Chordin forms a self-organizing morphogen gradient in the extracellular space between ectoderm and mesoderm in the *Xenopus* embryo

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This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2013.

Contributed by Edward M. De Robertis, October 22, 2013 (sent for review October 2, 2013)

The vertebrate body plan follows stereotypical dorsal–ventral (D-V) tissue differentiation controlled by bone morphogenetic proteins (BMPs) and secreted BMP antagonists, such as Chordin. The three germ layers—ectoderm, mesoderm, and endoderm—are affected coordinately by the Chordin–BMP morphogen system. However, extracellular morphogen gradients of endogenous proteins have not been directly visualized in vertebrate embryos to date. In this study, we improved immunolocalization methods in *Xenopus* embryos and analyzed the distribution of endogenous Chordin using a specific antibody. Chordin protein secreted by the dorsal Spemann organizer was found to diffuse along a narrow region that separates the ectoderm from the anterior endoderm and mesoderm. This Fibronectin-rich extracellular matrix is called "Brachet's cleft" in the *Xenopus* gastrula and is present in all vertebrate embryos. Chordin protein formed a smooth gradient that encircled the embryo, reaching the ventral-most Brachet cleft. Depletion with morpholino oligos showed that this extracellular gradient was regulated by the Chordin protease Tolloid and its inhibitor Sizzled. The Chordin gradient, as well as the BMP signaling gradient, was self-regulating and, importantly, was able to rescale in dorsal half-embryos. Transplantation of Spemann organizer tissue showed that Chordin diffused over long distances along this signaling highway between the ectoderm and mesoderm. Chordin protein must reach very high concentrations in this narrow region. We suggest that as ectoderm and mesoderm undergo morphogenetic movements during gastrulation, cells in both germ layers read their positional information coordinately from a single morphogen gradient located in Brachet's cleft.

The orchestration of tissue differentiation in the embryo to form a perfect individual time-after-time is a fascinating problem in developmental biology. The three germ layers—ectoderm, mesoderm and endoderm—are coordinately regulated to generate a well-organized body plan in which the various organs of the body develop. An experiment that opened the way for understanding embryonic cell differentiation was carried out in 1924 by Spemann and Mangold (1). They transplanted the dorsal side of the blastopore lip of an amphibian embryo, the region in which the involution of the mesoderm starts, into the ventral side of a host embryo and obtained Siamese twins. Their key discovery was the phenomenon of embryonic induction, in which the transplanted cells induced new cell fates on their neighbors, causing, for example, differentiation of central nervous system (CNS), somites, and kidneys. The inducing tissue was called the "organizer" because it induced the surrounding tissue to form a perfectly arranged secondary embryo. Spemann received the 1935 Nobel Prize in Physiology or Medicine for this work (reviewed in refs. 2 and 3).

Once molecular cloning became practical, a number of genes specifically expressed in Spemann's organizer were isolated, starting with the homeobox gene *goosecoid* (4). Through the work of several laboratories, it was found that organizer cells secrete a mixture of growth factor antagonists among which the

bone morphogenetic protein (BMP) antagonists Noggin, Follistatin, and Chordin are prominent (reviewed in refs. 5 and 6). BMPs are secreted growth factors of the TGF-β superfamily, first discovered by Urist at the University of California, Los Angeles as bone-inducing factors in decalcified bone matrix extracts (7). A morphogen gradient of BMP signaling plays the key role in the differentiation of cells into dorsal–ventral (D-V) tissue types in vertebrates and *Drosophila* (8, 9).

Embryos of the frog *Xenopus laevis* provide an excellent system to study the self-organizing properties of D-V patterning. When a blastula embryo is bisected, the ventral half forms a sphere consisting of ventral tissues (called a "belly-piece" by Spemann, ref. 1), whereas the dorsal half forms a well-proportioned embryo scaled to size. When the embryos are cut so that dorsal organizer tissue is present in both fragments, at low frequency, identical twins can be generated (10). These self-organizing properties of the embryo imply that cells can communicate with each other over very long distances. At the gastrula stage, the *Xenopus* embryo is 1.3 mm in diameter and consists of a single morphogenetic field of about 10,000 cells.

In the ectoderm, BMP signaling inhibition causes differentiation of the CNS, high levels of BMP induce epidermis, and intermediate concentrations induce neural crest. In the mesoderm, at low levels of BMP, signaling notochord is formed, and at progressively higher levels, kidney, lateral plate mesoderm (LPM), and blood tissues are induced (11, 12). Thus, histotypic differentiation depends on the graded activity of BMP signaling.

Significance

Cell differentiation in the embryo is regulated by diffusible substances called "morphogens," but these have never been directly visualized as endogenous components of the extracellular space. Chordin is an antagonist of the bone morphogenetic protein (BMP) pathway copiously secreted by a dorsal region of the *Xenopus* embryo called "Spemann's organizer" that has potent tissue-inducing activity. We report that Chordin protein forms a dorsal-to-ventral gradient in the embryo. This gradient is located in a narrow space containing extracellular matrix (ECM) that separates the ectoderm from the endomesoderm, which seems to serve as a highway for the diffusion of Chordin–BMP complexes over very long distances (2 mm) in the embryo. All vertebrate embryos have a similar ECM between ectoderm and mesoderm during gastrulation.

Remarkably, the three germ layers respond coordinately to changes in BMP signaling, which can cause dorsalization or ventralization of many tissues in the embryo (13). A key question investigated here, is whether a single signaling gradient or multiple ones are used to pattern D-V differentiation of the different germ layers.

BMP binding to its cell-surface Serine/Threonine kinase receptors leads to the phosphorylation of two carboxyl-terminal serines in the related transcription factors Smad1/5/8, causing them to translocate into the nucleus. The activation of these three proteins can be detected by a phosphospecific antibody (14, 15). In *Xenopus*, nuclei in the ventral side of the early gastrula are enriched for pSmad1/5/8 (16, 17). The transparency of the zebrafish embryo enabled the visualization of a gradient of BMP activity (18, 19). Despite the very extensive morphogenetic movements that take place during gastrulation (such as involution, epiboly, and convergence–extension), a surprisingly stable gradient of pSmad1/5/8 is maintained in the ventral side of the zebrafish early embryo (18).

The Chordin biochemical pathway plays a fundamental role in the formation of the self-organizing gradient of BMP activity in *Xenopus* (20). Chordin is a BMP antagonist secreted by Spemann organizer tissue (21, 22) that is produced in great excess: if uniformly distributed throughout the embryo, it would reach levels of 33 nM in the extracellular space during gastrulation, whereas BMPs would be present in the picomolar range (23). In the dorsal side, where it is produced, Chordin should reach much higher levels. Transplanted organizers depleted of Chordin with antisense morpholino oligos (MOs) lose all tissue-inductive power (24), and in the triple depletion of Chordin, Noggin, and Follistatin, all dorsal tissues are lost in *Xenopus* embryos (25). When bound to Chordin, BMPs cannot signal through BMP receptors, but this inhibition can be reversed by cleavage of Chordin at two specific sites by Tolloid metalloproteinases (26).

The degradation of Chordin–BMP complexes by Tolloid activity is rate limiting and tightly regulated. On the ventral side of the embryo, BMP signaling activates Smad1/5/8 through phosphorylation, causing the transcription of a secreted Frizzled-related protein (sFRP) called "Sizzled" that lacks Wnt-inhibiting activity (27). Sizzled is a competitive inhibitor of Tolloid activity, which binds to the enzyme active site and prevents the cleavage of Chordin (23, 28, 29). In addition, Tolloid protease activity is inhibited noncompetitively by direct binding of BMP proteins to its CUB (Complement 1r/s, Uegf, Bmp1) domains (30).

The opposite transcriptional control of D-V genes is a key feature of self-regulation. Ventral genes are activated by pSmad1/5/8, whereas dorsal components are transcribed when BMP signaling is low (31). The dorsal organizer secretes Chordin, two BMPs [called "BMP2" and "ADMP" (anti-dorsalizing morphogenetic protein)], the Olfactomedin-related Ont1 adaptor protein that bridges the binding of Chordin to Tolloid (32), and a close homolog of Sizzled called "Crescent" that inhibits both Tolloid and Wnt (33). The ventral side expresses BMP4 and BMP7, Sizzled, and Crossveinless 2 (CV2, also known as Bmper). CV2 binds BMPs and Chordin and has Cysteine-rich BMP-binding domains similar to those of Chordin but remains tethered to the surface of cells in which it is synthesized (34, 35).

Proteins of similar biochemical activities at opposite poles of the embryo can compensate for each other. For example, only when all four BMPs are depleted simultaneously does the D-V gradient collapse, turning the entire ectoderm into the CNS (31). In a similar way, Chordin and CV2, or Crescent and Sizzled, which are under reciprocal transcriptional regulation, compensate for the loss of each other (33, 35). Embryological experiments and mathematical modeling in *Xenopus* have led to the proposal that a D-V flux of BMPs bound to Chordin may increase the robustness of the self-organizing Chordin–BMP–Tolloid extracellular pathway (8, 31, 36). However, none of the

endogenous proteins of this morphogen pathway have ever been directly visualized in vertebrate embryos.

In the present study, we developed an improved immunolocalization method for *Xenopus* embryos and unexpectedly observed diffusion of overexpressed BMPs in a very narrow region of the gastrula embryo called "Brachet's cleft." This region of extracellular matrix (ECM) is rich in Fibronectin (37), and is well known in *Xenopus* (38) because it is used for the dissection of Keller mesodermal explants (39). An equivalent of Brachet's cleft is found in all vertebrate embryos, for this space corresponds to the ECM that separates the ectoderm from the endomesoderm. Using an affinity-purified Chordin antibody, we report here that endogenous Chordin protein forms a D-V gradient in the Brachet's cleft of gastrula embryos that extends to the ventral-most regions. The Chordin gradient was modified by depletion of components of the system such as Tolloid and Sizzled, and was complementary to the endogenous pSmad1/5/8-signaling gradient. Importantly, the gradient of Chordin protein was rescaled in dorsal half-embryos, and a second gradient formed in embryos with Spemann organizer transplants. The existence of a self-organizing Chordin gradient in Brachet's cleft suggests a unique mechanism by which an extracellular morphogen can coordinately regulate patterning in the ectoderm and endomesoderm during morphogenesis.

Results

Spemann organizer tissue secretes multiple growth factor antagonists such as the BMP antagonists Noggin, Follistatin, and Chordin; the Wnt antagonists Dkk1, Frzb, Crescent, and sFRP2; and the Nodal, Wnt and BMP antagonist Cerberus (8). Among these, Chordin plays an essential role, and a conserved biochemical pathway of Chordin-interacting extracellular proteins has been elucidated from studies in *Xenopus* (20), zebrafish (12), and *Drosophila* (9). As shown in Fig. 1A, a system of extracellular BMP regulators (black lines) is transcribed ventrally when BMP signaling is high and dorsally when it is low (blue lines),
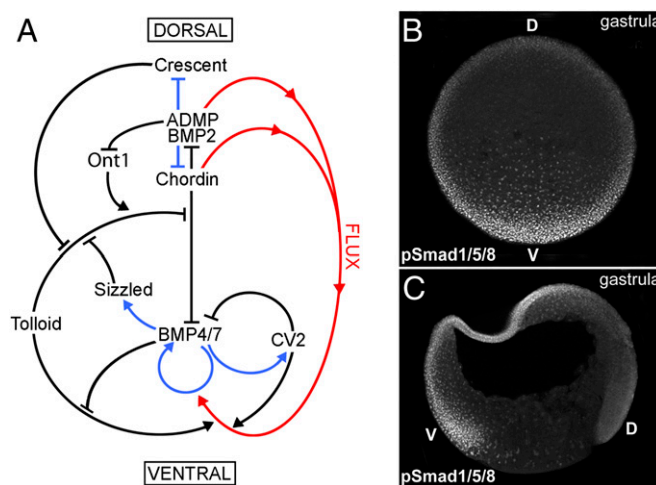
Fig. 1. A gradient of BMP activity patterns the D-V axis of the *Xenopus* gastrula. (*A*) BMP activity along the D-V axis results from a series of direct protein–protein interactions between Chordin and other partners (black arrows), transcriptional regulation (blue arrows), and protein flux (red arrows). The entire embryo participates in forming the BMP gradient, which results from the dueling activities of the dorsal and ventral signaling centers. (*B*) Transverse optical section at gastrula (stage 11) showing a ventral- (V) to-dorsal (D) gradient of BMP activity using anti-pSmad1/5/8 antibody as readout. (*C*) Gastrula (stage 11) embryo sectioned sagittally, showing higher BMP activity in the ventral animal cap and marginal zone nuclei as assessed by pSmad1/5/8 immunostaining.

and a flux of BMPs bound to Chordin (facilitated by proteolysis of Chordin by Tolloid) has been proposed (red lines) (8). The Chordin–BMP–Tolloid system is proposed to mediate cell–cell communication over long distances, so that for every action in the dorsal side there is a reaction in the ventral side of the gastrula. Despite extensive studies in *Xenopus* D-V patterning, the visualization of the endogenous distribution of any of the secreted proteins shown in Fig. 1*A* has not, to date, been possible.

Immunostaining the pSmad1/5/8 Gradient. We developed an improved protocol for immunostaining whole-mount *Xenopus* embryos, described in detail in *SI Materials and Methods*. Using this unique method, phosphorylated Smad1/5/8 was visualized as a continuous D-V gradient of nuclear protein in transverse optical sections (perpendicular to the animal–vegetal axis) of *Xenopus* embryos at mid- and late gastrula stages (Fig. 1*B*). In sagittal sections, nuclear pSmad1/5/8 was observed in the ventral marginal zone (future mesoderm) and animal cap (future epidermis), but only at low levels in the dorsal side (Fig. 1*C*). As expected, ventral BMP signaling was increased by the depletion of Chordin, a key BMP antagonist in *Xenopus* (Fig. S1 *A* and *B*). These results are in agreement with previous work in zebrafish showing a remarkably stable gradient of BMP signaling and maximal in the ventral side that is maintained throughout gastrulation (18).

Overexpressed BMPs Diffuse Along Brachet's Cleft. We next investigated whether overexpressed BMPs could diffuse through the embryo. We chose BMP2 and ADMP because these dorsally secreted BMPs (32, 40, 41) have been proposed to be transported by facilitated diffusion to the ventral side of the embryo bound to Chordin, where they are released by the cleavage of Chordin by Tolloid (Fig. 1*A*; ref. 31). Messenger RNAs for zADMP (42) and xBMP2 were tagged with GFP Venus protein and microinjected into a dorsal B1 blastomere at the 32-cell stage. At midgastrula, embryos targeted to dorsal ectoderm expressed high levels of protein in injected cells, and lower levels were found in nearby endomesoderm, as well (Fig. 2 *A* and *B*). Unexpectedly, we noticed that the injected fusion proteins also diffused along a narrow region of the embryo, indicated by arrowheads in Fig. 2 *A* and *B*. This space corresponded to Brachet's cleft (38), the thin extracellular region that separates the ectoderm from the anterior endoderm and mesoderm (Fig. 2*C*). Only BMP fusions were transported in this ECM, for a secreted construct of GFP alone (*Materials and Methods*) remained localized at the site of injection (Fig. S1*C*).

The diffusion of these secreted proteins specifically in Brachet's cleft suggested that the main morphogenetic gradient might be formed in the ECM between the ectoderm and the mesoderm, raising the possibility that a common gradient of growth factor activity may coordinately pattern two germ layers. During gastrulation, cells undergo extensive morphogenetic movements, and we propose that as they come in proximity to the ECM in Brachet's cleft, they read from it positional signals required for proper cell differentiation.

A Gradient of Chordin in Brachet's Cleft. We next asked whether endogenous components of the BMP signaling pathway could be detected in the ECM between the ectoderm and mesoderm. Chordin protein is very abundantly secreted (23), facilitating the study of its endogenous distribution in the embryo. A polyclonal antibody raised against the amino terminus of Chordin (26) was affinity purified and used to stain bisected *Xenopus* gastrulae (between stages 11 and 12). As shown in Fig. 3*A*, Chd staining was observed in dorsal endomesoderm (roof of the archenteron) and in Brachet's cleft. In the latter, a narrow line of Chordin staining, indicated by arrowheads, formed a gradient that extended ventrally. The Chordin signal was not limited to the
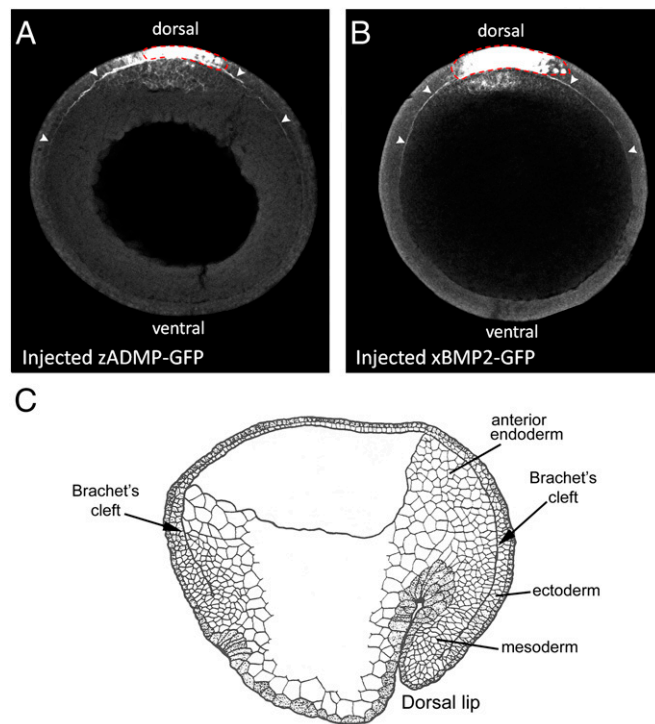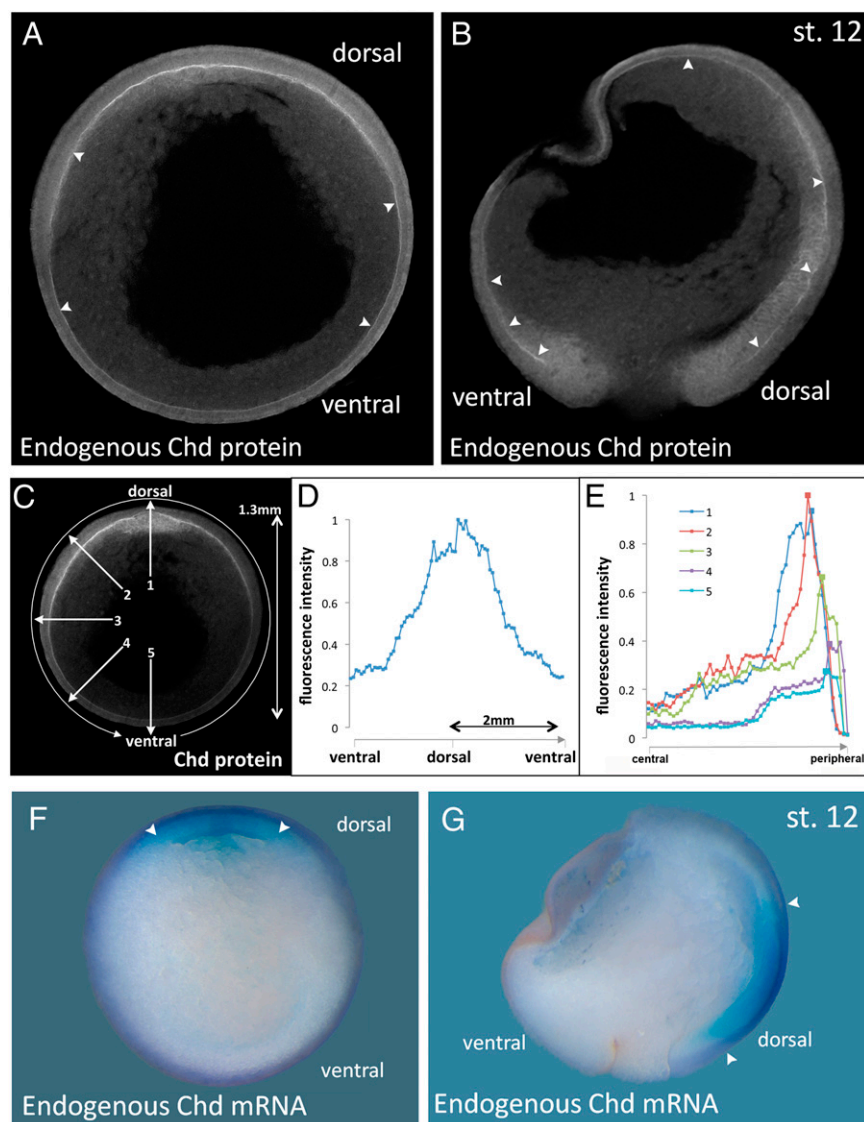

Fig. 2. Overexpressed BMP-GFP fusion proteins diffuse in Brachet's cleft. (*A* and *B*) Dorsally expressed zADMP-GFP and xBMP2-GFP fusion proteins diffuse within the narrow confines of Brachet's cleft (marked by arrowheads) away from the point of mRNA injection in ectodermal cells (indicated by a red dotted line) (*n* = 7 and *n* = 6, respectively). GFP fusion proteins were detected by GFP immunostainings of transverse optical sections of stage-12 embryos through the animal–vegetal axis. (*C*) Diagram of stage-12 sagittal section of a *Xenopus* embryo (after P. Nieuwkoop, ref. 38). Brachet's cleft is the narrow cavity that separates the mesodermal and anterior endodermal layers from the ectoderm and encircles the entire D-V axis.

narrow extracellular cleft and could be seen also staining, in a diffuse way, the adjoining ectoderm and mesoderm (Fig. 3 *A* and *C*). Although weak, fluorescence intensity in tissues next to Brachet's cleft could be quantified with ImageJ (http://rsbweb.nih.gov/ij/) in the radial direction (Fig. 3*E*), particularly compared with Chordin-depleted embryos (see Fig. S3). This orthogonal diffusion of Chordin–BMP may account for the uniformity of the pSmad1/5/8 gradient in cells that do not come in direct contact with Brachet's cleft. In sagittal sections, the extracellular staining was seen in dorsal mesoderm and in Brachet's cleft, which encircles the embryo, reaching its ventral-most side (arrowheads in Fig. 3*B*; anatomical structures shown in Fig. S2).

The gradient in Brachet's cleft could be traced around the entire circumference of the embryo using image analysis (Fig. 3*C*). Remarkably, the gradient extends over a long distance of about 2 mm, as indicated in Fig. 3*D*. When the Chordin signal was measured from the center toward the periphery (in the radial direction), peaks of Chordin protein could be detected in Brachet's cleft, progressively decreasing in the ventral direction but still clearly present even in the ventral-most cleft (Fig. 3 *C* and *E*). In contrast, *chordin* mRNA is transcribed only in dorsal regions (Fig. 3 *F* and *G*). These results indicate that Chordin protein is able to diffuse long range in the gastrula into regions that are distant from where it is produced.

Chordin staining in the ECM was entirely specific, for it was eliminated by depletion of Chordin with antisense MOs, as shown in Fig. S3. We conclude from these results that the Chordin antibody is specific, and that it detects an endogenous gradient of Chordin protein in Brachet's cleft that extends from the dorsal

to the ventral side of the embryo. Chordin was also released orthogonally from Brachet's cleft into neighboring tissues, probably explaining the smoothness of the nuclear pSmad1/5/8-signaling gradient.

Regulation of the Chordin and BMP Activity Gradients. We next tested how the D-V gradient was affected by depletion of regulators of Chordin stability. The Chordin protein gradient was reciprocal to the pSmad1/5/8 gradient both in sagittal (Fig. 4 *A* and *D*) and transverse (compare Fig. 4 *G* with *J*) optical sections. This was expected, as Chordin is a BMP signaling antagonist.

When Xolloid-related and BMP1, two of the Tolloid metalloproteinases that digest Chordin, were depleted with MOs, dorsalized (low-BMP) phenotypes were obtained (32). Tolloid depletion (Tld MO) resulted in decreased nuclear pSmad1/5/8 signal in the dorsal side (compare Fig. 4 *A* with *B* and *G* with *H*), and increased Chordin protein staining in Brachet's cleft (compare Fig. 4 *D* with *E* and *J* with *K*). Chordin stabilization was particularly striking in the ventral part of Brachet's cleft (indicated with arrowheads in Fig. 4 *E* and *K*) upon Tolloid knockdown.

Sizzled is an inhibitor of Tolloid activity expressed at high BMP-signaling levels in the ventral side. When Sizzled is depleted, Tolloid proteases become more active, causing Chordin

degradation and ventralized (high-BMP) phenotypes (23). Sizzled depletion resulted in a strong increase in nuclear pSmad1/5/8 accumulation, particularly in the ventral side of the embryo (compare Fig. 4 *A* with *C* and *G* with *I*), whereas Chordin protein was reduced in Brachet's cleft (but increased in dorsal ectoderm) (Fig. 4 *F* and *L*). Taken together, these results suggest that the depletion of genes that degrade (Tolloid) or stabilize (Sizzled) Chordin have strong effects on the levels of Chordin in Brachet's cleft and on the pSmad1/5/8-activity gradient.

The Chordin Gradient Rescales in Half-Embryos and Organizer Grafts. A fundamental question is whether the observed gradient of Chordin protein is able to self-organize after bisection of embryos, rescaling in the same way embryonic patterning does. In addition, some have expressed doubts that the D-V signaling gradient is self-regulating (43). An obstacle in carrying out half-embryo experiments was that our method for the visualization of the Chordin gradient requires embryos devoid of pigment. However, the darker pigmentation of the ventral side is what enables one to distinguish the dorsal from the ventral side to perform bisections at blastula stage 9, before the dorsal blastopore lip appears. We noticed that at the eight-cell stage, ventral blastomeres are not only darker, but adopt a unique butterfly-like
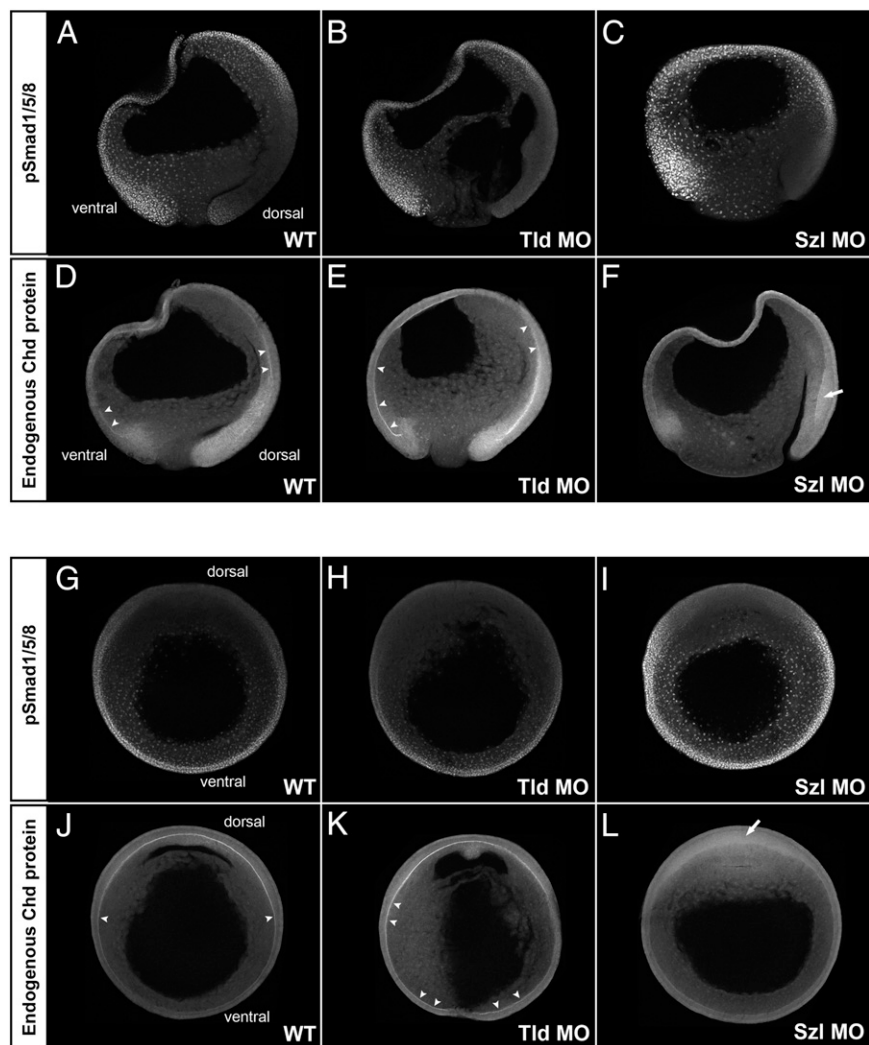
Fig. 4. Analysis of BMP signaling and Chordin protein localization in embryos depleted of Tolloid (Tld MO) or Sizzled (Szl MO). All embryos were siblings allowed to develop for the same period; all images were processed identically. (*A–F*) pSmad1/5/8 (*Upper*) and Chordin (*Lower*) immunostainings of sagittal optical sections. The gradient of BMP activity was complementary to that of Chordin localization in WT embryos (*Left*, $n = 6$ and $n = 4$, respectively). When translation of the Chordin-degrading enzyme Tolloid was inhibited in Tld MO-injected embryos (*Center*, $n = 5$ and $n = 3$, respectively), BMP activity was decreased on the dorsal side and increased accumulation of Chordin was observed in Brachet's cleft (arrowheads). Conversely, when Sizzled was depleted (Szl MO), BMP activity (nuclear pSmad1/5/8) was greatly increased in the embryo, and Chordin failed to accumulate in the Brachet's cleft (*Right*, $n = 3$). Diffuse accumulation of Chordin in dorsal ectoderm is indicated by the arrow. (*G–L*) pSmad1/5/8 (*Upper*) and Chordin (*Lower*) immunostainings of embryos sectioned transversely through the animal–vegetal axis at late gastrula (stage 12). pSmad1/5/8 staining was decreased in Tld MO embryos (*H*, $n = 6$) and increased in Szl MO embryos (*I*, $n = 4$) compared with WT (*G*, $n = 6$). Tld MO increased (*K*, $n = 7$) and Szl MO decreased (*L*, $n = 5$) Chordin staining in Brachet's cleft compared with WT (*J*, $n = 7$).

pattern extending toward the sides, whereas the dorsal blastomeres extend toward the equator (Fig. S4*A*). These peculiar shapes are observed only in regularly cleaving embryos (44) and, importantly, can be seen very distinctly in albino embryos (Fig. S4*B*). This allowed us to mark the ventral side by microinjection of a lineage tracer, such as Nile Blue, to carry out D-V dissections as accurately as in pigmented embryos (Fig. S4). We think that this simple observation, which allows one to reliably identify D-V polarity at a very early stage, will be of considerable benefit to the *Xenopus* field.

We examined endogenous pSmad1/5/8 and Chordin protein distribution using this method. Embryos were bisected at the blastula and allowed to develop until whole control embryos reached stage 12. As can be seen in Fig. 5 *A* and *B*, the gradient of pSmad1/5/8 was regenerated in dorsal halves. In ventral halves, which form a belly-piece consisting exclusively of ventral tissues, BMP signaling was uniform and unrestrained due to the lack of organizer signals, with nuclear pSmad1/5/8 reaching much higher levels (Fig. 5*C*) than those seen in normal embryos. Chordin protein formed a reciprocal gradient in Brachet's cleft of dorsal half-embryos (although not as uniform as in whole embryos) (Fig. 5*E*), whereas only background staining was seen in ventral halves (Fig. 5*F*). We conclude from these experiments that the pSmad1/5/8 and Chordin gradients can be rescaled according to embryonic size.

We next tested whether Chordin diffusion could be detected in embryos receiving Spemann organizer transplants (Fig. 6). To distinguish the grafted organizer, albino donor embryos were microinjected with mRNA encoding a GFP targeted to the cell membrane via a palmitoylation signal (membrane GFP or mGFP) (45). Albino embryos received the transplant in the ventral side at stage 10, as soon as the blastopore lip became distinguishable. Immunostainings of transversely bisected embryos were performed at late gastrula stage 12. The Chordin signal was detected in Brachet's cleft diffusing long-range from the GFP-positive grafted tissue, of which two examples are shown (Fig. 6 *B–E*, arrowheads) ($n = 19$). The results suggest that the Brachet cleft ECM provides a highway for the long-distance-facilitated diffusion of Chordin from ventrally transplanted organizer tissue.

Discussion

Localization of the Chordin Gradient in Vivo. A gradient of BMP signaling patterns the embryo in many species (15, 20), and it has been proposed that Chordin facilitates the D-V flux of BMPs in a Tolloid-regulated way (8, 9). However, the physical location of endogenous gradient-forming signaling components remains unknown. In this study we developed a unique method that improved immunolocalization of proteins in *Xenopus*. When the distribution of microinjected BMP2-GFP and ADMP-GFP was examined with anti-GFP antibodies, we were surprised to detect long-range diffusion of these proteins in Brachet's cleft, the
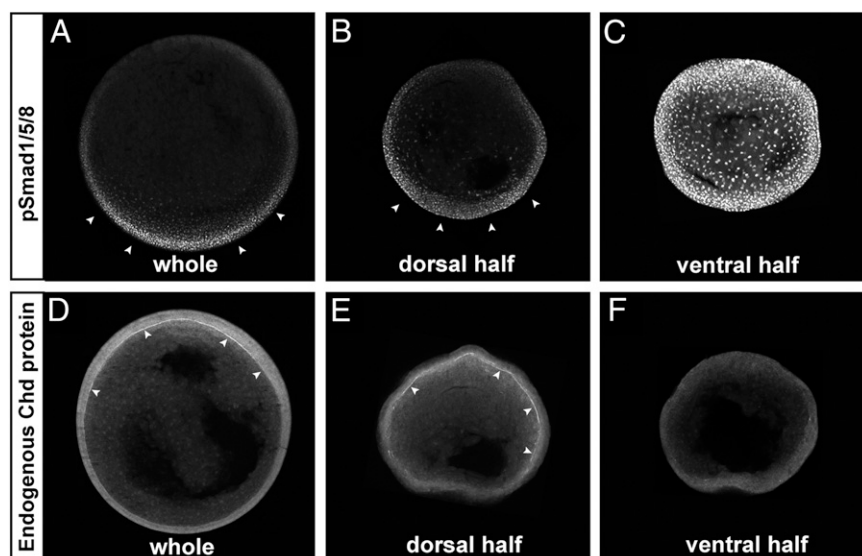
Fig. 5. The Chordin and pSmad1/5/8 gradients self-regulate in dorsal half-embryos. The ventral side of albino embryos was marked at the 8-cell stage using the method described in Fig. S4. Embryos were bisected at stage 9, cultured until stage 12, fixed, and sectioned transversely. All embryos were siblings and images were processed identically. (*A–C*) pSmad1/5/8 immunostaining of whole embryo (*A*, *n* = 3), dorsal (*B*, *n* = 6) or ventral (*C*, *n* = 3 of 4 showing the phenotype) half-embryos. Note that the BMP gradient was reestablished in the dorsal half-embryo, while very strong uniform pSmad1/5/8 was present in the ventral half-embryo. (*D–F*) Chordin immunostaining of whole embryo (*D*, *n* = 3), dorsal (*E*, *n* = 10) or ventral (*F*, *n* = 7) half-embryo. The Chordin gradient was regenerated in the Brachet's cleft of dorsal half-embryos (in 8 of 10), while no Chordin expression was detected in the ECM of the ventral half-embryo (in 6 of 7). Similar results were obtained in two independent experiments.

ECM that separates the ectoderm from the mesoderm and anterior endoderm (Fig. 2*C*). Using an affinity-purified polyclonal antibody against Chordin, we showed that an endogenous gradient of Chordin protein formed in Brachet's cleft at mid- and late gastrula, extending from dorsal to the most ventral regions of the embryo. The endogenous gradient of Chordin was the mirror image of the pSmad1/5/8 gradient of BMP activity. The Chordin staining was specific because it was absent from embryos depleted of Chordin.

Depletion of components of the Chordin biochemical pathway modified the gradients in the ways predicted by the pathway in Fig. 1*A*. Tolloid is an enzyme that degrades Chordin (26), and its depletion by Tld MOs (32) resulted in a shallower pSmad1/5/8 gradient. This was accompanied by the accumulation of endogenous Chordin protein in Brachet's cleft, which formed a more intense gradient that extended to the ventral-most ECM. Conversely, depletion of Sizzled (an inhibitor of Tolloid; ref. 23) increased pSmad1/5/8 signaling and decreased Chordin staining in Brachet's cleft. In an elegant recent study, Inomata et al. showed that a microinjected Chordin-GFP fusion was able to rapidly diffuse between neighboring cells in *Xenopus* ectodermal animal cap explants (29). Using fluorescence recovery after photobleaching, they showed that when Chordin is stabilized by the overexpression of Sizzled, it is able to rapidly diffuse. A similar diffusion constant was measured for Sizzled-GFP (29). Thus, these key proteins can efficiently diffuse in-between cells. Like Chordin, Sizzled is very abundantly secreted at gastrula stages (23), and it would be interesting to determine in the future the endogenous distribution of this ventrally produced protein.

A Self-Organizing Chordin Gradient. Since the classical work of Spemann, we know that the amphibian embryo has the remarkable property of a self-organizing pattern, one of the most mysterious properties of living organisms (2). The dorsal half of a bisected blastula embryo gives rise to embryos containing the normal allocations of tissue differentiations but scaled to a smaller size. We found that dorsal halves regenerated a Chordin protein gradient in Brachet's cleft, as well as a ventral gradient of pSmad1/5/8 BMP signaling. Ventral half-embryos, which lack a Spemann's organizer and differentiate into ventral tissues (31), contained very high, uniform levels of pSmad1/5/8 signaling, and did not stain with Chordin antibody. In embryos transplanted with Spemann organizer tissue in the ventral side, a second gradient of Chordin was formed in Brachet's cleft. The Chordin protein diffused a long distance from the lineage-marked

organizer graft. The Chordin gradient observed in ECM is self-regulating and resilient after experimental manipulations. In the future, it will be interesting to manipulate the formation of the cleft, by direct microinjection of proteins into Brachet's cleft or by depletion of components required for its formation, such as Frizzled-7, PAPC, and RhoA (46).

Chordin is secreted in large amounts (23), so it must reach extremely high concentrations within the narrow confines of Brachet's cleft. This unexpected endogenous graded localization could have important consequences for how we view embryonic patterning. The gradient of BMP activity that patterns both the ectoderm and the mesoderm may result from a single Chordin gradient, helping explain how dorsalizing or ventralizing treatments can affect both germ layers coordinately (13). It is possible that ectodermal and mesodermal cells in contact or in proximity to the Chordin/BMP gradient in the ECM receive Chordin/BMP signals released orthogonally in both directions, enabling cells to read their D-V positional cues. During gastrulation, the germ layers become patterned while simultaneously undergoing very extensive and rapid morphogenetic movements. Perhaps embryonic cells sense their positional information as they move along the ECM surface instead of depending solely on signals relayed in a "bucket-brigade" fashion from cell to cell.

Comparison with Other Systems. There is a precedent in the literature in which the ECM has been considered as a highway for long-range facilitated diffusion of growth factors. Studying left–right asymmetry in *Xenopus* embryos, Marjoram and Wright discovered that the ECM flanking the LPM provides a principal surface for the accumulation and facilitated transport of Nodal and its antagonist Lefty at the tailbud stage (47). Using animal cap grafts injected with tagged mRNAs, they demonstrated long-range diffusion of both Nodal and Lefty along the ECMs that separate the ectoderm and endoderm from the LPM. It was found that Nodal moved rostral-ward through the ECM highway, whereas the inhibitor Lefty diffused faster in ECM and also orthogonally into deep adjacent tissue, eventually reaching the opposite side of the embryo. The ECM regions flanking LPM contain Fibronectin and heparan sulfate proteoglycans (HSPGs) (47). Brachet's cleft is the direct precursor to the ECM that separates the epidermis from the LPM at tailbud stages. In our own study, at the neurula stage, Chordin protein was also detected in the space between the presomitic mesoderm/LPM and the endoderm. Thus, the long-range diffusion of morphogens along the ECM separating cell layers might be a general
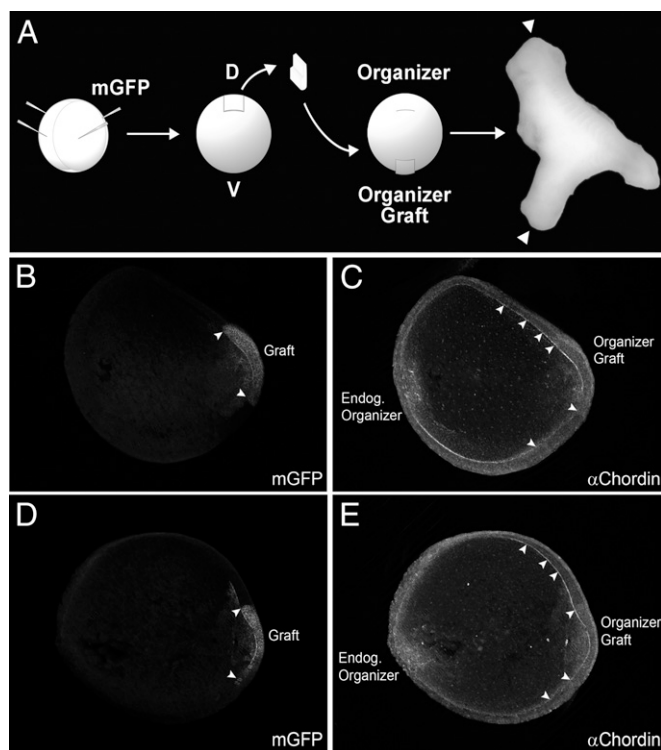
Fig. 6. Chordin protein diffuses long-range in Brachet's cleft of embryos transplanted with Spemann organizer tissue. (*A*) Diagram of experimental procedure. mGFP was injected in the donor embryo (four injections at the four-cell stage) to trace the lineage of grafted tissue. Secondary axes were complete (with heads, indicated with arrowheads in this photograph) in 75% of the cases (*n* = 6/8). (*B–E*) Two embryos transplanted at stage 10 and fixed at stage 12 stained with GFP and Chordin antibodies are shown in optical transverse sections. *B* and *D* show localization of the grafted organizer tagged with mGFP; borders are indicated by arrowheads. In *C* and *E*, a second gradient of Chordin was observed, with Chordin protein staining in Brachet's cleft extending a considerable distance from the graft (arrowheads) (*n* = 19). This indicates that Chordin protein diffuses from the graft through Brachet's cleft.

phenomenon in growth-factor signaling in embryonic development. Syndecan-1, a transmembrane HSPG that has been shown to bind Chordin (48), is expressed in the inner layer of the ectoderm immediately adjacent to Brachet's cleft at the gastrula stage in *Xenopus* (49) and could regulate diffusion.

The Chordin/BMP pathway is ancestral and conserved in organisms as diverse as *Drosophila*, spiders, amphioxus, hemicordates, and vertebrates (50). The *Drosophila* homolog of Chordin is called "Short gastrulation" (Sog) and is expressed in the ventral side (low-BMP, where the CNS is formed), for the D-V axis was inverted in the course of evolution. Sog is required to shuttle BMPs (called "Dpp" and "Screw") to the dorsal side to achieve maximal signaling (51, 52). Endogenous Sog protein has been shown to diffuse at a distance from its source during *Drosophila* D-V patterning (53). However, Sog protein was only detected in dorsal blastoderm cells after its endocytosis, as the method used did not allow direct staining in the outer ECM itself.

Facilitated diffusion, driven by Tolloid proteolysis, during D-V patterning may take place in the perivitelline space separating the embryonic membrane from the innermost layer of the egg shell. Indeed, Sog protein is enriched in the apical side (toward the outside) of the cells that secrete it in the blastoderm embryo (53). In another study, anti-GFP antibodies microinjected into the perivitelline space were shown to be transported together with Dpp-GFP and concentrated on BMP receptors in the dorsal-most cells of the embryo (54). The requirement for a functional ECM

during D-V patterning has been demonstrated in *Drosophila*, as Type IV Collagen is necessary for patterning by Sog/Dpp (55). However, because Type IV Collagen accumulates in both the basal (basal lamina) and apical (perivitelline) surfaces of the blastoderm epithelium, it is unclear where the gradient is actually formed. It will be important in the future to determine directly whether a Sog gradient diffuses in the perivitelline ECM or is generated by a cell-to-cell relay mechanism. If the Sog gradient were formed in the perivitelline ECM, this would raise the possibility that this region is the topological homolog of Brachet's cleft.

All vertebrate embryos contain an ECM equivalent to Brachet's cleft in the space between the ectoderm and the endomesoderm; perhaps a Chordin/BMP gradient is formed in this region in other embryos such as zebrafish, chick, and mouse. We have analyzed here the primary embryonic morphogenetic field, yet there are many other later "secondary" self-organizing fields that are formed during organogenesis (e.g., eye, olfactory, pituitary, and limb fields) and regeneration (2, 56). Perhaps long-range-signaling gradients of growth factors and their regulators, emanating from opposite poles of the fields and facilitated by diffusion in the ECM, might be worthwhile investigating in these systems, as well.

Materials and Methods

Antibodies. An anti-Chordin rabbit polyclonal antiserum raised against the 19 amino-terminal residues of *Xenopus* Chordin (22) was affinity-purified as described in *SI Materials and Methods* and used at 1:100. Other antibodies used were rabbit polyclonal anti-pSmad1/5/8 (Cell Signaling 9511L, 1:100), rabbit anti-GFP (Molecular Probes 1:200), and mouse anti-GFP (Santa Cruz Biotechnology 9996, 1:100). Cy3-conjugated goat anti-rabbit antibody (Jackson Immunoresearch, 111–166-003) was used at 1:500 for Chordin staining. For lineage tracing of Spemann's grafts injected with mGFP mRNA, mouse anti-GFP was used and visualized using Dylight 488-conjugated donkey anti-mouse as the secondary antibody (Jackson Immunoresearch 715–485-150, 1:500).

Immunostaining and in Situ Hybridization. A step-by-step protocol of the immunostaining procedure is provided in *SI Materials and Methods*. Some of the steps used to decrease nonspecific background in optical sections included bisection of fixed embryos, treatment of albino embryos in methanol/H_2O_2 to reduce autofluorescence, Guanidinium·HCl for antigen retrieval, reduction of disulfide bonds with DTT followed by blocking of SH groups with iodoacetate to increase signals of extracellular proteins, treatment with sodium borohydride to reduce aldehydes and ketones to alcohols to further decrease autofluorescence, and use of Murray's solution (benzyl alcohol/benzyl benzoate) to render embryos transparent. Chordin and pSmad1/5/8 were visualized using a Cy3-conjugated secondary antibody (the red channel has lower autofluorescence in *Xenopus*) and Dylight 488-conjugated secondary antibody was used for GFP staining. Images were acquired and processed identically with an Apotome or a confocal microscope (Zeiss) using a 5× objective and AxioVision 4.8 software (Zeiss). The gradient was quantified using ImageJ, as explained in *SI Materials and Methods*. For in situ hybridizations, see www.hhmi.ucla.edu/derobertis/index.html.

Embryological Methods. Antisense MOs used for knockdown of Chordin (24), Tolloids (32), and Sizzled (27) were as described. The zADMP and xBMP2-GFP constructs are described in *SI Materials and Methods*. For DNA injections, 25 pg of plasmid were injected in a single animal cell into the B1 dorsal blastomere at the 32-cell stage in albino embryos. The dorsal location of the injected cell in ectoderm was confirmed by fluorescent confocal imaging after immunostaining. Marking of the presumptive dorsal or ventral side of the embryo was done by subcortical injection of Nile Blue dye into a single cell at the 8-cell stage using the morphological recognition criteria as described in the legend to Fig. S4. Bisection experiments were done at the blastula stage as described (31), and half-embryos were allowed to develop until control siblings reached stage 12. Spemann grafts were performed as described (24), except that transplantation was performed in 1× Steinberg solution and grafted embryos were transferred after 1 h into 0.1× Steinberg for culture until stage 12. Research using *Xenopus* embryos has been approved by the University of California, Los Angeles Office of Animal Research Oversight and the Chancellor's Animal Research Committee.

1. Spemann H, Mangold H (1924) Induction of embryonic primordial by implantation of organizers from a different species. *Roux Arch Entw Mech* 100:599–638. [Reprinted and translated in De Robertis EM, Arechaga J, eds. (2001) *Int J Dev Biol* 45(1):13–31].
2. Spemann H (1938) *Embryonic Development and Induction* (Yale Univ Press, New Haven, CT). [Reprinted (1962) (Hafner, New York)].
3. Hamburger V (1988) *The Heritage of Experimental Embryology: Hans Spemann and the Organizer* (Oxford Univ Press, Oxford).
4. Cho KWY, Blumberg B, Steinbeisser H, De Robertis EM (1991) Molecular nature of Spemann's organizer: The role of the *Xenopus* homeobox gene *goosecoid*. *Cell* 67(6): 1111–1120.
5. Harland R, Gerhart J (1997) Formation and function of Spemann's organizer. *Annu Rev Cell Dev Biol* 13:611–667.
6. De Robertis EM, Kuroda H (2004) Dorsal-ventral patterning and neural induction in *Xenopus* embryos. *Annu Rev Cell Dev Biol* 20:285–308.
7. Urist MR (1965) Bone: Formation by autoinduction. *Science* 150(3698):893–899.
8. De Robertis EM (2009) Spemann's organizer and the self-regulation of embryonic fields. *Mech Dev* 126(11-12):925–941.
9. Umulis D, O'Connor MB, Blair SS (2009) The extracellular regulation of bone morphogenetic protein signaling. *Development* 136(22):3715–3728.
10. De Robertis EM (2006) Spemann's organizer and self-regulation in amphibian embryos. *Nat Rev Mol Cell Biol* 7(4):296–302.
11. Heasman J (2006) Patterning the early *Xenopus* embryo. *Development* 133(7): 1205–1217.
12. Langdon YG, Mullins MC (2011) Maternal and zygotic control of zebrafish dorso-ventral axial patterning. *Annu Rev Genet* 45:357–377.
13. Kao KR, Elinson RP (1988) The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. *Dev Biol* 127(1):64–77.
14. Massagué J (2012) TGFβ signaling in context. *Nature* 13(10):616–630.
15. Ramel MC, Hill CS (2012) Spatial regulation of BMP activity. *FEBS Lett* 586(14): 1929–1941.
16. Faure S, Lee MA, Keller T, ten Dijke P, Whitman M (2000) Endogenous patterns of TGFbeta superfamily signaling during early *Xenopus* development. *Development* 127(13):2917–2931.
17. Schohl A, Fagotto F (2002) Beta-catenin, MAPK and Smad signaling during early *Xenopus* development. *Development* 129(1):37–52.
18. Tucker JA, Mintzer KA, Mullins MC (2008) The BMP signaling gradient patterns dorsoventral tissues in a temporally progressive manner along the anteroposterior axis. *Dev Cell* 14(1):108–119.
19. Hashiguchi M, Mullins MC (2013) Anteroposterior and dorsoventral patterning are coordinated by an identical patterning clock. *Development* 140(9):1970–1980.
20. Plouhinec JL, De Robertis EM (2009) Systems biology of the self-regulating morphogenetic gradient of the *Xenopus* gastrula. *Cold Spring Harb Perspect Biol* 1(2): a001701.
21. Sasai Y, et al. (1994) Xenopus *chordin*: A novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* 79(5):779–790.
22. Piccolo S, Sasai Y, Lu B, De Robertis EM (1996) Dorsoventral patterning in Xenopus: Inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* 86(4):589–598.
23. Lee HX, Ambrosio AL, Reversade B, De Robertis EM (2006) Embryonic dorsal-ventral signaling: Secreted frizzled-related proteins as inhibitors of tolloid proteinases. *Cell* 124(1):147–159.
24. Oelgeschläger M, Kuroda H, Reversade B, De Robertis EM (2003) Chordin is required for the Spemann organizer transplantation phenomenon in Xenopus embryos. *Dev Cell* 4(2):219–230.
25. Khokha MK, Yeh J, Grammer TC, Harland RM (2005) Depletion of three BMP antagonists from Spemann's organizer leads to a catastrophic loss of dorsal structures. *Dev Cell* 8(3):401–411.
26. Piccolo S, et al. (1997) Cleavage of Chordin by Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. *Cell* 91(3): 407–416.
27. Collavin L, Kirschner MW (2003) The secreted Frizzled-related protein Sizzled functions as a negative feedback regulator of extreme ventral mesoderm. *Development* 130(4):805–816.
28. Muraoka O, et al. (2006) Sizzled controls dorso-ventral polarity by repressing cleavage of the Chordin protein. *Nat Cell Biol* 8(4):329–338.
29. Inomata H, Shibata T, Haraguchi T, Sasai Y (2013) Scaling of dorsal-ventral patterning by embryo size-dependent degradation of Spemann's organizer signals. *Cell* 153(6): 1296–1311.
30. Lee HX, Mendes FA, Plouhinec JL, De Robertis EM (2009) Enzymatic regulation of pattern: BMP4 binds CUB domains of Tolloids and inhibits proteinase activity. *Genes Dev* 23(21):2551–2562.
31. Reversade B, De Robertis EM (2005) Regulation of ADMP and BMP2/4/7 at opposite embryonic poles generates a self-regulating morphogenetic field. *Cell* 123(6):1147–1160.
32. Inomata H, Haraguchi T, Sasai Y (2008) Robust stability of the embryonic axial pattern requires a secreted scaffold for chordin degradation. *Cell* 134(5):854–865.
33. Ploper D, Lee HX, De Robertis EM (2011) Dorsal-ventral patterning: Crescent is a dorsally secreted Frizzled-related protein that competitively inhibits Tolloid proteases. *Dev Biol* 352(2):317–328.
34. Serpe M, et al. (2008) The BMP-binding protein Crossveinless 2 is a short-range, concentration-dependent, biphasic modulator of BMP signaling in Drosophila. *Dev Cell* 14(6):940–953.
35. Ambrosio AL, et al. (2008) Crossveinless-2 Is a BMP feedback inhibitor that binds Chordin/BMP to regulate Xenopus embryonic patterning. *Dev Cell* 15(2):248–260.
36. Ben-Zvi D, Shilo BZ, Fainsod A, Barkai N (2008) Scaling of the BMP activation gradient in Xenopus embryos. *Nature* 453(7199):1205–1211.
37. Marsden M, DeSimone DW (2001) Regulation of cell polarity, radial intercalation and epiboly in Xenopus: Novel roles for integrin and fibronectin. *Development* 128(18): 3635–3647.
38. Nieuwkoop PD, Florschütz PA (1950) Quelques caractères spéciaux de la gastrulation et de la neurulation de l'œuf de *Xenopus laevis*, Daud. et de quelques autres Anoures. *Arch Biol (Liege)* 61:113–150.
39. Keller R (1991) Early embryonic development of Xenopus laevis. *Methods Cell Biol* 36: 61–113.
40. 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(12):4293–4301.
41. Inui M, et al. (2012) Self-regulation of the head-inducing properties of the Spemann organizer. *Proc Natl Acad Sci USA* 109(38):15354–15359.
42. Willot V, et al. (2002) Cooperative action of ADMP- and BMP-mediated pathways in regulating cell fates in the zebrafish gastrula. *Dev Biol* 241(1):59–78.
43. François P, Vonica A, Brivanlou AH, Siggia ED (2009) Scaling of BMP gradients in Xenopus embryos. *Nature* 461(7260):E1, discussion E2.
44. Klein SL (1987) The first cleavage furrow demarcates the dorsal-ventral axis in *Xenopus* embryos. *Dev Biol* 120(1):299–304.
45. Kim SH, Yamamoto A, Bouwmeester T, Agius E, Robertis EM (1998) The role of paraxial protocadherin in selective adhesion and cell movements of the mesoderm during Xenopus gastrulation. *Development* 125(23):4681–4690.
46. Gorny AK, Steinbeisser H (2012) Brachet's cleft: A model for the analysis of tissue separation in Xenopus. *Wiley Interdiscip Rev Dev Biol* 1(2):294–300.
47. 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(3):475–485.
48. Jasuja R, Allen BL, Pappano WN, Rapraeger AC, Greenspan DS (2004) Cell-surface heparan sulfate proteoglycans potentiate chordin antagonism of bone morphogenetic protein signaling and are necessary for cellular uptake of chordin. *J Biol Chem* 279(49):51289–51297.
49. Teel AL, Yost HJ (1996) Embryonic expression patterns of *Xenopus* syndecans. *Mech Dev* 59(2):115–127.
50. De Robertis EM (2008) Evo-devo: Variations on ancestral themes. *Cell* 132(2):185–195.
51. Holley SA, et al. (1996) The Xenopus dorsalizing factor noggin ventralizes Drosophila embryos by preventing DPP from activating its receptor. *Cell* 86(4):607–617.
52. Ashe HL, Levine M (1999) Local inhibition and long-range enhancement of Dpp signal transduction by Sog. *Nature* 398(6726):427–431.
53. Srinivasan S, Rashka KE, Bier E (2002) Creation of a Sog morphogen gradient in the Drosophila embryo. *Dev Cell* 2(1):91–101.
54. Wang YC, Ferguson EL (2005) Spatial bistability of Dpp-receptor interactions during Drosophila dorsal-ventral patterning. *Nature* 434(7030):229–234.
55. Wang X, Harris RE, Bayston LJ, Ashe HL (2008) Type IV collagens regulate BMP signalling in Drosophila. *Nature* 455(7209):72–77.
56. De Robertis EM, Morita EA, Cho KWY (1991) Gradient fields and homeobox genes. *Development* 112(3):669–678.

Supporting Information

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SI Materials and Methods

***Xenopus laevis* Immunostaining Protocol.** In general, for all steps, embryos were handled in 4-mL glass screw cap vials (Fisherbrand catalog no. 03–339-25B). Unless otherwise noted, a volume of 2.5–3 mL of solution was used per vial, and during incubations, tubes were left gently rocking horizontally on an orbital shaker. A benchtop vacuum pump connected to a 200-µL plastic pipet tip or Pasteur pipet can be used for removal of solutions from vials. Samples should not be left to dry between washes.

Fixation and Storage of Embryos.

i) Fix albino embryos at desired stage in MEMFA (MOPS, EGTA and Magnesium sulfate salts and Formaldehyde) (containing half the amount of salts than usual, see *Solutions*) overnight or longer at 4 °C.
ii) Wash in PBS for 5 min.
iii) Transfer embryos to an agarose-coated Petri dish filled with PBS.
iv) Cut embryos in half along the intended imaging plane with a scalpel blade. Holes previously imprinted in the agarose with a heated pasteur pipette can be useful to keep embryos in the desired orientation during sectioning. Hemisectioning of embryos improves penetration of antibodies into deeper tissues and prevents antibody trapping inside internal cavities.
v) Dehydrate in methanol (MeOH) 25% (vol/vol), 50%, and 70% in H_2O for 5 min each, wash twice in MeOH 100%, and store at −20 °C overnight or up to a few weeks.

Immunostaining Day 1.
Bleach to remove autofluorescence.

i) Rinse embryos in MeOH 100%.
ii) Bleach embryos in 2:1 MeOH: 30% H_2O_2 for 1.5–2 h.
iii) Rehydrate embryos in MeOH 70%, 50%, and 25% in H_2O for 5 min each.

Further remove autofluorescence by reduction with sodium borohydride (optional).

i) Wash twice in Tris Buffered Saline (TBS) for 5 min.
ii) Incubate embryos in 100 mM sodium borohydride ($NaBH_4$) in TBS for 1.5–2 h in a fume hood. $NaBH_4$ releases hydrogen gas when mixed with water and should be handled with caution. Because reduction of aldehydes and ketones into alcohols by $NaBH_4$ produces bubbles, tubes should be left upright with the cap partially unscrewed to prevent pressure build up, and shaking should be avoided. Cutting embryos in half prevents trapping of bubbles inside the embryos.
iii) $NaBH_4$ solution should be carefully removed using a Pipetman (Gilson) as embryos tend to stick to bubbles and float. Do not allow embryos to dry.
iv) Wash embryos two to three times in TBS+0.1% Tween 20 (TBST) for 5 min, adding the buffer dropwise and tapping the tube to prevent embryos from floating.

Antigen retrieval (optional; recommended for detection of extracellular proteins containing disulfide bridges).

i) Wash twice in TBS for 5 min.
ii) Remove all buffer from the tube using a pipetman.
iii) Add 1 mL of Guanidinium·HCl antigen retrieval buffer and let embryos sink.
iv) Add 20 µL of DTT 1 M (20 mM final), mix well, and incubate for 1 h at room temperature (RT). Keep tubes in upright

position with occasional manual swirling. DTT will open disulfide bridges.
v) Add 1.6 mL of iodoacetate 500 mM pH 8.5 (toxic), and incubate for 1 h at 37 °C. Keep tubes upright with occasional swirling. Iodoacetate will react with sulfhydryl groups present in cysteines in an irreversible manner and prevent disulfide bridges from reforming.
vi) Wash twice in TBS for 5 min.

Block embryos and add primary antibody.

i) Wash twice in TBST containing 0.2% BSA for 10 min.
ii) Incubate embryos in blocking solution (TBST containing 5% BSA) for 1 h at RT.
iii) Incubate embryos in primary antibody diluted in blocking solution overnight at 4 °C, with rocking. At least 500 µL to 1 mL of antibody solution should be used so that embryos are covered at all times. If the amount of antibody is limited, embryos can be transferred to 500-µL glass vials (Fisher) using 100–200 µL of antibody solution. The optimal dilution of the primary antibody should be determined by empirical tests. Antibody solution may be recovered after incubation, stored at 4 °C for a few days or at −20 °C for a few weeks, and reused once or twice. Antibody solution should always be centrifuged before use for 10 min at top speed in a benchtop centrifuge (Eppendorf centrifuge model 5415C) to remove protein aggregates and transferred to a fresh tube. A protocol for preblocking of primary antibodies to decrease nonspecific binding is provided below.

Immunostaining Day 2.

i) Wash embryos seven times in TBST containing BSA 0.2% (quick wash + 5 min + 30 min + 4 × 45 min to 1 h).
ii) Incubate embryos in blocking solution for 1 h at RT.
iii) Incubate embryos with a fluorescent secondary antibody (1:1,000) in 1 mL blocking solution overnight at 4 °C, and rocking in the dark (aluminum foil). Cy3-conjugated (red) secondary antibodies are preferred to Dylight 488-conjugated (green) antibodies because less autofluorescence is observed in the red channel. However, Dylight 488-conjugated secondary antibodies were used successfully for double immunostainings. Cy5-conjugated (or equivalent far-red or infrared antibodies) have not been tested but could display less autofluorescence. Antibody solution should always be centrifuged before use to remove protein aggregates and transferred to a fresh tube. Preblocking of secondary antibodies is usually not required.

Immunostaining Day 3. Note: All steps should be carried out in the dark (aluminum foil) to prevent bleaching of the fluorescent antibody signal.
Washes.

i) Wash embryos seven times in TBST containing BSA 0.2% (quick wash + 5 min + 30 min + 4 × 45 min to 1 h).
ii) Wash embryos in TBS for 10 min.
iii) Wash in MeOH 70% in H_2O for 5 min.
iv) Wash in MeOH 100% quickly, then three times for 10 min. At this point, embryos can be stored at RT for the day, or overnight at 4 °C without significant fading of the signal.

Clear embryos.

i) When ready to image, remove MeOH and quickly add 3 mL of Murray's clear. Do not let embryos dry up.

ii) Leave tubes standing upright in the dark for 10–20 min until embryos are clear and sink. At this point, embryos are mostly invisible, but might be observed as a faint translucent reddish color when the tube is held toward a light source. Embryos should be handled with care because Murray's solution makes them very brittle. Many embryos are lost at this stage.

iii) Discard most of the Murray's solution and replace with 2 mL fresh Murray's solution. While keeping the tubes upright, carefully remove the MeOH–Murray's solution mixture that sits at the top of the tube (because Murray's clear is denser than MeOH), until ∼500 μL of solution is left. Do not store embryos in Murray's clear over multiple days because fluorescent signal fades quickly in this solution.

Imaging. Embryos are imaged using a slide with an open chamber of the following design:

i) Clean coverslips (20 × 50 mm) with a Kimwipe (Kimberly-Clark).

ii) Prepare coverslips by adding small spheres of modeling clay (pastel color Claytoon, Van Aken) at each corner. Enough clay should be added to prevent the coverslip from crushing the embryos. If too much is used, Murray's will flow off. Because bisected embryos are about 700-μm thick, the aim for thickness should be 1–1.5 mm.

iii) Clean slides carefully before use with ethanol and a Kimwipe.

iv) Carefully transfer embryos onto a microscope slide. A wide enough plastic pipet should be used to prevent damage to the embryos. Embryos are spotted by looking through the tube oriented toward a light source and aspirated by bringing the pipet close enough to the embryos but without touching them. To ensure embryos have been transferred onto the slide, ensure slide is slightly tilted and excess Murray's aspirated with the plastic pipet. Embryos will appear as small bumps.

v) Add the coverslip carefully.

vi) Slowly add Murray's solution to fill the space between slide and coverslip. It takes about 1 mL for a 20 × 50-mm coverslip.

vii) Embryos are imaged using an apotome or confocal microscope. Slides should be handled carefully. Murray's solution might leak and is not easily cleaned off from microscopes and objectives and may dissolve plastics. This setting has not been tested on an inverted microscope.

Affinity-Purification of Anti-Chordin Antibody. Anti-Chordin antibody was raised against the 19 amino-terminal residues that follow the signal sequence of *Xenopus* Chordin, conjugated to KLH carrier protein (1); and 1.5 mL of antiserum was affinity-purified using nitrocellulose-bound baculovirus recombinant xChordin (1). Recombinant xChordin (500 μL of serum-free conditioned medium) was run in a Novex 4–20% SDS Tris·Glycine gel (Invitrogen) using standard loading buffer without DTT or β-Mercaptoethanol and electroblotted to nitrocellulose membrane. Membrane was stained for 1 h in Ponceau S (0.1% Ponceau S, 1% acetic acid) and destained in water. The xChordin band was excised with a razor blade and a nitrocellulose strip was further destained in 1% acetic acid, followed by water and TBST washes, and blocked overnight in 5% BSA in TBST; 0.5 mL of antiserum were incubated for 3 h at RT with the nitrocellulose strip (cut in half), after adding 200 μL 10x TBST, 1 mL 10% BSA and 300 μL water, in a 2-mL Eppendorf tube placed on a rotating wheel. Following washes in 1x TBST (1 h followed by four times 5 min), low pH elution was performed with 1 mL 0.2 M Glycine·HCl pH 2.5, 0.01% Tween for 2 min and neutralized with 0.4 mL 1 M Tris·HCl pH 8. Purified antibodies were desalted twice in Amicon 30 kDa ultra-0.5 centrifugal filters (Millipore), using 1–5 dilutions in TBS. Final antibody volume was 100 μL in TBS and storage was at 4 °C or −20 °C.

Embryo Powder for Blocking Primary Antibody. Embryo powder blocks nonspecific epitopes that may be recognized by a primary polyclonal antibody and will compete nonspecific binders. The following procedure may not be necessary for monoclonal antibodies which target specific antigens.

Preparing embryo powder.

i) Fix a large batch of *Xenopus* embryos in 50 mL MEMFA in a Falcon tube (BD Biosciences) overnight at 4 °C. The embryos should be collected at a stage where the protein targeted by the antibody is not expressed: for an antibody directed against a protein exclusively expressed after midblastula transition, such as Chordin, stage-8 embryos work well; for an exogenous protein like GFP, embryos from the same stages used for the immunostaining are best (gastrula-to tailbud-stage embryos dissociate easily).

ii) Remove all MEMFA and wash once in 50 mL PBS and once in 50 mL PBS+0.1% Tween 20 (PBST) for 30 min each.

iii) Leave just enough buffer to cover the embryos and pipet up and down with a 1-mL pipet tip with a wide opening (2 mm) until the embryos are reduced to a fine powder.

iv) Wash twice with 50 mL PBST.

v) Rock the tube for 1 h at RT, let tube stand upright until powder has sedimented, and discard the supernatant. Repeat washes until the supernatant is clear and the milky aspect has disappeared.

vi) Wash twice in 50 mL MeOH 100% (same procedure as the previous step). Repeat washes until the supernatant is clear and the milky aspect has disappeared. The resulting embryo powder can be stored in aliquots in the last MeOH wash for a few months at −20 °C.

vii) Wash once in 50 mL TBST, then in TBST containing BSA 0.2% for 15 min at RT rocking.

viii) Transfer powder to a 15-mL Falcon tube and wash with 15 mL of blocking solution.

ix) Leave just enough buffer to cover powder. The powder is now ready to use (excess powder can be stored at −20 °C, although it is not optimal for its preservation).

Preblocking primary antibody.

i) On the day before the start of immunostaining, preblock the primary antibody in 100–500 μL of embryo powder with 2 mL of primary antibody diluted in blocking solution. Proportions can be adjusted empirically. The antibody can be diluted at 5–10x its working concentration to prepare a concentrated stock for use the next day.

ii) On the next day, spin tube for 2 min at 2,000 rpm to sediment the powder, recover the supernatant, spin again for 10 min at 12,000 rpm to remove any remaining aggregates, and recover the supernatant that can be used as is or further diluted in blocking solution.

Solutions.

TBS. 50 mM Tris·HCl pH 7.6, 150 mM NaCl. From 10x stock.

TBST. TBS with 0.1% Tween 20. From 10x stock.

Blocking solution. TBST containing 5% BSA.

BSA 10%. Dissolve 5 g BSA fraction V (Sigma) in 30 mL H_2O. Adjust total volume to 50 mL Filter with a 0.2-μm filter and store at 4 °C.

PBS. Dissolve 8 g NaCl, 0.2 g KCl, 1.78 g sodium phosphate dibasic, 0.27 g potassium phosphate monobasic in 100 mL H_2O final for 10x stock, pH 7.4.

MEMFA. Prepare a 10x MEMFA salt solution containing 1 M MOPS pH 7.4, 20 mM EGTA, and 10 mM magnesium sulfate. On the day of use, mix 8 parts H_2O, 0.5 part 10x MEMFA salts

stock solution, and 1 part formaldehyde 37%. Note that to prevent collapse of the *Xenopus* gastrula blastocoel roof, we use MEMFA with half the usual proportion of salts.

NaBH₄/TBS solution. In a fume hood, dilute NaBH₄ 3 M in tetraglyme (Sigma 556351) into TBS to a final concentration of 100 mM. Be careful when mixing; NaBH₄ releases hydrogen gas in water.

Antigen retrieval buffer. Add 250 μL of 0.6 M Tris pH 8.0, 750 μL 8 M Guanidium·HCl.

DTT 1 M. Dissolve 0.15 g of DTT in 0.8 mL H₂O. Adjust total volume to 1 mL; 100-μL aliquots were stored at −20 °C.

Iodoacetate 500 mM. Weigh 4.65 g of iodoacetic acid (toxic! 186 g/mol, pK_a = 3.18) under fume hood. Add 25 mL of H₂O and invert several times to dissolve. Add an equimolar amount (500 mM final) of sodium hydroxide (5 mL NaOH 5 N) to neutralize iodoacetic acid (pH ≥ 8.5, so iodoacetic acid is in the active iodoacetate form).

Murray's clear. Benzyl benzoate:Benzyl alcohol 2:1 vol/vol solution. Store in a glass bottle as it can dissolve some plastics.

Agarose-coated Petri dishes. Prepare 2%-agarose solution in H₂O in a microwave oven. Pour a ∼5-mm deep layer in 5-cm Petri dishes.

**Quantification of the Endogenous Chordin Gradient.** Digital images of Chordin immunostaining were exported to ImageJ software (http://rsbweb.nih.gov/ij/) to convert visual data into pixel coordinates and obtain an intensity-value table. This table was processed using a custom R script (www.r-project.org), in which Cartesian pixel coordinates were translated into polar coordinates

(the pole being the center of the embryo). The average pixel intensity value was measured along the radial and polar axis of an 80 × 80-polar grid. Radial profiles (Fig. 3*E*) were obtained by plotting this average pixel intensity along the radial axis at a fixed angle. The Chordin concentration gradient in the cleft was obtained by plotting the average pixel intensity along the cleft (Fig. 3*D*).

Plasmid Constructs. zADMP and xBMP2-GFP fusions were constructed following the guidelines used for a Dpp-GFP fusion (2). Venus fluorescent protein sequence (3) lacking an initiating Methionine was inserted by PCR in frame into the full coding sequence of the secreted bone morphogenetic protein (BMP) domain (1-aa before the first cysteine), and the resulting DNA fragment cloned into the pCS2 plasmid. Sequences at the fusion junction were RKRLKS-SG(Venus)SG-SCRRHP for BMP2, and ERGEKM-SG(Venus)SG-ACQRQP for ADMP, where BMP2 (4) and zADMP (5) sequences are underlined. Secreted GFP was obtained by insertion of the Venus protein sequence into the pCS2-NChordin-Flag vector (6). All constructs were expressed in 293T cells to determine that proper secretion and processing took place. To test for biological activity, 25 pg of plasmid DNA were injected four times in all four blastomeres of four-cell stage *Xenopus laevis* embryos. Injection of the secreted GFP construct produced no phenotype. ADMP-GFP and BMP2-GFP injected embryos were severely ventralized (to a similar extent as embryos injected with full-length BMP2 plasmid DNA), indicating that the fusion proteins retain biological activity.

1. Piccolo S, Sasai Y, Lu B, De Robertis EM (1996) Dorsoventral patterning in Xenopus: Inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* 86(4):589–598.
2. Teleman AA, Cohen SM (2000) Dpp gradient formation in the Drosophila wing imaginal disc. *Cell* 103(6):971–980.
3. Nagai T, et al. (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat Biotechnol* 20(1):87–90.
4. Inomata H, Haraguchi T, Sasai Y (2008) Robust stability of the embryonic axial pattern requires a secreted scaffold for chordin degradation. *Cell* 134(5):854–865.
5. Willot V, et al. (2002) Cooperative action of ADMP- and BMP-mediated pathways in regulating cell fates in the zebrafish gastrula. *Dev Biol* 241(1):59–78.
6. Piccolo S, et al. (1997) Cleavage of Chordin by Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. *Cell* 91(3):407–416.
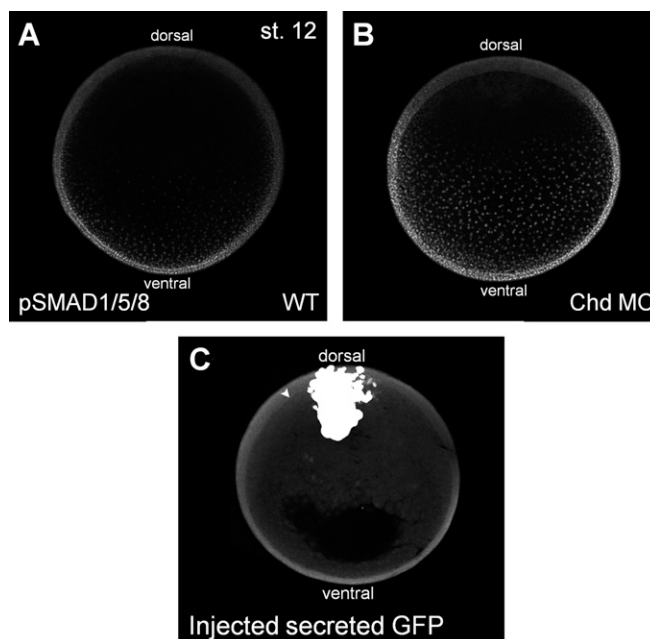
Fig. S1. Chordin (Chd) depletion increases the BMP signaling (D-V) gradient. (*A* and *B*) pSmad1/5/8 immunostainings of injected embryos at stage 12 sectioned transversely between the animal–vegetal axis. pSmad1/5/8 signal is increased in the ventral side in Chd morpholino oligo (MO)-injected embryos (*n* = 6) compared with wild type (WT) (*n* = 6). (*C*) A secreted form of GFP does not diffuse in Brachet's cleft (arrowhead) after overexpression (*n* = 5); this indicates that the observed long-distance diffusion of BMP–GFP fusions (zADMP-GFP and xBMP2-GFP) in Brachet's cleft requires the BMP moiety. ADMP, anti-dorsalizing morphogenetic protein.
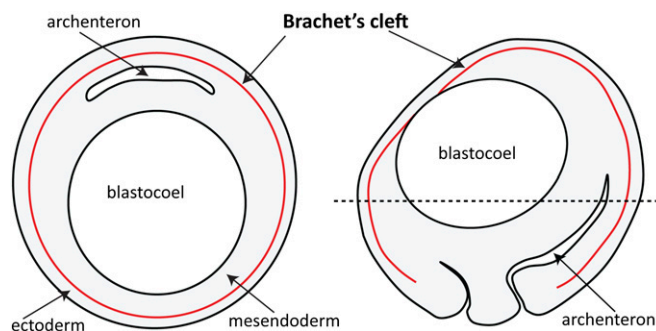
Fig. S2. Schematic drawing of the anatomical structures observed in the embryos shown in Fig. 3. On the left, diagram of stage-12 embryo sectioned transversely between the animal–vegetal axis. On the right, a sagittally sectioned embryo; the dotted line indicates the plane of section used in the transverse sections shown throughout this study. Note that the archenteron cavity (definitive gut) provides an excellent reference point for the dorsal side. The red line indicates the Fibronectin-rich extracellular matrix (ECM) that separates the ectodermal and endomesodermal layers, called the "Brachet's cleft" in *Xenopus*.
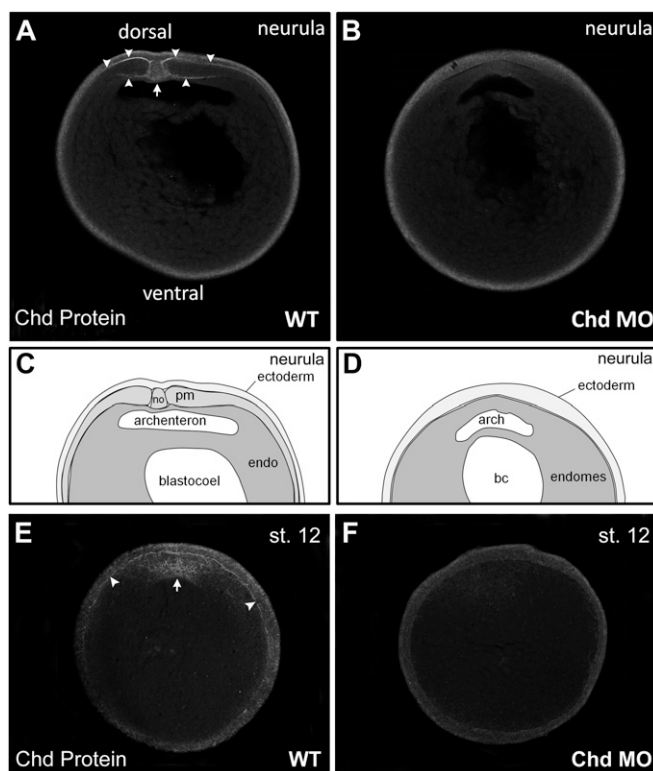
Fig. S3. The Chordin antibody staining of ECM is specific. (*A*) Immunostaining with Chordin antibody of neurula (stage 14) wild-type (WT) embryo sectioned transversely. Signal is observed on the dorsal side in the notochord (where Chordin is transcribed, arrow) and in the spaces separating the presomitic mesoderm (pm) from ectoderm (upper arrowheads) and dorsal endoderm (lower arrowheads), in regions where Chordin is not actively transcribed (*n* = 7). (*B*) Transverse section of Chordin-depleted embryo at neurula (Chd MO) stained with Chordin antibody. Note that the lack of signal in ECM indicates that anti-Chordin staining is specific (*n* = 5). (*C* and *D*) Diagrams of the anatomical structures seen in neurula stage WT or Chd MO-injected embryos shown in *A* and *B*, respectively. Arch, archenteron; bc, blastocoel; endomes, endomesoderm; no, notochord. (*E*) Chordin staining in transverse section of WT embryo (*n* = 5) showing Chordin protein in Brachet's cleft (arrowheads) and dorsal mesoderm (arrow). (*F*) Immunostaining of stage-12 embryo injected with Chd MO showing that Chordin signal was eliminated in both dorsal mesoderm and Brachet's cleft, confirming the specificity of the antibody; note that some background staining remains in the ectoderm (*n* = 5).
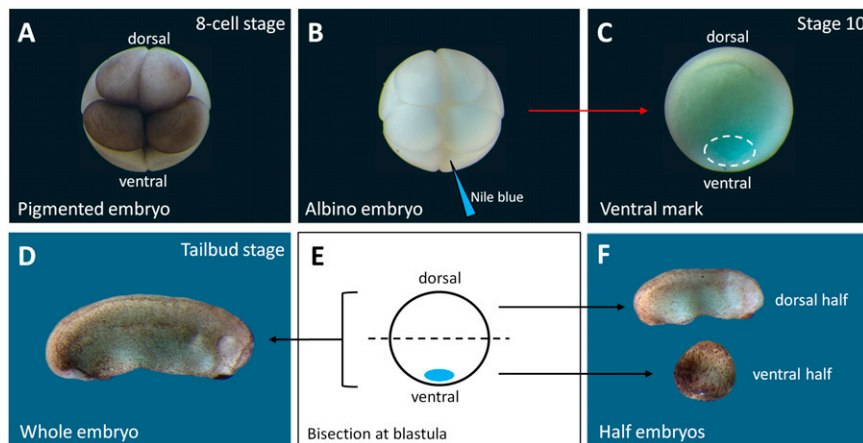
Fig. S4. The D-V polarity of the embryo can be predicted from the shape of the animal blastomeres at the eight-cell stage. Use of the vital dye Nile Blue sulfate allows marking of the ventral side of an albino embryo in preparation for bisection at blastula. (*A*) Animal (top) view of a pigmented eight-cell embryo showing the shape of the ventral cells spreading in a butterfly pattern and dorsal cells extending toward the equator. (*B*) A similar cell-shape pattern was observed in albino embryos, allowing a ventral blastomere to be marked with 20 mg/mL Nile Blue sulfate. (*C*) This pattern allows reliable marking of the ventral (or dorsal) side at the eight-cell stage as illustrated by this stage-10 embryo showing a ventral Nile Blue mark. (*D–F*) Bisection experiment in which pigmented or albino embryos were bisected at the blastula stage into dorsal and ventral halves (pigmented embryos are shown here). The dorsal half rescales to form a smaller but perfectly patterned embryo, whereas the ventral half lacks axial structures. In albino bisected embryos, all presumptive dorsal halves formed an axis ($n = 20$), whereas in presumptive ventral halves, only one ($n = 12$) formed an axis. The amount of Nile Blue sulfate should be kept to a minimum as it can quench fluorescence. Although Nile Blue sulfate was used in this study, other lineage tracers may be preferable in future studies.