *Drosophila* embryos (first in the ventral two thirds of the blastoderm, then in neurogenic ventral ectoderm, and finally in ventral midline cells), whereas Chordin is expressed dorsally in the vertebrates. An argument against homologous roles for Chordin and Sog was that one was expressed in mesoderm and the other in neuroectoderm. The realization that Chordin is expressed in the BCNE center, which gives rise to the brain and floor plate (Kuroda et al. 2004), now removes this objection because in both animals the initial expression is found in neuroectoderm. A number of recently identified

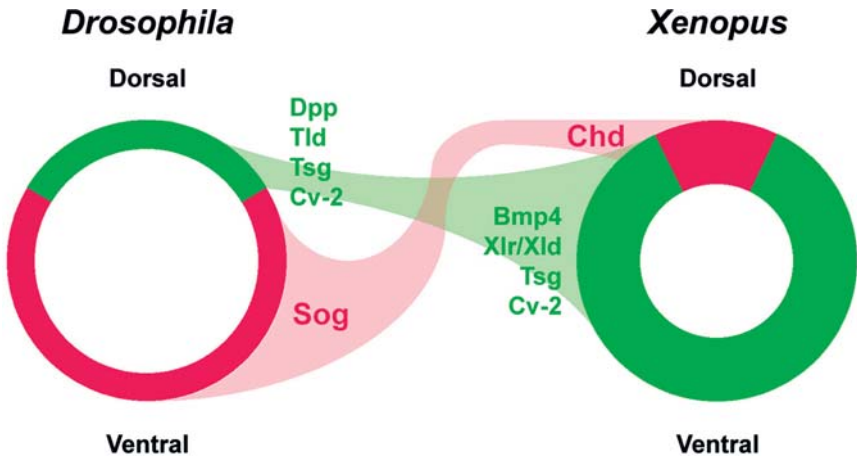


Figure 7 Inversion in the course of evolution of a conserved network of extracellular regulators of BMP/Dpp signaling involved in D-V patterning.

extracellular regulators of BMP signaling—Xld, CV-2 and Tsg—are expressed in the ventral side of the *Xenopus* embryo as part of the BMP4 synexpression group. Their *Drosophila* counterparts—Dpp, Tld, CV-2, and Tsg—are expressed on the dorsal side (Figure 7). As additional genes are discovered in future, the case for a unified mechanism for D-V patterning in evolution will become more persuasive. At later stages, once the ventral nerve cord or the neural plate is formed, additional D-V conservations become apparent between *Drosophila* and the vertebrates. These include Netrin and Slit in the midline, and three D-V columns of homeobox gene expression in the neurogenic regions (*vnd/Nkx2.2*, *ind/Gsh* and *msh/Msx*) (Arendt & Nubler-Jung 1999, von Ohlen & Doe 2000). Although considerable similarities exist between the zygotic D-V patterning systems, the initial signals that set up these patterns of gene expression appear to be very different: In *Drosophila* the maternal signal is provided by a Dorsal/NF κ B signal and in *Xenopus* by the early β -Catenin signal (Lall & Patel 2001). Detailed promoter studies, perhaps on the Chordin BCNE enhancer, may help determine whether any common upstream signals exist.

Whereas the extracellular Chd/Sog BMP/Dpp system has been remarkably conserved, other vertebrate D-V genes are not conserved in *Drosophila*. The sFRP Wnt antagonists, Dkk, Cerberus, and Bambi are not present in the *Drosophila* genome. It is possible that in the course of evolution growth factor antagonists may be more easily generated than, say, new signaling pathways. Within the vertebrates, the ventral gene *sizzled* and its dorsal homologue *crescent* are found in zebrafish, *Xenopus*, and chick but have not been detected in the mouse, rat, or human genomes. An extreme example of innovation within the vertebrates is the case of *Xnr3*, which is only found in *Xenopus*.

Communicating Dorsal and Ventral Signals

During development the dorsal and ventral sides of the embryo must be able to talk to each other over many cell diameters. As seen in Figure 1, when transplanted at gastrula (about 10,000-cell stage), a small organizer graft will induce a perfectly patterned second embryo. Similarly, if a gastrula is divided into two halves with a hair loop, two smaller but well-proportioned embryos can be obtained (Spemann 1938). This problem of regulation in a developing field of cells that tend to re-form normal structures after experimental perturbations constitutes one of the unsolved mysteries of developmental biology. The realization that the *Xenopus* gastrula has dorsal and ventral signaling centers that secrete related growth factor antagonists (such as Chd/CV-2 and crescent/sizzled) under opposite transcriptional control by BMP signals, now sets the stage for investigating the molecular nature of these cell-cell communications between the dorsal and ventral poles of the embryo. The use of loss-of-function morpholino reagents in combination with the cut-and-paste embryological experiments that are possible in *Xenopus*, holds promise for further advances in our understanding of D-V patterning regulation, morphogenetic fields, and neural differentiation.

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