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Molecular mechanisms of cell-cell signaling by the Spemann-Mangold organizer

EDDY M. DE ROBERTIS*, OLIVER WESSELY, MICHAEL OELGESCHLÄGER, BRENDA BRIZUELA, EDGAR PERA, JUAN LARRAÍN, JOSÉ ABREU and DANIEL BACHILLER[1]

Howard Hughes Medical Institute and Department of Biological Chemistry, University of California, Los Angeles, CA, USA and [1]Victor Goodhill Ear Center. Division of Head and Neck Surgery, UCLA School of Medicine, Los Angeles, CA, USA

ABSTRACT We review how studies on the first Spemann-Mangold organizer marker, the homeobox gene *goosecoid*, led to the discovery of secreted factors that pattern the vertebrate embryo. Microinjection of *goosecoid* mRNA formed secondary axes and recruited neighboring cells. These non-cell autonomous effects are mediated in part by the expression of secreted factors such as *chordin*, *cerberus* and *Frzb-1*. Unexpectedly, many of the molecules secreted by the Spemann-Mangold organizer turned out to be antagonists that bind growth factors in the extracellular space and prevent them from binding to their receptors. The case of *chordin* is reviewed in detail, for this molecule has provided biochemical insights into how patterning by Spemann's organizer can be regulated by diffusion and proteolytic control. The study of the BMP-binding repeats of Chordin, which are present in many extracellular proteins, may provide a new paradigm for how cell-cell signaling is regulated in the extracellular space not only in embryos, but also in adult tissues.

KEY WORDS: *Spemann-Mangold organizer, chordin, cerberus, Frzb-1, tolloid, collagen, BMP, TGFβ.*

Introduction

Since the original experiment of Spemann and Mangold (1924), isolating the molecules responsible for the inductive activities of organizer grafts has been the Holy Grail of vertebrate embryologists. After a number of premature attempts (related in Holtfreter and Hamburger, 1955; Nakamura and Toivonen, 1978), the advent of recombinant DNA technology opened the organizer problem to experimentation. The molecular exploration of the Spemann-Mangold organizer proved a gold mine for new genes and included unexpected surprises. The main surprise was that the organizer is a source of secreted antagonists of growth factors, which they bind in the extracellular space. These secreted antagonists can act as inhibitors that prevent binding to the cognate growth factor receptors, as well as modulators of signaling, in which a growth factor that was inactive can be brought back to signaling by the action of specific proteases that degrade the inhibitor. The principles of signaling regulation in the extracellular space learned from studies on the vertebrate Spemann-Mangold organizer may serve as a useful paradigm for understanding homeostasis of adult tissues and organs as well. This review is concerned with the homeobox gene *goosecoid*, which provided the first organizer gene marker, and with *chordin* and other secreted antagonists isolated in the course of efforts to identify genes transcriptionally activated by *goosecoid*.

Goosecoid and the isolation of organizer specific genes

The isolation of the homeobox gene *goosecoid* in 1991 provided the first organizer-specific gene (Cho *et al.*, 1991). This was an important landmark, because before it had not been possible to visualize Spemann-Mangold organizer tissue, and its existence could only be inferred from the results of transplantation experiments. *goosecoid* is a homeobox-containing gene, and the fact that overexpression of its mRNA in ventral cells led to the formation of secondary axes implicated, right from the outset, homeobox transcription factors in the execution of Spemann-Mangold organizer activity (Cho *et al.*, 1991). Microinjection of *goosecoid* mRNA was able to recruit neighboring cells into a secondary body axis and to trigger anteriorward cell movements in the injected cells (Niehrs *et al.*, 1992). As this single mRNA could mimic many of the properties of organizer cells, it came as a surprise that later on it was found that a great many other transcription factors have similar or even more potent activities than *goosecoid* (Fig. 1). In time it became clear that the Spemann-Mangold organizer is composed by multi-

Abbreviations used in this paper: BMP, Bone Morphogenetic Protein; *gsc, goosecoid*; *Xtwn, Xenopus Twin* homeobox gene; Dpp, Decapantaplegic; CR, cysteine rich domain of the chordin type; *fkh, forked-head* gene.

***Address correspondence to:** Eddy De Robertis. Howard Hughes Medical Institute and Department of Biological Chemistry, University of California, Los Angeles, CA 90095-1662, USA. FAX: +1-310-206-2008. e-mail: derobert@hhmi.ucla.edu

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ple populations of cells that are controlled by a plethora of transcription factors. These transcription factors in turn regulate the production of downstream secreted factors that mediate the inducing activities of the organizer (Fig. 1; reviewed in De Robertis *et al.*, 1997; Harland and Gerhart, 1997; Nieto, 1999).

Studies on *goosecoid* expression helped identify the corresponding homologous regions of the Spemann-Mangold organizer in other vertebrates, such as mouse, chick and zebrafish (reviewed in De Robertis *et al.*, 1993). Together with *Brachyury* – which provides a marker of all trunk mesoderm - *goosecoid*, which marks the Spemann-Mangold organizer and subsequently the prechordal plate, is widely used to define anatomical points of reference of the various vertebrate embryos during gastrulation. Studies in the mouse showed that organizer formation starts with the initial appearance of primitive streak cells in the posterior of the embryo. Organizer cells are then found in the anterior primitive streak (Blum *et al.*, 1992) before becoming located in the definitive node (more properly called the

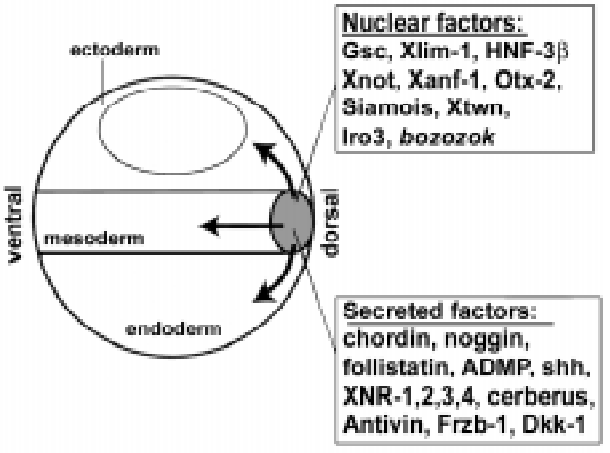

Fig. 1. Organizer-specific genes that pattern the early *Xenopus* embryo. *Schematic representation of a gastrula stage embryo showing the localization of the organizer in the dorsal marginal zone and its effect on patterning of all three germ layers, the endo-, meso- and ectoderm. The boxes show secreted and nuclear factors that are expressed in this region and that have been suggested to contribute to the function of the organizer.*

Hensen's node of the mouse), head process and prechordal plate. Transplantation experiments in mouse have confirmed this allocation of organizer tissue to the anterior primitive streak (Beddington, 1994; Tam and Steiner, 1999), which was initially supported only by crude Einsteck experiments (transplantation into the blastocoel cavity) using mouse day 6.5 embryo fragments transplanted into *Xenopus* gastrulae (Blum *et al.*, 1992).

Studies using chick *goosecoid* showed, in addition to expression in the tip of the progressing primitive streak and Hensen's node, a much earlier phase of expression in the posterior of the embryo (Izpisúa-Belmonte *et al.*, 1993). This expression is seen in the recently laid chicken egg, in cells located just beneath the epiblast in a structure called Koller's sickle. Although Koller's sickle had been known for over a hundred years, its role could only be analyzed when a molecular marker became available. As has proven often the case, the availability of novel markers coupled to lineage tracing of cell fates can lead to new insights. In the case of the *goosecoid* positive cells of Koller's sickle, they were shown to mark the initial precursors of what will become, after extensive cell

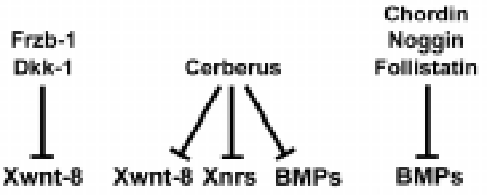

Fig. 2. The organizer is a source of secreted antagonists that bind growth factors in the extracellular space. *Three different types of extracellular modulators were been discovered in the organizer. All three have been shown to encode secreted inhibitors. Chordin, Noggin and Follistatin bind to BMPs and thereby inhibit them from activating their receptor. Frzb-1 and Dkk-1 antagonize Xwnt-8 in the extracellular space. Cerberus is a multivalent inhibitor of three different signals, BMPs, Xwnt-8 and the Xenopus Nodal-related mesoderm-inducing molecules (Xnr1, 2 and 4).*

migrations, the organizer region in the anterior of the primitive streak (Izpisúa-Belmonte *et al.*, 1993).

The study of *goosecoid* provided the first visualization of Spemann-Mangold organizer cells and of their dynamic changes during gastrulation. A disappointment was that the knockout of the *goosecoid* gene was lethal but lacked severe effects on gastrulation (Yamada *et al.*, 1995; Rivera-Pérez *et al.*, 1995). Subsequent studies showed that the region that develops in association with the prechordal plate is indeed affected, reflecting early defects in the formation of the midline of the base of the cranium (Belo *et al.*, 1997). Transplantation experiments of mouse node into chick gastrulae indicate that *gsc*$^{-/-}$ nodes are severely reduced in their neural-inducing strength (Zhu *et al.*, 1999a). Importantly, Filosa *et al.* (1997) have shown that in gsc$^{-/-}$;*HNF-3*β$^{+/-}$ compound mutants, dorso-ventral patterning of the CNS is severely disrupted in day 8.5 embryos. This is a recurring theme in organizer studies; with so many genes involved, double mutant studies are required to uncover redundant compensatory functions. We also now know that vertebrates contain at least three genes of the *goosecoid* family (reviewed in Belo *et al.*, 1997), which could contribute to the relatively weak phenotypes observed in homozygous mutant mice.

Mouse homologues of the *Xenopus* organizer specific-genes *Siamois* and *Xtwn* (Lemaire *et al.*, 1995; Laurent *et al.*, 1997) have not yet been cloned in the mouse and therefore have not been mutated.

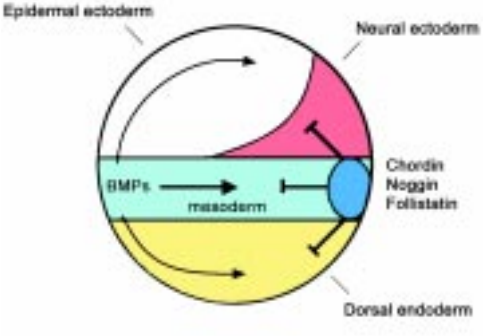

Fig. 3. Spemann-Mangold organizer secreted factors antagonize ventral signals provided by BMPs. *BMPs are secreted by a wide region at the ventral side of the embryo and are antagonized by organizer secreted factors such as Chordin, Noggin and Follistatin (blue oval). These factors directly bind to BMPs in the extracellular space of ectoderm, mesoderm and endoderm and thereby pattern these three germ layers.*

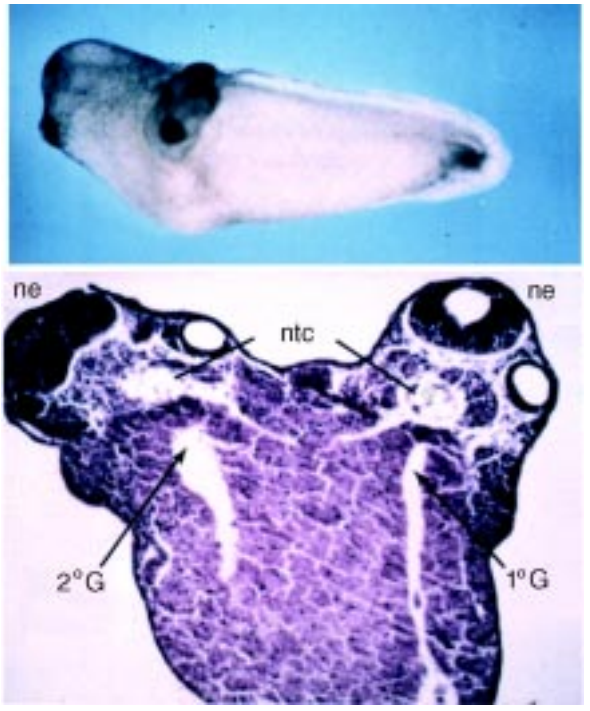

Fig. 4. *Chordin* mRNA induces secondary axes. (Upper panel) *Ventral injection at the 8-cell stage lead to the formation of a twinned embryo, which contains eyes and cement gland.* **(Lower panel)** *A sagittal histological section of this embryo shows that it contains two notochords (ntc), neural tubes (ne) and gut (1° G and 2° G). Thus, injection of a single molecule can recapitulate the inductions mediated by organizer grafts.*

Another homeobox gene in zebrafish (variously designated *dharma* or *nieuwkoid*; Yamanaka *et al.*, 1998; Koos and Ho, 1998 and 1999) has a strong mutant phenotype, leading to cyclopia in zebrafish mutants known as *bozozok* (Fekany *et al.*, 1999). Interestingly, the *dharma/bozozok* gene is more related in sequence to *goosecoid* than to *Siamois* or *Xtwn*, and functions upstream of *goosecoid*, whose expression is severely inhibited in zebrafish *bozozok* mutants (Fekany *et al.*, 1999). From these studies we now know that *goosecoid* is not the initial organizer homeobox gene to be activated, but rather part of a second wave of gene expression that takes place in dorsal mesoderm once the gastrula organizer is induced. Unraveling the respective contributions of the many genes involved in Spemann-Mangold organizer activity (Fig. 1) will undoubtedly require detailed genetic analyses in the future.

Secreted antagonists as patterning molecules

During the course of differential screens of a cDNA library prepared from Spemann-Mangold organizer tissue, we have identified several secreted factors (Sasai *et al.*, 1994; Bouwmeester *et al.*, 1996). As shown in Fig. 2, these include *Frzb-1*, a Wnt inhibitor (Leyns *et al.*, 1997; Wang *et al.*, 1997; Mayr *et al.*, 1997), *cerberus*, a multivalent inhibitor of Nodal, Wnt and BMP signals (Bouwmeester *et al.*, 1996; Piccolo *et al.*, 1999), and *chordin*, a BMP inhibitor thought to play a central role in organizer function (Sasai *et al.*, 1994; 1995; Piccolo *et al.*, 1996). Other groups used different methods to isolate additional organizer-specific secreted factors such as the BMP inhibitor Noggin (Smith *et al.*, 1992; Lamb *et al.*, 1993; Zimmerman *et al.*, 1996); the Activin and BMP inhibitor Follistatin (Hemmati-Brivanlou and Melton, 1994; Sasai *et al.*, 1995; Fainsod *et al.*, 1997) and the Wnt inhibitor Dickkopf (Glinka *et al.*, 1998).

In overexpression experiments, molecules such as *chordin* and *noggin* can induce neural tissue in ectodermal explants and dorsal mesoderm in ventral mesoderm explants (Lamb *et al.*, 1993; Sasai *et al.*, 1995; Piccolo *et al.*, 1996). In addition to their neural inducing activity, *chordin* and *noggin* can induce endoderm, in particular dorsal endoderm, in animal cap explants (Sasai *et al.*, 1996). This lead to the current model by which BMP antagonists emanating from the organizer would pattern all three germ layers of the embryo (Fig. 3). This view is in agreement with the result of injecting *chordin* mRNA into a ventral blastomere of the *Xenopus* embryo. As can be seen in Fig. 4, secondary axes are induced that contain, as in Spemann and Mangold's original experiment, a secondary neural tube, dorsal mesoderm (somites) and a secondary gut. Thus, the organizer phenomenon can be reproduced by the injection of a single molecule. Similar results can be obtained with *noggin*, another BMP antagonist, or with *short-gastrulation* mRNA. *Short-gastrulation* is the *Drosophila* homologue of *chordin* (Holley *et al.*, 1996; Schmidt *et al.*, 1995) and was known to act as a genetic antagonist of *decapentaplegic* (*Dpp*, a *BMP* homologue) signaling in *Drosophila* (Ferguson and Anderson, 1992). This gave us the clue that *chordin* might function as a BMP antagonist (reviewed in De Robertis and Sasai, 1996).

Unraveling the mechanism of action of Chordin

In microinjection experiments, the neural inducing activity of *chordin* could be inhibited by co-injection of BMP-4 mRNA (Sasai *et al.*, 1995). In what came initially as a surprise, the neuralizing activities of *noggin* and *follistatin* could also be blocked by BMP-4 (Sasai *et al.*, 1995). In the converse experiment, blocking BMP signaling with a dominant-negative BMP receptor (tBR, Suzuki *et al.*, 1994; Graff and Melton, 1994) or with antisense BMP-4 RNA (Steinbeisser *et al.*, 1995) resulted in the induction of anterior neural tissue in animal cap ectodermal explants in this groundbreaking study by Sasai *et al.* (1995).

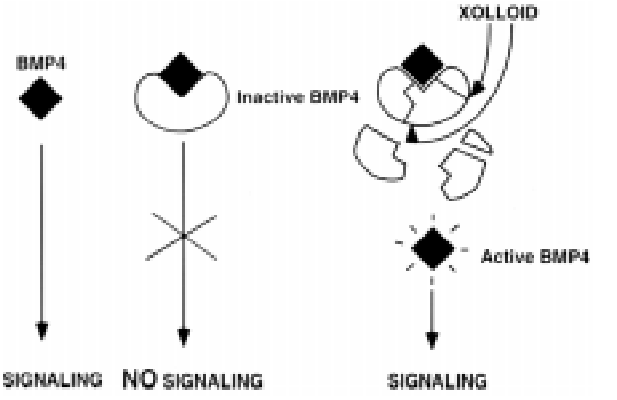

Fig. 5. Model of BMP signal re-activation by Xolloid cleavage. *BMP-4 binds to BMP receptors inducing the ventral pathway. Binding to Chordin blocks this signaling, whereas cleavage of Chordin by the Xolloid metalloprotease at two specific sites releases active BMP-4, re-establishing the ventralizing signal.*

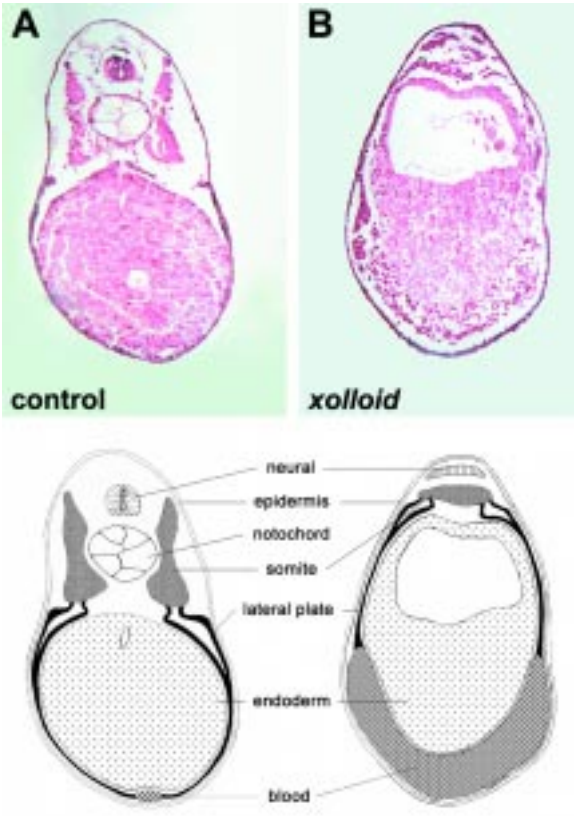

Fig. 6. *Xolloid* mRNA ventralizes mesodermal pattern. *Sagittal sections of tail bud stage embryos at the trunk level. The various dorso-ventral tissues are indicated in the lower panel.* **(A)** *Control.* **(B)** *Embryo after radial injection of xolloid mRNA at the four cell stage. Note the ventralization of the entire mesodermal layer in embryos expressing ectopic Xolloid metalloprotease.*

The molecular mechanism by which these neural inducers work was resolved with the purification of Chordin (Piccolo *et al.*, 1996) and Noggin (Zimmerman *et al.*, 1996) proteins, and the demonstration that they bind BMPs, preventing binding to BMP receptors (Fig. 5). The equilibrium dissociation constant (K_D) of Chordin for BMP-4 is 3 x 10^{-10} M, which is about the same as the affinity of BMP for its cognate receptors on cell membranes (Piccolo *et al.*, 1996). The affinity of Noggin for BMP is higher, of the order of 1.5 x 10^{-11} M (Zimmerman *et al.*, 1996). A concentration of 1 nM Chordin protein is sufficient to induce *NCAM* in ectodermal animal cap explants or α-*actin* in ventral marginal zone explants. In the case of Noggin, 1 nM will dorsalize mesoderm, but concentrations in the 10 nM range are required to induce neural tissue (Harland and Gerhart, 1997). Thus, embryonic cells have biological responses to the various neural inducers that are not solely dependent on *in vitro* affinities.

Chordin inhibition can be reversed by the Xolloid metalloprotease

In *Drosophila*, the gene *tolloid* functions genetically to increase *dpp* (BMP) signaling (Ferguson and Anderson, 1992). In *Xenopus*, a related gene called *xolloid* was isolated by Leslie Dale's laboratory (Goodman *et al.*, 1998; Piccolo *et al.*, 1997). Microinjection of *xolloid* mRNA causes an almost textbook-like ventralization of *Xenopus* embryos (loss of notochord, decrease in somite, increase in blood

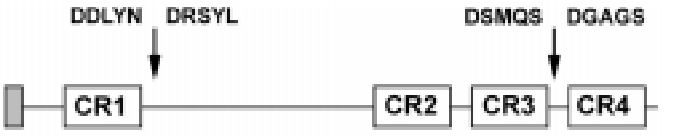

Fig. 7. Chordin is cleaved by Tolloid/Xolloid/mTll-1 at two specific sites. *Schematic drawing of Xenopus Chordin showing its signal peptide (gray box) and the BMP binding modules, the four cysteine-rich domains (CR1-4). The metalloprotease Tolloid/Xolloid/mTll-1 cleaves the mature protein at two specific sites 29 amino acids downstream of CR1 and 16 amino acids downstream of the third CR repeat and thereby inactivates the protein. The recognition sequences for the protease and the relative positions of the cleavage sites are indicated. After experiments of Piccolo et al., 1997 and Scott et al., 1999.*

islands) as shown in Fig. 6. This phenotype is similar to what one observes by injecting intermediate doses of BMPs, or would expect to find from a partial inhibition of Spemann-Mangold organizer activity.

Using a biochemical approach it was shown that the Xolloid zinc metalloprotease can cleave Chordin, but not Noggin, at two specific sites (Piccolo *et al.*, 1997). Furthermore, it was demonstrated that the cleavage of Chordin is able to reactivate previously inactive BMP, which is once again able to signal (Fig. 5) in *Xenopus* assays (Piccolo *et al.*, 1997). Thus, the way in which Xolloid works is through the specific inactivation of Chordin and the ensuing release of reactivated BMPs. In parallel studies in *Drosophila*, Marqués *et al.* (1997) found that *Drosophila* Tolloid cleaves Short-gastrulation at three sites, two of which correspond to the cleavage sites of Xolloid on Chordin.

More recent studies have revealed additional vertebrate homologues of Tolloid and that some of them, in particular BMP-1 and mouse tolloid-like-1, are effective at inactivating Chordin as well (Wardle *et al.*, 1999; Scott *et al.*, 1999). The exact location of the two sites at which Chordin is cleaved by Tolloid metalloprotesases (Piccolo *et al.*, 1997; Scott *et al.*, 1999) is of interest. As shown in Fig.

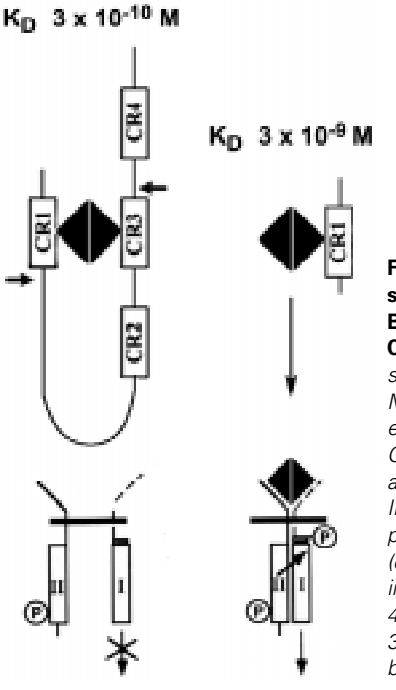

Fig. 8. Hypothetical model showing that Chordin binds BMP with higher affinity than CR1. *Chordin blocks BMP signaling efficiently (K_D 3 x 10^{-10} M) probably because the presence of CR1 and CR3 provide a Chordin monomer with two high affinity sites for each BMP dimer. In contrast, the CR1 fragment produced by Xolloid digestion (cleavage sites on Chordin are indicated by arrows) binds BMP-4 with a 10-fold lower affinity (K_D 3 x 10^{-9} M) and is less efficient in blocking BMP signaling.*

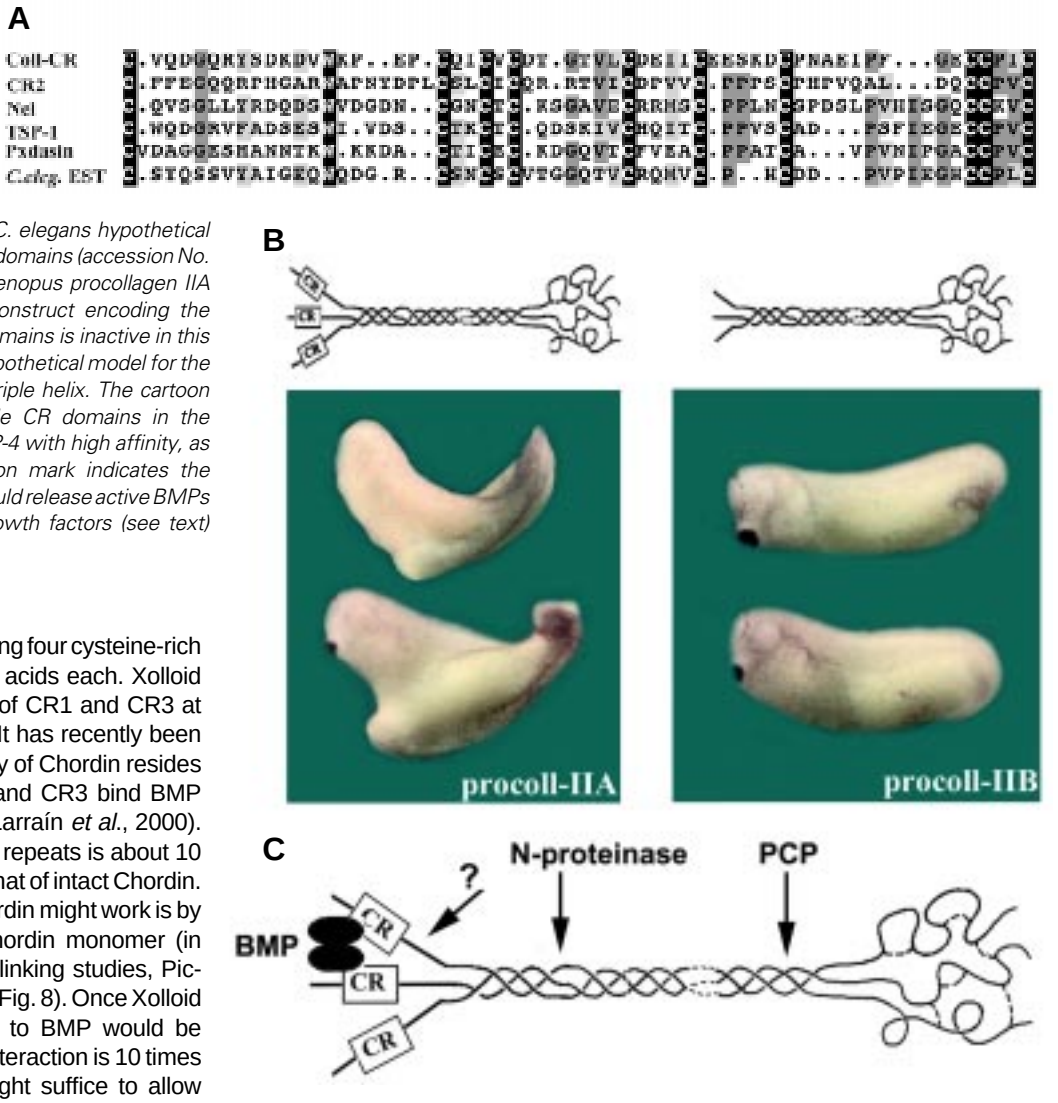

Fig. 9. CR domains present in procollagen IIA modulate BMP signaling. (A) *Sequence comparison of CR domains contained in different extracellular matrix proteins. Coll-CR, type IIA Xenopus procollagen; CR2, murine Chordin second repeat; Nel, rat nel; Pxdasin, Drosophila peroxidasin; C. eleg. EST, C. elegans hypothetical protein containing five procollagen-like domains (accession No. CAA94866).* **(B)** *Ventral injection of Xenopus procollagen IIA mRNA induces secondary axes. A construct encoding the splice variant Coll IIB lacking the CR domains is inactive in this assay (after Larraín et al., 2000).* **(C)** *Hypothetical model for the binding of BMP-4 to procollagen IIA triple helix. The cartoon shows how the presence of multiple CR domains in the procollagen triple helix could bind BMP-4 with high affinity, as is the case for Chordin. The question mark indicates the proposed protease that, like Tolloid, would release active BMPs from this extracellular reservoir of growth factors (see text) when required for tissue homeostasis.*

7, Chordin is a large protein containing four cysteine-rich domains (CRs) of about 70 amino acids each. Xolloid cleaves Chordin just downstream of CR1 and CR3 at conserved aspartic acid residues. It has recently been shown that the BMP-binding activity of Chordin resides in the CR repeats and that CR1 and CR3 bind BMP much better than CR2 and CR4 (Larraín *et al*., 2000). The binding affinity of CR1 or CR3 repeats is about 10 times lower (K_D of 3×10^{-9} M) than that of intact Chordin. Thus, a possible model of how Chordin might work is by binding a BMP dimer to each Chordin monomer (in agreement with results from crosslinking studies, Piccolo *et al*., 1996) via CR1 and CR3 (Fig. 8). Once Xolloid cleaves Chordin, CRs still bound to BMP would be released. Since the affinity of this interaction is 10 times lower, this decrease in affinity might suffice to allow binding of BMP to its receptors (Fig. 8), or perhaps additional components may be required to liberate BMP from the CRs.

Genetic studies discussed below indicate that the interaction of Chordin with BMPs is of central importance for generating dorsoventral pattern. The binding of BMP to Chordin could permit the diffusion of BMPs to distant sites without being sequestered by BMP receptors that are present at high concentrations in adjoining cell membranes. Once the Chordin-BMP complex meets the metalloprotease, active BMPs can be released at a distance (reviewed by Weinmaster, 1998). In *Drosophila*, it is known from genetic mosaic and other studies that Short-gastrulation can affect Dpp/Screw signaling many cell diameters away (Zusman, 1988; Ashe and Levine, 1999). In conclusion, mechanistic studies on the Chordin protein have provided important insights into how gradients of dorsoventral positional information are generated by protein interactions in the extracellular space.

Many proteins contain CR domains

The Chordin CR domains contain a series of conserved cysteines and hydrophobic residues that are present in a number of other proteins (Fig. 9A). CR repeats are present in Thrombospondin, von Willebrand factor and fibrillar procollagens (Bornstein, 1992; Sasai *et al*., 1994; François *et al*., 1994). Other proteins with such repeats include Nel-like proteins with four CRs and six EGF repeats (Watanabe *et al*., 1996), a C. elegans EST (Larraín *et al*., 2000) homologous to a chick transmembrane protein called CRIM1 with five CRs in the extracellular portion (Kolle *et al*., 2000), and *Drosophila* peroxidasin (Nelson *et al*., 1994).

The recent demonstration that at least some of these CRs are also implicated in BMP/TGFβ signaling, may have important consequences for the regulation of extracellular signals not only in the embryo, but also in adult tissues and organs. *Collagen IIA* mRNA is able to induce secondary axes in microinjected *Xenopus* embryos (Larraín *et al*., 2000) and this dorsalizing activity requires the CR repeats (Fig. 9B). The isolated CR repeat of Collagen II has BMP and TGFβ binding capacity (Zhu *et al*., 1999b; Larraín *et al*., 2000) but is devoid of biological activity. Presumably this is because, as in the case of Chordin, multiple CRs are required for high affinity binding. As shown in Fig. 9C, three CRs can come into close proximity in the NH_2-propeptide of procollagen trimers. This could provide binding sites for BMPs or other growth factors of the TGFβ superfamily at

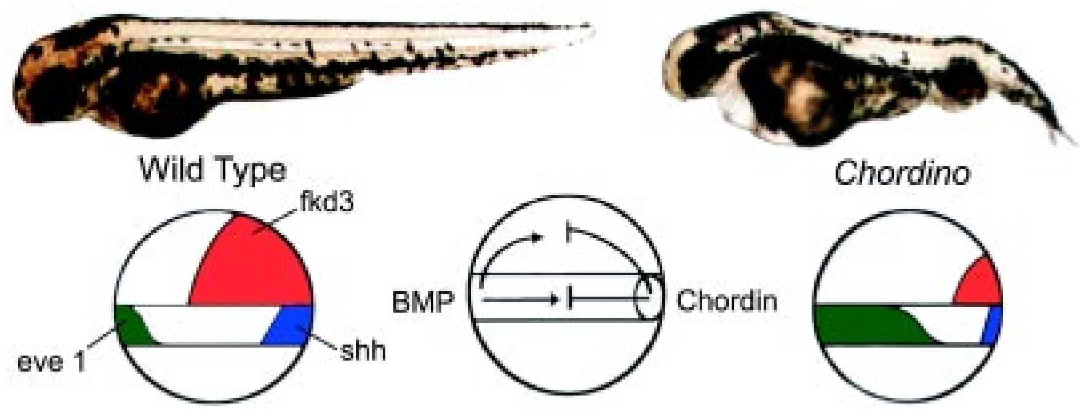

Fig. 10. Chordin is required for dorsal-ventral patterning in zebrafish. *The Chordino mutant phenotype is due to a loss-of-function of zebrafish chordin. In mutant embryos the tail is enlarged at the expense of head and anterior trunk. In situ analyses of mutant embryos demonstrated a reduction of the neural plate (marked by fkd3) and dorsal mesoderm (marked by shh), and an expansion of ventral mesoderm (marked by eve1) (Hammerschmidt et al., 1996). Therefore the antagonism between the Spemann-Mangold organizer secreted protein Chordin and BMP is required for the establishment of the dorsal-ventral polarity of ectoderm and mesoderm in zebrafish. Zebrafish photographs courtesy of Dr. Stefan Schulte-Merker (Tübingen).*

places where they could be most required, such as developing prerichondrium, tendon or bone, all of them rich in fibrillar collagens (Sandell *et al*., 1991; Su *et al*., 1991; Cheah *et al*., 1991; Zhu *et al*., 1999b). In order for these growth factors to become active, one would have to propose the existence of a protease that, like Tolloid, would cleave the complexes close downstream of the CRs (indicated in Fig. 9C). Thus, Chordin has provided a paradigm for understanding extracellular signaling regulation in adult tissues. This model may perhaps also apply to other Spemann-Mangold organizer secreted factors that are members of large multigene families also expressed in adult tissues (Rattner *et al*., 1997; Hsu *et al*., 1998; Pearse *et al*., 1999).

Chordin mediates dorso-ventral patterning

Since multiple BMP inhibitors are secreted by the Spemann-Mangold organizer (Fig. 3), loss-of-function studies are required to determine their individual functions and whether they compensate for each other. In zebrafish extensive genetic screens have been carried out, and two ventralized (as one would expect from loss or reduction of the Spemann-Mangold organizer) mutants have been isolated (Hammerschmidt *et al*., 1996a). The strongest mutation, *chordino*, was a loss-of-function of *chordin* (Schulte-Merker *et al*., 1997). As indicated in Fig. 10, at the gastrula stage neural (*fkh*) and dorsal mesodermal (*shh*) markers are reduced, and ventral mesodermal markers (*eve-1*) are expanded in *chordino* mutants (Hammerschmidt *et al*., 1996b). *Chordino* embryos regulate, and despite the much reduced initial neural plate, eventually a CNS develops, but the embryos have smaller heads and for the most part die at the stage shown in Fig. 10. These loss-of-function studies showed that the organizer-specific gene *chordin* is required for patterning both the ectodermal and mesodermal germ layers, as had been predicted from *Xenopus* overexpression studies.

The opposite class of mutations, the dorsalized mutants, are also of interest in the context of organizer function. The strongest mutant, *swirl*, is a mutation in *BMP-2* (Mullins *et al*., 1996; Kishimoto *et al*., 1997; Nguyen *et al*., 1998). *BMP-2* has a strong maternal component, and in its absence transcription of zygotic BMP-4 is also prevented in the ventral side (Kishimoto *et al*., 1997). Double mutants of *swirl;chordino* have a *swirl* phenotype (Hammerschmidt *et al*., 1996b); these epistatic studies show that *chordin* is a dedicated BMP antagonist. Other dorsalized mutations affect additional components of the BMP signaling pathway such as Smad-5 and BMP-7 (Hild *et al*., 1999; Dick *et al*., 2000; Schmid *et al*., 2000).

These genetic results in zebrafish highlight that a large part of the dorso-ventral patterning by the Spemann-Mangold organizer is effected through BMP signaling (Fig. 3). Last but not least, the most frequently isolated dorsalized mutation, *mini-fin*, has been identified as the zebrafish homologue of *tolloid/xolloid* (Connors *et al*., 1999), confirming the importance of proteolytic control in establishing gradients of dorso-ventral polarity.

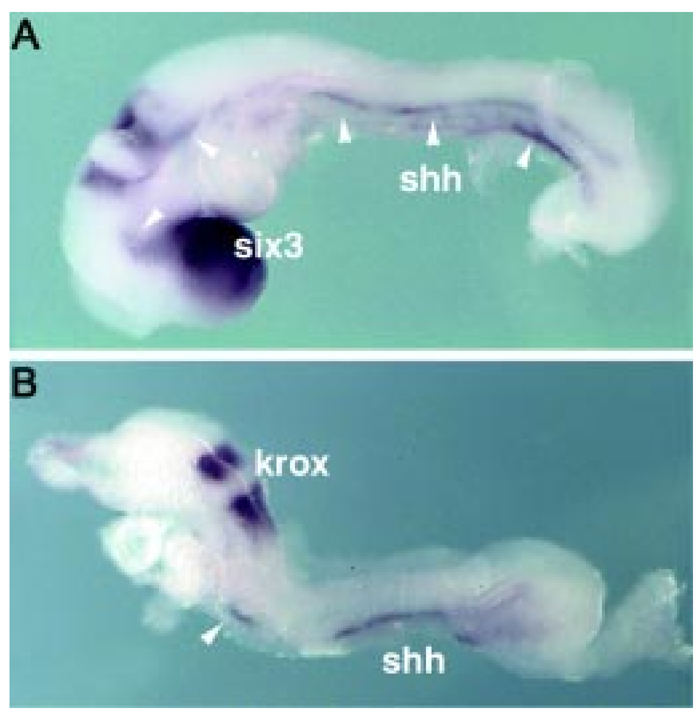

Fig. 11. Loss of prosencephalon in *chordin* and *noggin* double mutants (A,B) *wild-type and chordin-/-; noggin-/- day 8.5 mouse embryos hybridized with Six3, Krox-20 and Shh. Note that forebrain tissue expressing Six3 is almost completely absent, whereas Krox-20 is expressed in the double mutant at the expected position. The white arrowhead in* B *marks the level of anterior-most expression of Shh. These double mutant mouse embryos lose the prosencephalon as well as the anterior notochord.*

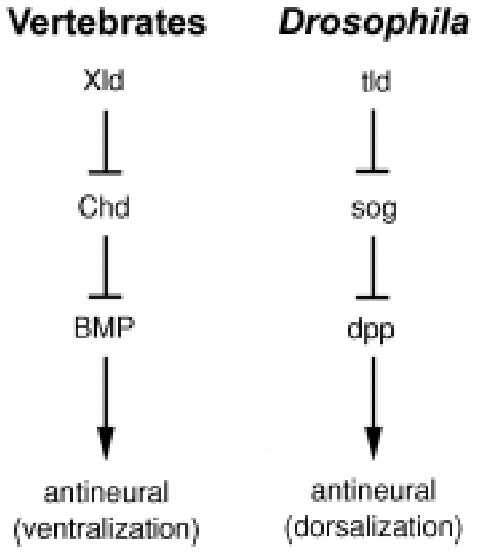

Fig. 12. Vertebrates and invertebrates share a common dorsal-ventral patterning system. *Vertebrate Xolloid blocks Chordin suppression of BMP-4 allowing ventralization (antineural); likewise in Drosophila, Tolloid blocks short-gastrulation suppression of Dpp allowing dorsalization (antineural) differentiation. This is a powerful argument in favor of the last common ancestor of protostomes and deuterostomes, the Urbilateria, having this dorso-ventral patterning system in place. During the course of evolution the dorsal axis has become inverted, as first proposed by French Zoologist Etienne Geoffroy Saint-Hilaire (1822).*

In the case of mouse embryos, mutation of *chordin* results in milder phenotypes and neural induction is normal (Bachiller *et al*., 2000, and unpublished results). Similarly, in *noggin* mutants development is relatively normal until embryonic day 8.5, although strong posterior axial deficits are seen at later stages (McMahon *et al*., 1998; Brunet *et al*., 1998). Double mutant studies have shown that these two BMP antagonists, which are co-expressed in the mouse Hensen's node, compensate for each other. In *chordin*$^{-/-}$;*noggin*$^{-/-}$ embryos, the prosencephalic vesicle is essentially absent at day 8.5 (Fig. 11, note the lack of expression of the Six-3 forebrain marker). This antero-posterior deficit can be traced back to the early neural plate stages (Bachiller *et al*., 2000). In addition, dorso-ventral phenotypes are observed such as the lack of notochord and *sonic hedgehog* expression in the anterior of the embryo (Fig. 11B, arrowhead). Finally, the left-right axis is also affected, with randomization of the heart situs (Bachiller *et al*., 2000). Therefore, genetic studies in the mouse demonstrate that the BMP antagonists Chordin and Noggin are required for correct patterning of the three main body axes of the mammalian embryo.

Conserved patterning mechanisms in evolution

Our interest in homeobox genes started with the isolation of the first Hox gene from vertebrates (Carrasco *et al*., 1984). As is now clear, the antero-posterior patterning system uses an intricate system of conserved genes in all bilateral animals (e.g., de Rosa *et al*., 1999). The isolation of a homeobox gene from a Spemann-Mangold organizer cDNA library, *goosecoid*, opened a new avenue of exploration for dorso-ventral patterning (Cho *et al*., 1991). Since microinjection of *goosecoid* mRNA had non-cell autonomous effects recruiting neighboring cells into secondary axes, this led to the search for downstream targets of *goosecoid* that could mediate these inductive activities. One such secreted target gene activated at the transcriptional level by injection of *goosecoid* mRNA was identified with the cloning of *chordin*, a gene that can mimic Spemann-Mangold organizer transplantation when overexpressed (Sasai *et al*., 1994). As shown in Figure 12, Chordin is part of a conserved dorsal-ventral patterning system involving *dpp*/*sog*/*tolloid* in *Drosophila* and *BMPs*/*chd*/*tolloid* in vertebrates (Sasai *et al*., 1994; François *et al*., 1994; Holley *et al*., 1995; Schmidt *et al*., 1995; Piccolo *et al*., 1997; Marqués *et al*., 1997). The molecular machinery required for the generation of gradients of BMP signaling is intricate and involves activation of BMP signaling via a double inhibition mechanism requiring a novel proteolytic control step in the extracellular space. There is one important difference, however, and that is that the dorsal-ventral axis has been reversed during the course of evolution (reviewed in De Robertis and Sasai, 1996; De Robertis, 1997). The last common ancestor of protostomes and deuterostomes, called the Urbilateria, had both a dorsal-ventral and an antero-posterior patterning system in place. Current phylogenetic analyses place Urbilateria very deep in evolutionary times, even before the split of molting animals (such as arthropods and nematodes) from the other protostomes (such as mollusks, and annelids, Aguinaldo *et al*., 1997; de Rosa *et al*., 1999). Therefore, the body patterns of all bilateral animals are constructed using conserved dorso-ventral and antero-posterior patterning systems, raising the question of whether this placed any constraints in the evolution of body plans.

The Spemann and Mangold experiment provided embryologists with an intellectual framework for asking questions about pattern development in vertebrates. It is remarkable that more than 75 years later a simple transplantation experiment still continues to stimulate new research, as can be seen throughout this volume.

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