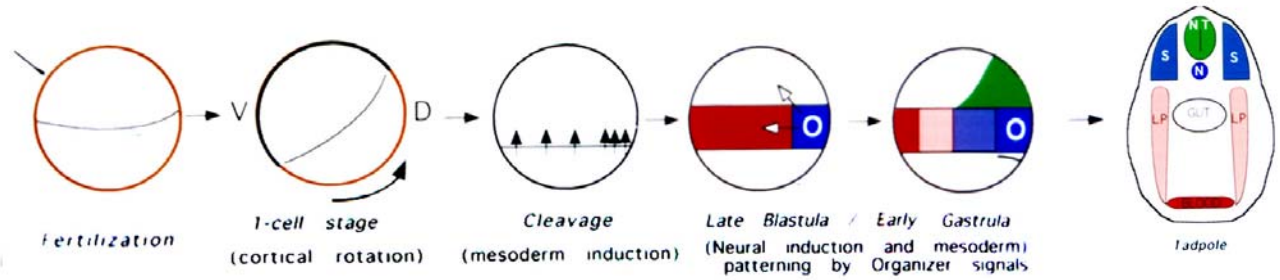
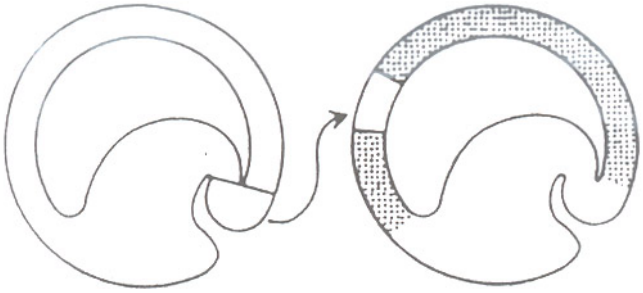
MOLECULAR BIOLOGY OF SPEMANN'S ORGANIZER AND NEURAL INDUCTION - Lecture 5

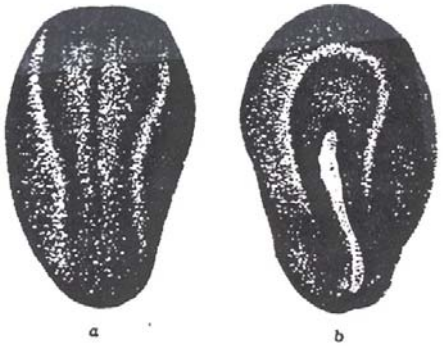
Having discussed the early events in mesoderm induction, we now turn to signaling events that take place during gastrulation.



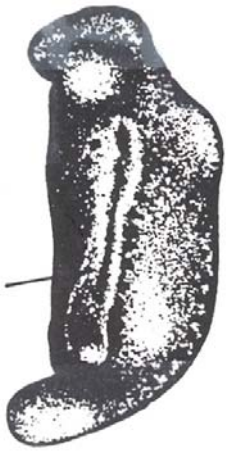
1. Dorsalization of mesoderm and neural induction by Spemann's Organizer during gastrulation

The "Organizer" experiment (Spemann and Mangold, 1924) is the best known experiment in embryology. It has led, more than any other, to the current view that development occurs through a cascade of cell-cell interactions. If the dorsal lip (the site where gastrulation starts) of the blastopore is transplanted to the opposite side of the embryo, it is able to recruit host cells organizing them into a secondary (twinned) body axis containing many histotypes and complex structures.

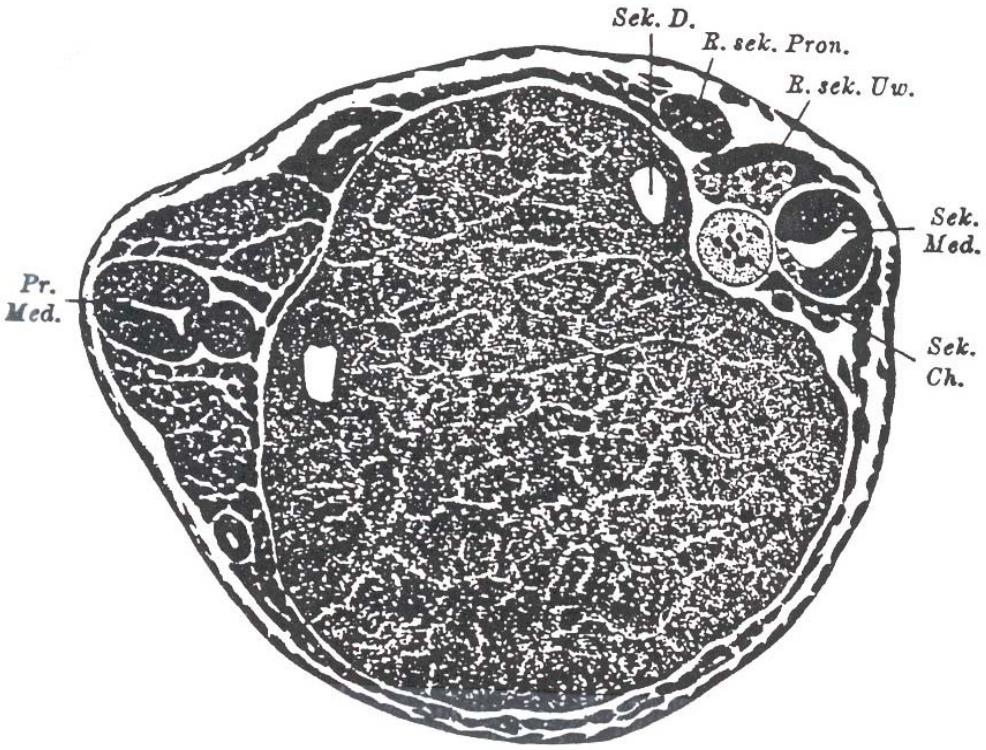




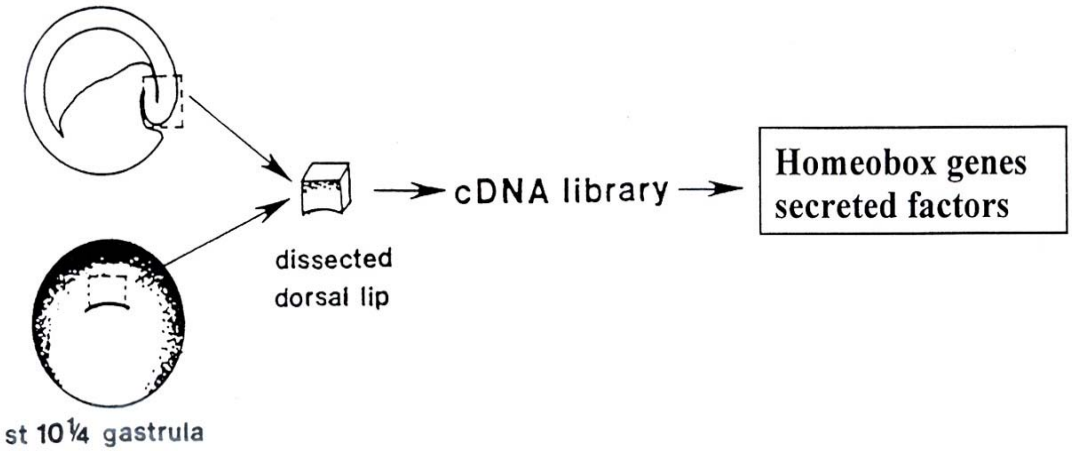
a and b. Early neurula of *Triton taeniatus* (a) with an induced secondary neural plate (b). (After H. Spemann and Hilde Mangold, 1924.)



Embryo of *Triton taeniatus* with optic cup, ear vesicles and tail bud. On its left side a secondary embryo, with ear vesicles at the fore end of the medullary tube and two rows of somites. (After H. Spemann and Hilde Mangold, 1924.)

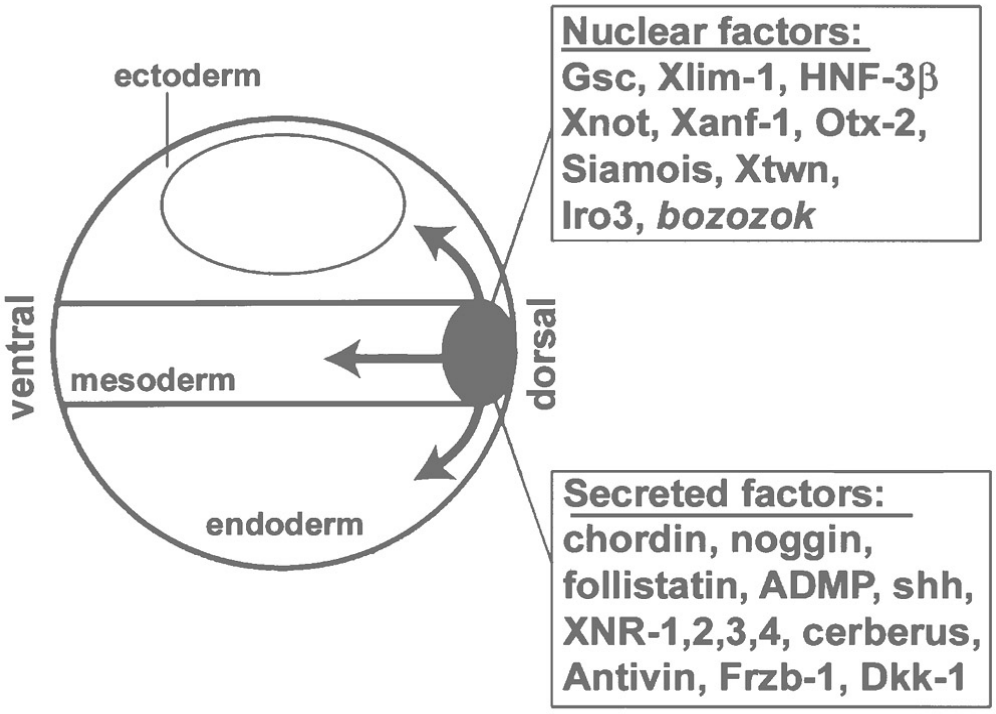


The organizer has three main properties: 1) it induces neural tissue on the overlying ectoderm, 2) imparts more dorsal characteristics to the mesoderm of the marginal zone (i.e., "dorsalizes mesoderm"), leading to the formation of somites and trunk muscles, and 3) it induces a secondary gut ("dorsalization of the endoderm"). This small region of the gastrula has been a goldmine for the isolation of new molecules involved in cell signaling. We made organizer-specific libraries and screened them for cDNAs expressed in the organizer (e.g., identification of *goosecoid*, *chordin*, *cerberus*, *Frzb-1*). Other labs used functional injection assays, injecting pools of synthetic mRNAs into the ventral side of embryos and then doing sib-selection until a single gene is identified (e.g., identification of *noggin*, *Siamois*, *Xtwn*, *dickkopf*).



91 *Xenopus* dorsal-specific cDNAs isolated by differential screening
(Bouwmeester et al., 1996)

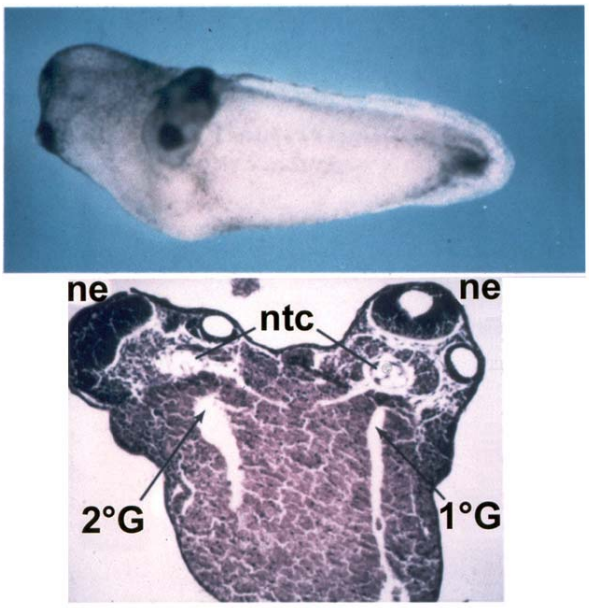
Gene	Product	# of isolates
chordin	novel secreted protein	70
cerberus	novel secreted protein	11
goosecoid	homeobox/transcription factor	3
pintallavis/XFKH-1	transcription factor	2
PAPC	protocadherin/structural gene	2
Xnot-2	homeobox/transcription factor	1
Xlim-1	homeobox/transcription factor	1
Frzb-1	novel secreted protein	1

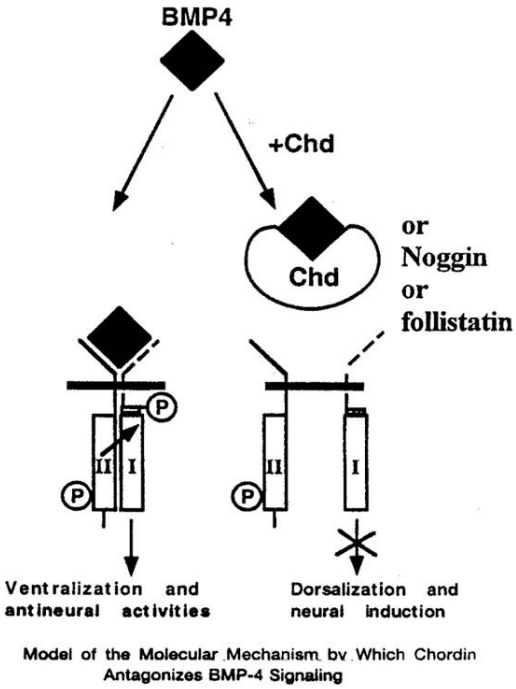


Most of the molecules isolated were transcription factors, especially homeobox genes, or secreted proteins. Ventral microinjection of these homeobox-containing mRNAs (*goosecoid, Siamois, Xtwn, Xlim-1, boz, Xnot*) can cause secondary axes and recruit neighboring uninjected cells into them, as in Spemann's experiments. Since they encode DNA binding proteins, the inductive effects on neighboring cells are mediated by changes in the expression of secreted proteins. The factors secreted by the organizer pattern the three germ layers. The effects of the organizer-specific factors are opposed by ventralizing genes that are expressed in ventro-lateral regions of the embryos. Notable among the latter are *BMP-2*, *BMP-4* and *Xwnt-8*.

2. Chordin, noggin and follistatin antagonize BMP ventralizing signals.

Microinjection of *chordin, noggin* or *follistatin* mRNA will induce secondary axes, rescue UV embryos, dorsalize mesoderm in ventral marginal zone explants and induce CNS differentiation in animal caps.

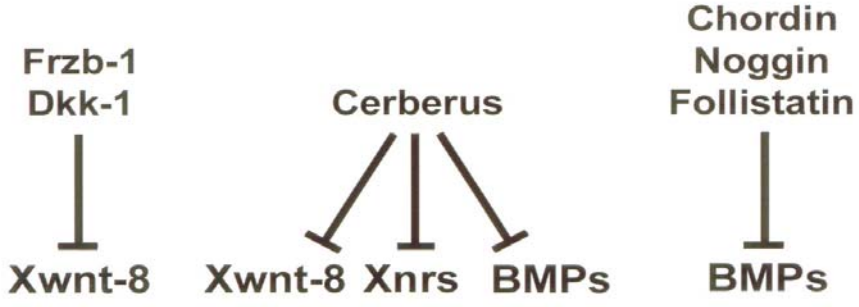


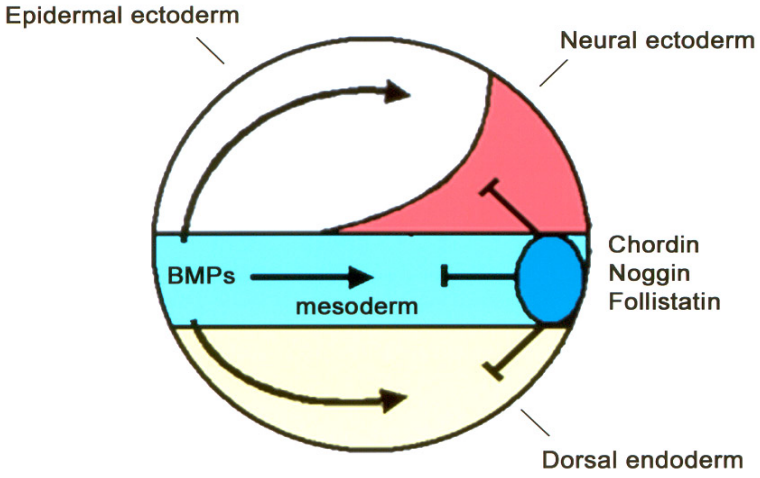


Model of the Molecular Mechanism by Which Chordin Antagonizes BMP-4 Signaling

To our surprise, co-injection of *BMP-4* abolished neural induction by chordin, by noggin, and by follistatin, which are secreted proteins of entirely different structures. This antagonism takes place in the extracellular space (as indicated by injecting different blastomeres). Production of the proteins in tissue culture showed that both noggin and chordin bind to BMPs and prevent their binding to BMP receptors. The lack of BMP signaling in turn leads to the dorsalization of mesoderm and neuralization of ectoderm. (The same result can be obtained using a DN-BMPReceptor construct). Thus, the surprising finding was that neural induction and the dorsalization of mesoderm (and also of endoderm) had the same molecular basis: antagonism of ventral BMP signals.

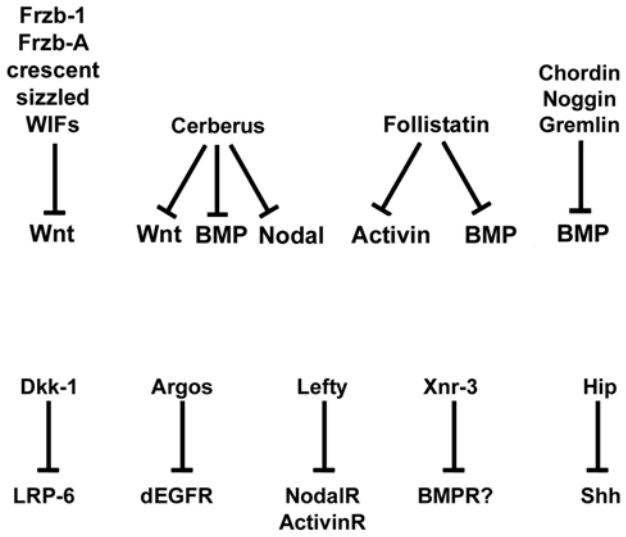
The biggest surprise from these studies on Spemann's organizer was that patterning by the organizer is effected through secreted antagonists of growth factors:





Model indicating that the same set of regulatory signals may provide the positional information that patterns ectoderm, mesoderm and endoderm in *Xenopus*. On the dorsal side (right), the organizer (oval) provides dorsal positional values to ectodermal (animal cap, top) and mesodermal (marginal zone, middle) tissues by secreting organizer factors such as *chd*, *noggin* and *follistatin*. On the opposite side (left), ventralizing factors such as *BMP-4* and presumably other signals give ventral positional values to the tissues, antagonizing organizer signals. High dorsal values promote neural differentiation in the ectoderm and formation of notochord and muscle in mesoderm, while high ventral values lead to epidermogenesis in ectoderm and differentiation of blood island and mesenchymes in the mesoderm.

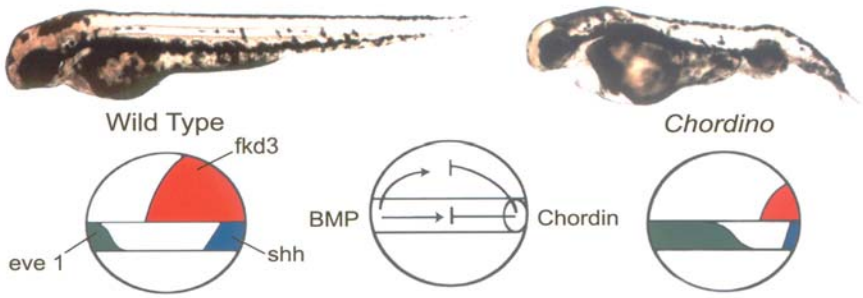
Spemann's organizer is full of inhibitors. Some think that it may have been easier, perhaps, to produce inhibitors during the course of evolution to modulate pathways ("a spanner in the works") than to evolve entirely new signaling pathways. A large number of secreted antagonists have been discovered in various tissues:



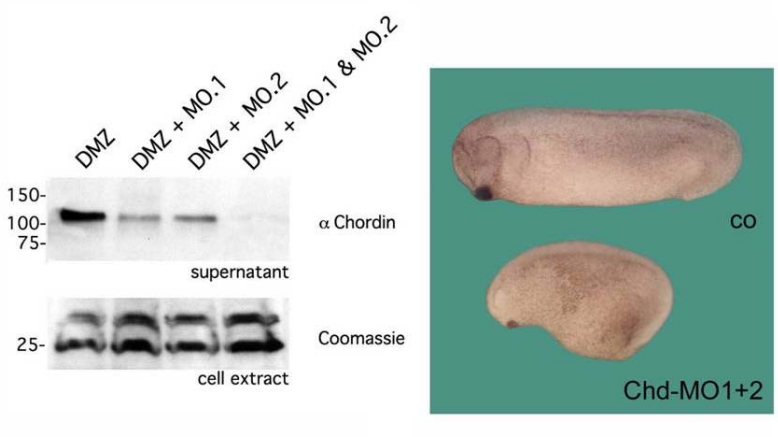
3. The Chordino mutation in zebrafish.

A large screen for mutations affecting zebrafish development has been carried out. Two ventralized mutants (less notochord and somites, more blood and small head) were identified. A ventralized mutant is what one would expect from a Spemann organizer mutant.

The strongest one, *dino*, was found to be a loss of function of *chordin* and renamed *chordino*. At the gastrula stage *chordino* embryos have less neural plate (marked by forked-head 3), less dorsal mesoderm (marked by sonic hedgehog) and more ventral mesoderm (marked by *eve-1*, a homeobox gene). At later stages the fish recover somewhat. The lethality can be rescued by injecting *chordin* (or *noggin* or *DN-BMPR*) mRNA into the embryo.



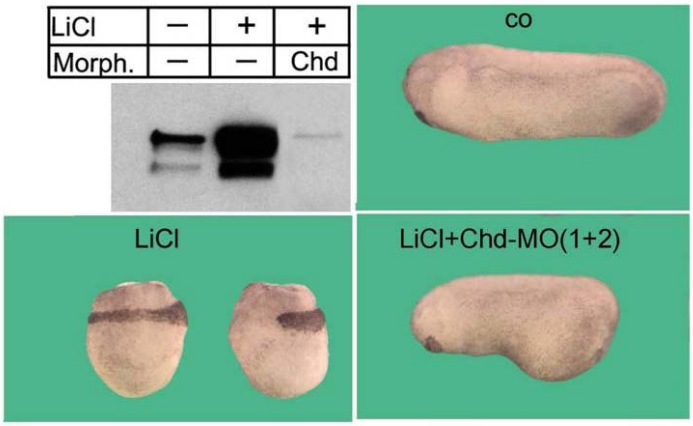
These studies validate the view that a single gene can pattern the ectoderm and the mesoderm. Other zebrafish mutations (at least 6) have a dorsalized phenotype (more notochord, somites, less blood). The strongest one of these, called *swirl*, is a mutation in *BMP-2*. (Mutation of *BMP-2* secondarily also reduces expression of *BMP-4* in ventral regions of the gastrula). Double mutants *swirl*$^{-/-}$; *chordino*$^{-/-}$ have a *swirl* phenotype (i.e., *swirl* is epistatic to *dino*). This indicates that the function of *chordin* is to antagonize BMPs. Other zebrafish dorsalized mutations have been mapped to BMP-7, Smad5, a BMP receptor and Tolloid.



4. In *Xenopus* Chordin is required for Spemann's organizer phenomenon.

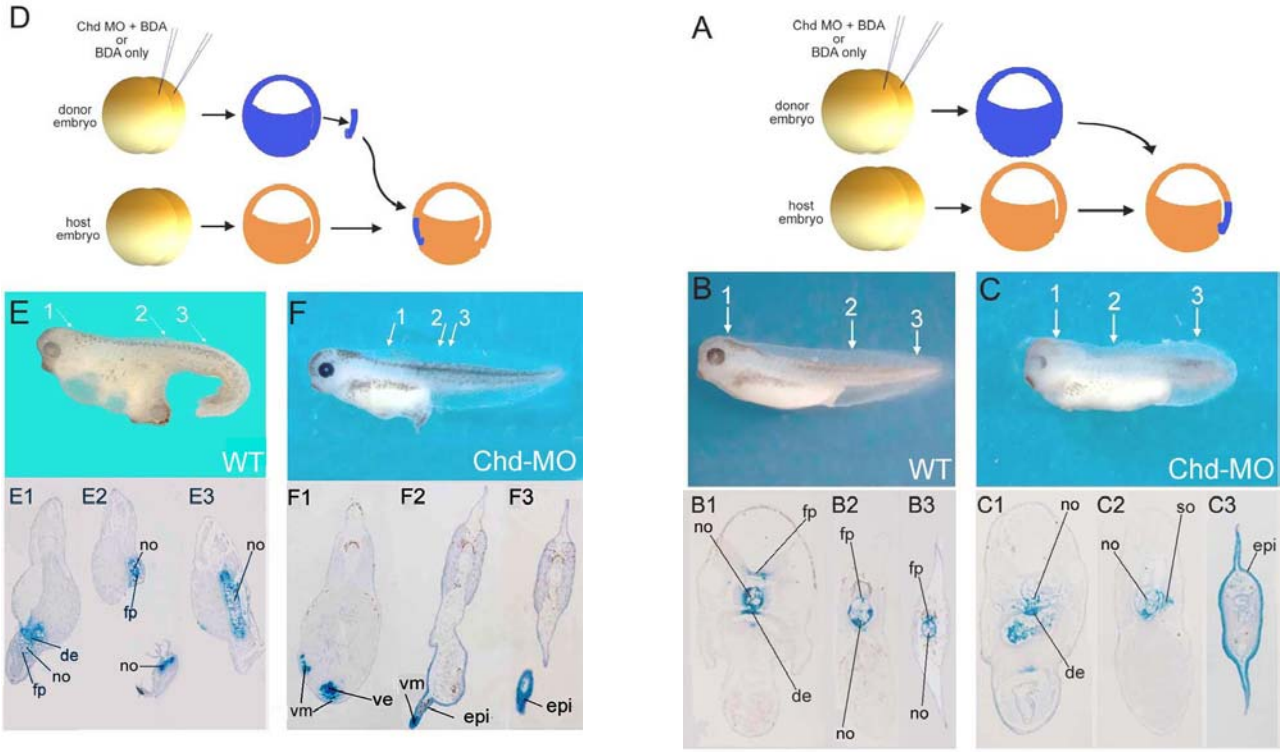
In *Xenopus*, antisense morpholinos produce a moderately ventralized phenotype very similar to Chordino.

However, by experimentally manipulating the embryo, much stronger requirements are seen. For example, the LiCl effect has an absolute requirement for Chordin.



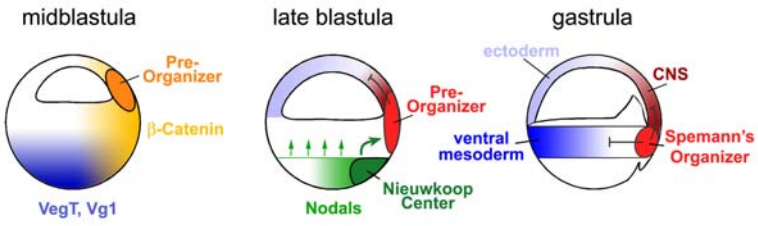
Organizer grafts to the ventral side demonstrate that Chordin is required for Spemann's organizer to induce a CNS.

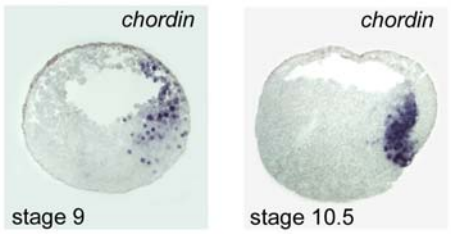
In control grafts of dorsal lip into the dorsal region, an unexpected cell-autonomous requirement for Chordin was revealed. The embryo prefers to make CNS from cells that express Chordin. Cells injected with Chd-MO remain in the skin ectoderm instead of differenciating into CNS. Why?



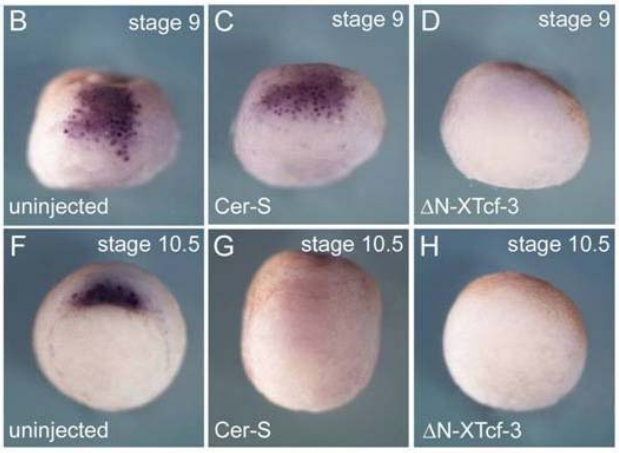
5. An early phase of Chordin expression – The Preorganizer

Right after midblastula there is an early phase of Chordin expression in a region called the preorganizer, which gives rise to the CNS later on.



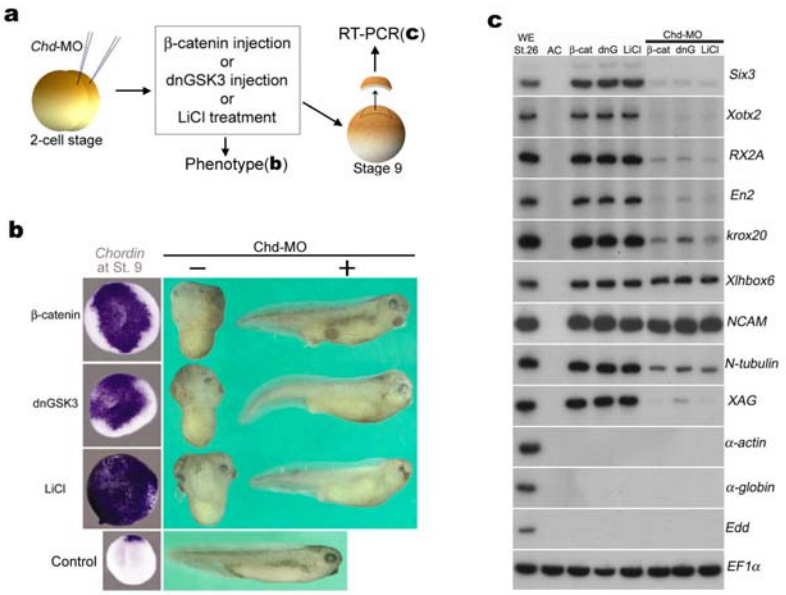


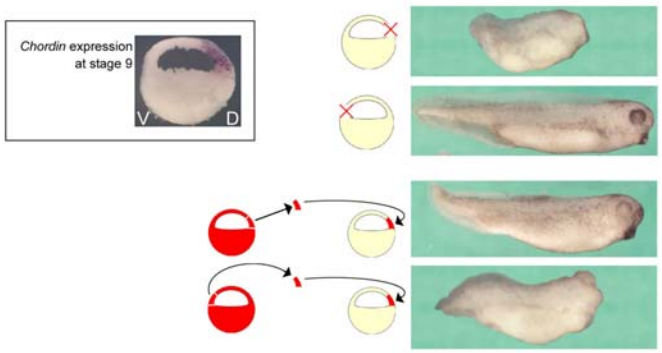
O. Wessely et al. Dev. Biol. 234, 161-173 (2001)



At gastrula Chd requires Nodal signaling and can be inhibited by Cerberus-short mRNA. The blastula preorganizer expression requires β-Catenin signals since it is blockec by ΔN-TCF-3.

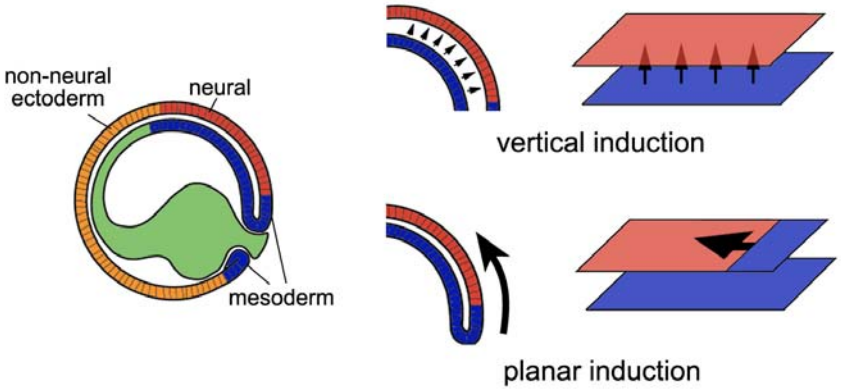
Injection of β-catenin or GSK-3 mRNA, or treatment with LiCl will induce CNS formation in animal caps (neural differentiation marked by Six3, Otx-2, Rx2a, En2, Krox20, NCAM and Neuro-tubulin. All anterior neural markers require Chordin (and Noggin). Thus β-catenin induces neural tissue through BMP inhibitors such as Chordin and Noggin expressed at the blastula stage.





The preorganizer is required for anterior CNS formation, explaining findings by Embryologists of the 1920's. Brain tissue can be induced even if the formation of the gastrula organizer is blocked with the inhibitor Cer-Short. These findings help clarify old observations in which planar signals (diffusing in the plane of the ectoderm) versus vertical

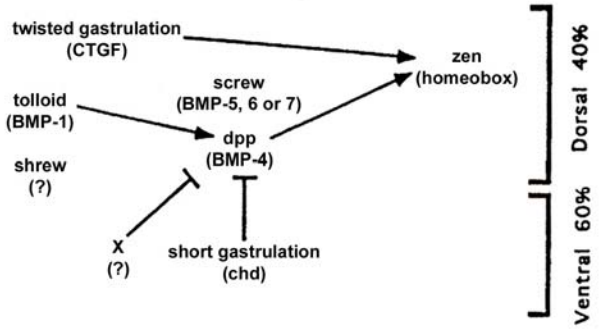
signals (emanating from the mesoderm) were analyzed. The early β-catenin signal that takes place in the future CNS predetermines the position of the CNS. Signals from the mesoderm (Chordin, Noggin and Cerberus), that are also required, explain the vertical induction discovered by organizer grafts. I will show a few additional slides to demonstrate this.



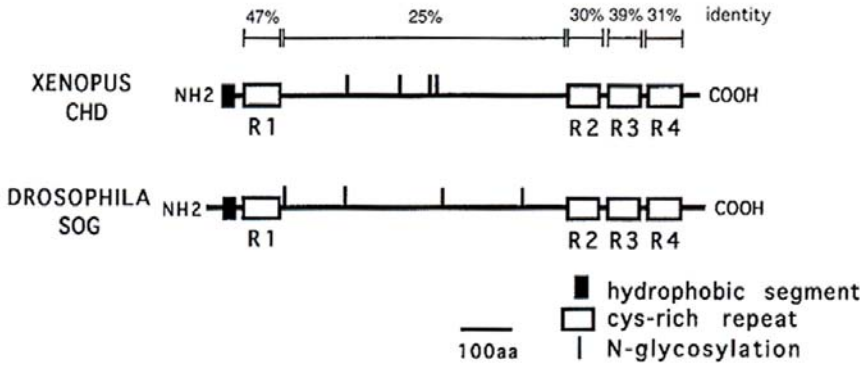
5. *Short-gastrulation* antagonizes *dpp*.

In *Drosophila* there are several zygotic genes involved in dorso-ventral patterning. They are controlled by the maternal morphogen *dorsal*.

Zygotic genes involved in dorsoventral patterning of the ectoderm in *Drosophila* embryos, and their vertebrate relatives. Several genes are expressed in the dorsal 40% of the embryo; *tolloid* encodes a metalloprotease required for *dpp* activity, and is similar in sequence to vertebrate *Bmp-1*; *twisted-gastrulation* encodes a protein related to vertebrate connective tissue growth factor; *screw* is a secreted growth factor related to either *Bmp-5, 6 or 7* that might form heterodimers with *dpp*; *zen* encodes a homeobox gene for which a vertebrate counterpart has not been isolated.

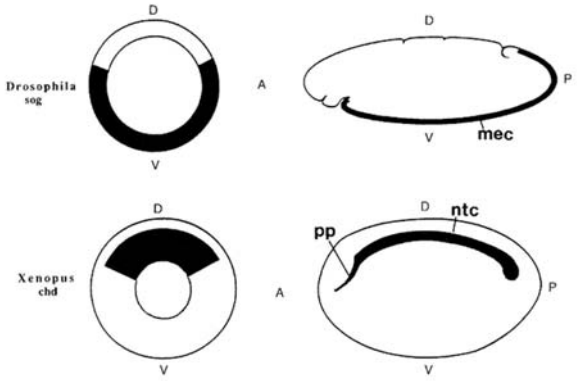


A breakthrough came when *Xenopus chd* and *Drosophila sog* were found to be similar, and to functionally substitute for each other.



The amino acid sequences of *Xenopus chd* and *Drosophila short-gastrulation* share similarities. Both proteins have a secretory signal sequence, or hydrophobic segment, at the amino (NH_2) end (dark box), several putative N-glycosylation sites (vertical lines) and four cysteine-rich repeats. The first repeat (R1) of *chd* is more similar to R1 of *sog* than to any of

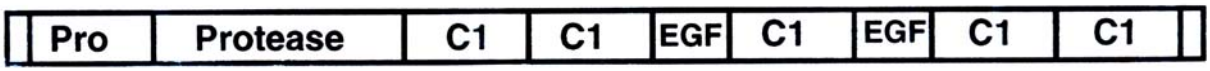
the other repeats in *chd*. The same is true for R4, indicating that both genes are derived from a common ancestor.

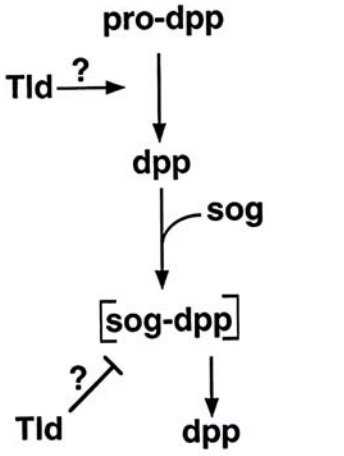


The expression of *Drosophila short-gastrulation* (*sog*) (top) and *Xenopus chordin* (*chd*) (bottom) is reversed with respect to the dorso-ventral axis. In *Drosophila sog* is first activated in the ventral 60% of the blastoderm, is then switched off in the mesoderm and persists in the neurogenic ectoderm, and by late gastrulation the expression resolves into two rows of cells located in the ventral midline, the mesectodermal cells (*mec*). In *Xenopus*, *chd* is expressed initially in the dorsal side of the blastopore, while by the end of gastrulation it is resolved to the mesoderm of the dorsal midline, occupying the head mesoderm of the prechordal plate (*pp*) and the notochord (*ntc*). *sog* is ventral in *Drosophila* while *chd* is dorsal in *Xenopus*. This provided support for an old zoological hypothesis that protostomes and deuterostomes had undergone a dorso-ventral inversion during evolution.

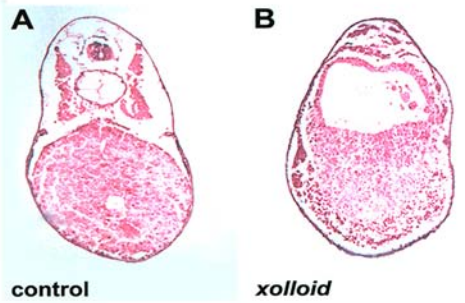
7. *Tolloid/Xolloid* proteases cleave *sog/chd*. Geoffroy's victory.

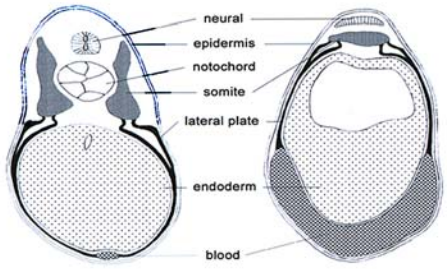
Tolloid, and its *Xenopus* homologue, *Xolloid,* encodes a secreted zinc metalloprotease that has five protein-protein interaction repeats present in complement C1 subunits and two EGF repeats. The zebrafish tolloid homologue is mutated in the *mini-fin* dorsalized mutant.





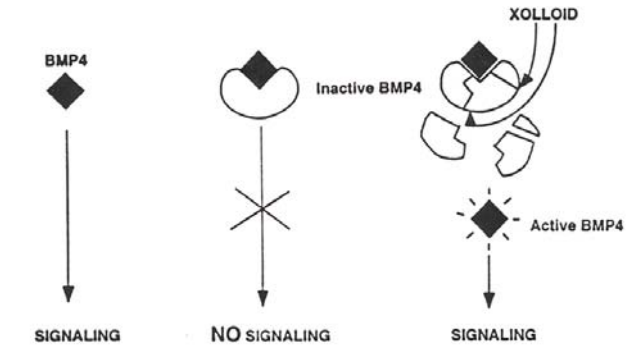
Tolloid was thought to increase *dpp* activity by proteolytically processing *dpp*. However, *tolloid* could also act by degrading the inactive *sog/dpp* complex.

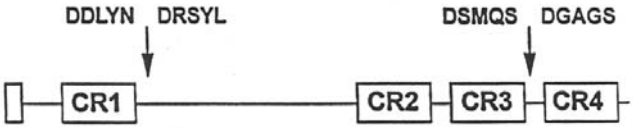


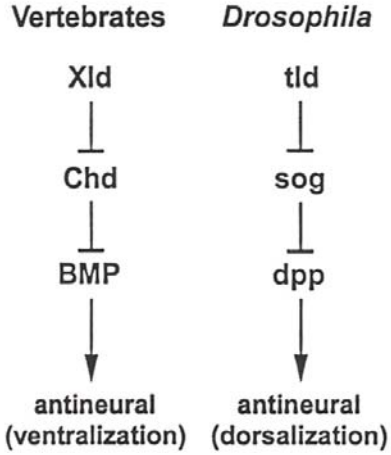


The *Xenopus* homologue *Xolloid* will ventralize the mesodermal layer when its mRNA is microinjected. Note the loss of notochord and increased blood.

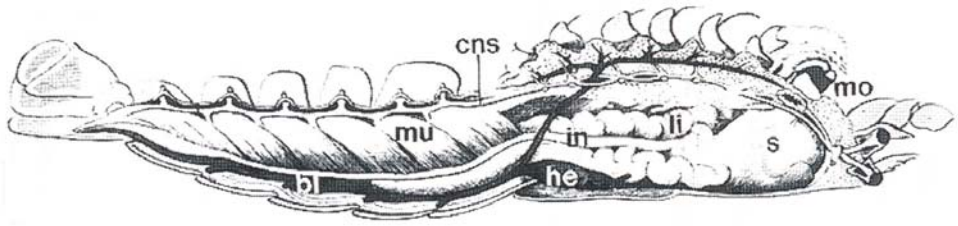
This proposition was tested by direct biochemical experiments by Piccolo et al. In co-injection experiments *Xolloid* inhibited the secondary axis-forming ability of *chordin* but not that of *noggin, follistatin* or *DN-BMPR*. Conditioned medium from cells transfected with *Xolloid* cleaved recombinant *chordin*, but not *noggin*, at two sites. *Xolloid* also cleaves *chordin-BMP* complexes at the same sites. Cleavage of *chordin* inactivates its activity and releases active *BMP-4* from inactive [Chordin-*BMP*] complexes.





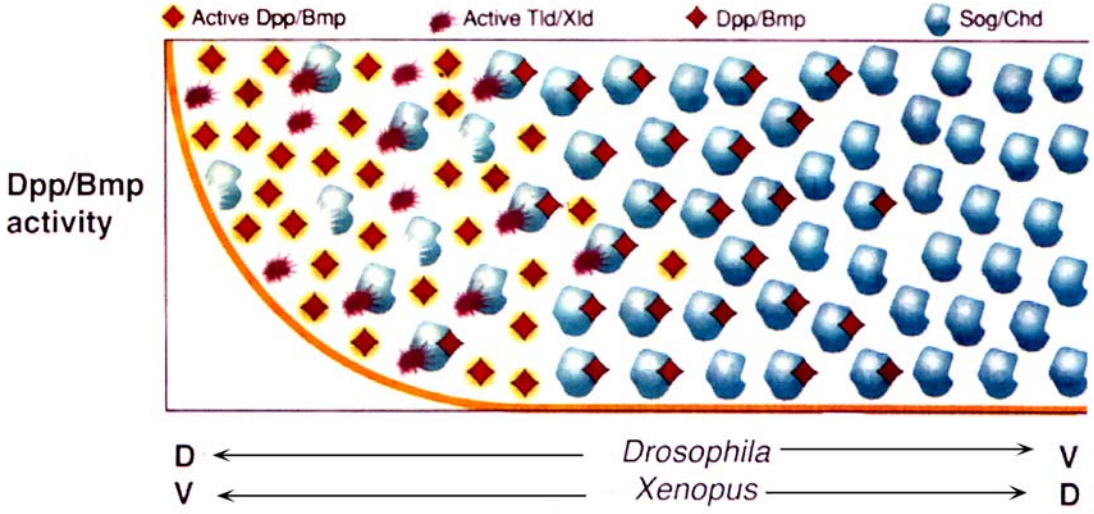


In parallel studies M. O'Connor showed that *Drosophila tolloid* cleaves the *sog/dpp* complex. Conclusion: these studies indicate that the entire of dorso-ventral patterning system was inverted as proposed first by Geoffroy Saint-Hilaire in 1822, forty years before Darwin.



Geoffroy Saint-Hilaire's famous lobster. In this dissection the animal is presented in the orientation opposite to that it would normally have with respect to the ground. The central nervous system (cns or nerve cord) is above, and is traversed by the mouth (mo). Underneath is the digestive tract, with the stomach (s), liver (li) and intestine (in). Below the gut are the heart (he) and main blood vessels (bl). Muscles (mu) flank the CNS. In this orientation the body plan of the arthropods resembles that of the vertebrate. From ref. 1, by courtesy of the History and Special Collection Division, Louise M. Darling Biomedical Library, UCLA.

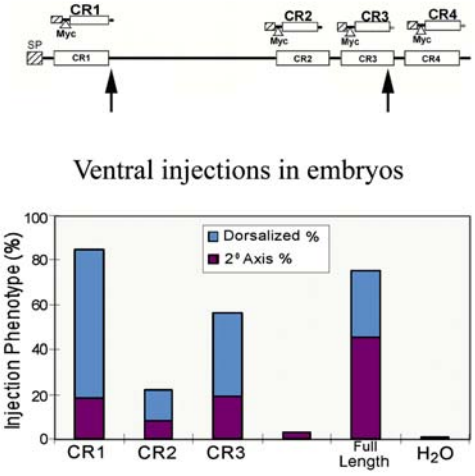
Proteolytic control plays a crucial role in the formation of gradients of growth factor activity.



Gerry Weinmaster SCIENCE · VOL. 279 · 16 JANUARY 1998 ·

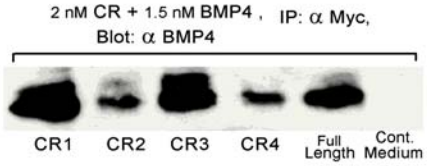
8. The anti-BMP activity of chordin resides in the cysteine-rich modules

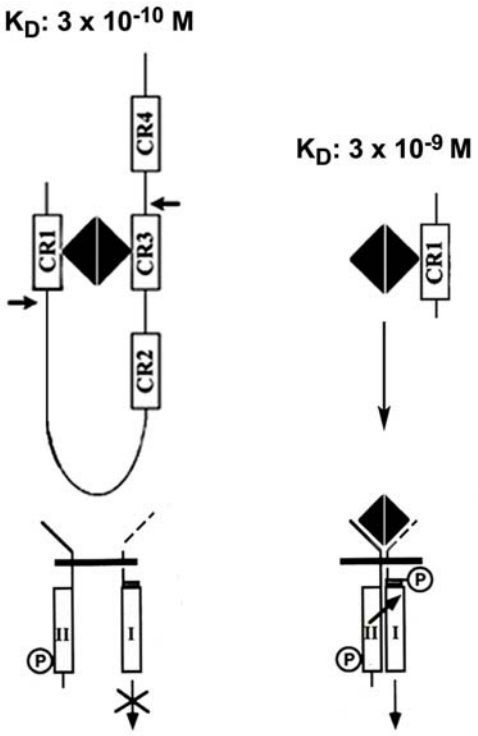
It has been found that the cysteine rich domains (CRs) are BMP-binding modules. Constructs containing individual CRs (of about 80 amino acids), in particular CR1 and CR3, are able to bind BMPs. Xolloid cuts Chordin just downstream of CR1 and CR3. Although the CR domain can bind BMP, it does so with 10-fold lower affinity than intact Chordin.

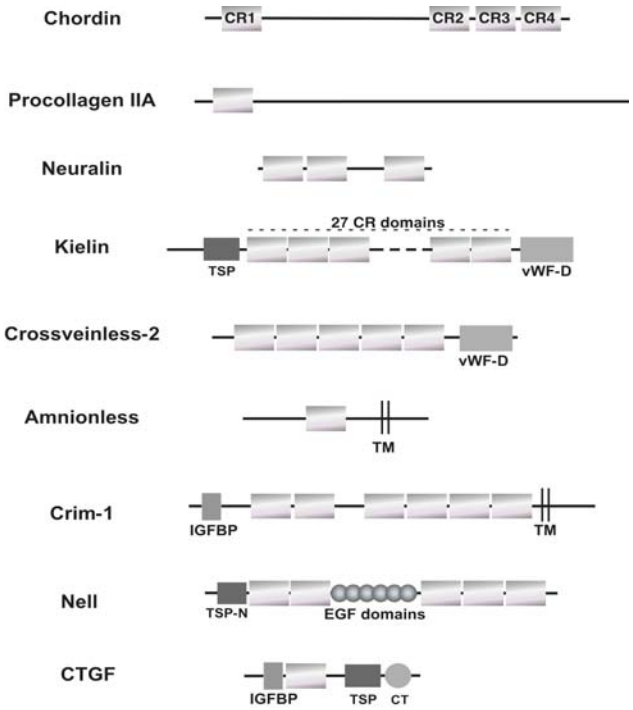


Ventral injections in embryos

Immunoprecipitation of BMP4 with CRs







CRs are found in many extracellular matrix proteins such as fibrillar procollagens (the most abundant proteins in the body), thrombospondin, von Willebrand factor, Neuralin-1, Crossveinless-2, Keilin, CTGF (connective tissue growth factor) and other proteins.

In the case of collagen the CR modules bind BMPs and TGFβ. The bound growth factors in principle might also be released by metalloproteases that cut close downstream of the module (as in the case of chordin), providing active signaling factors when required for tissue homeostasis in the adult.

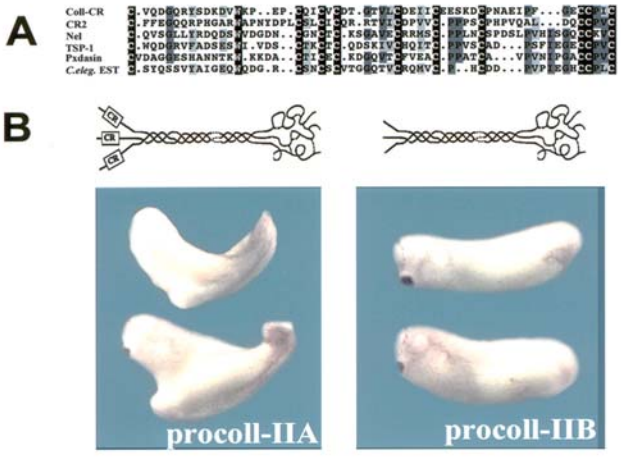
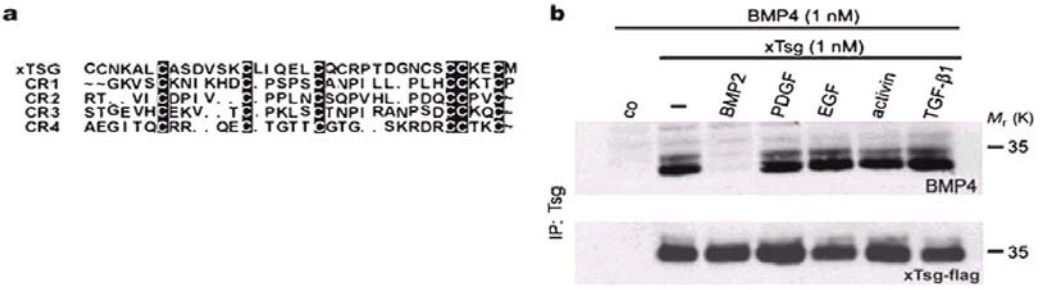


Fig. 9. CR domains present in procollagen IIA modulate BMP signaling. (A) *Sequence comparison of CR domains contained in different extracellular matrix proteins. Coll-CR, type IIA Xenopus procollagen; CR2, murine Chordin second repeat; Nel, rat nel; Pxdasin, Drosophila peroxidasin; C. eleg. EST, C. 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 Larrain 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.*

9. An additional player: Twisted Gastrulation

The *Drosophila* gene Twisted-gastrulation (dTsg) encodes a secreted protein that is required for the formation of the amnioserosa, the tissue that requires the highest levels of dpp/screw activity. Thus, dTsg functions to promote maximal BMP signaling. Oelgeschläger et al. (2000) noted that xTsg shared some sequence similarity to the part of the CR domains of chordin. This suggested that xTsg might be a BMP-binding protein, which it was.



xTsg binds BMP, but it does not compete with full-length chordin for the binding of BMP. On the contrary, it stimulated it, because xTsg is also a chordin-binding protein capable of forming a stable ternary complex of chordin, BMP and xTsg. However, when the proteolytic product of chordin digestion by Xolloid, CR1, was used the results were very different: the residual BMP binding activity of CR1 was dislodged by xTsg in cross-linking experiments.

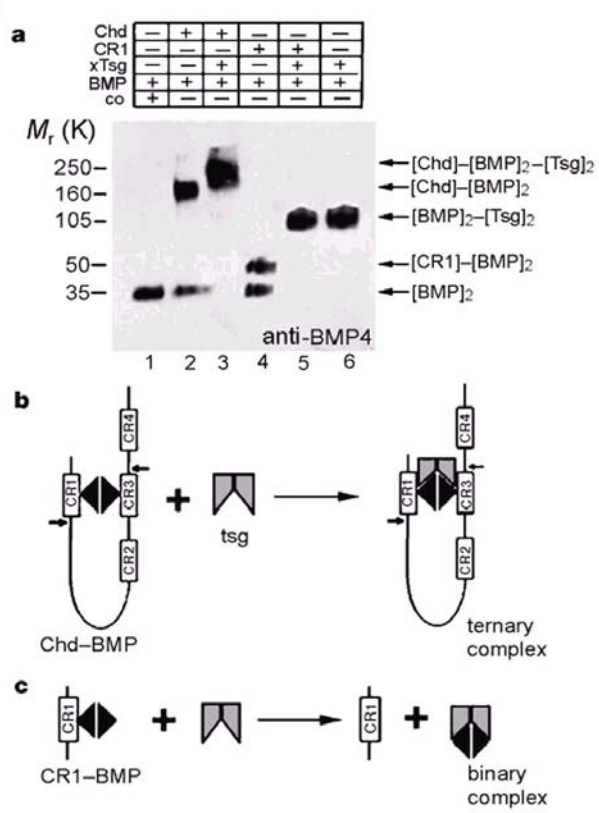
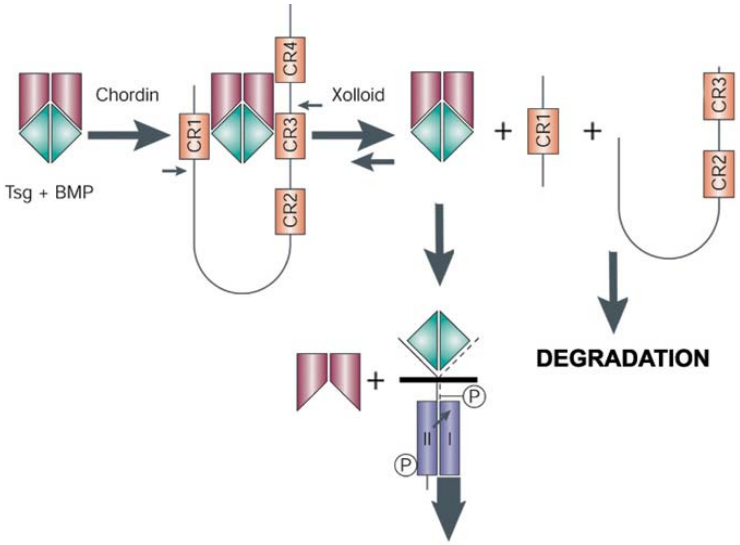


Figure 6 xTsg competes for binding of BMP-4 with CR1 but not with full-length Chordin. Crosslinking analyses were performed using DSS. **a**, Lane 1, BMP-4 (1 nM). 1 nM Chd and 1 nM BMP-4 form a complex of about 150K (lane 2) that shifts to ~220K in the presence of 1 nM xTsg (lane 3). When 1 nM CR1 was crosslinked to BMP-4 a 50K complex was detected (lane 4). When xTsg was added a complex of around 100K was produced exclusively (lane 5), which was identical to the $[BMP]_2–[xTsg]_2$ complex (lane 6). **b, c**, Diagrams summarizing the crosslinking results. The small arrows in Chordin indicate the Xolloid cleavage sites.

The ternary complex is a much better inhibitor of BMP signaling and the proteolytic cleavage of Chordin subsequently provides the molecular switch that permits BMP/xTsg to be released, allowing binding of BMP to its receptor.



Conclusion: A finely regulated molecular pathway involving Chordin, Xolloid and Twisted-gastrulation regulates the dorsal-ventral activity gradient of bone morphogenetic protein in *Xenopus*.

10. Head induction I: Dickkopf

Dickkopf-1 (German for big-head, stubborn) was isolated by Christof Niehrs by screening pools of 150 synthetic mRNAs co-injected with truncated (dominant-negative) BMP receptor. This was followed by sib selection. He knew that BMP inhibition gives trunk organizer and BMP and Wnt double inhibition head organizer:

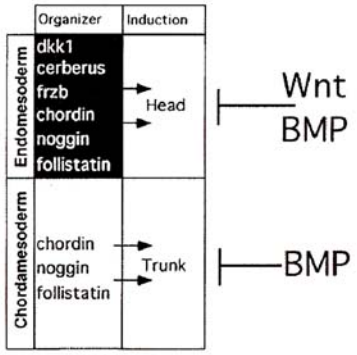
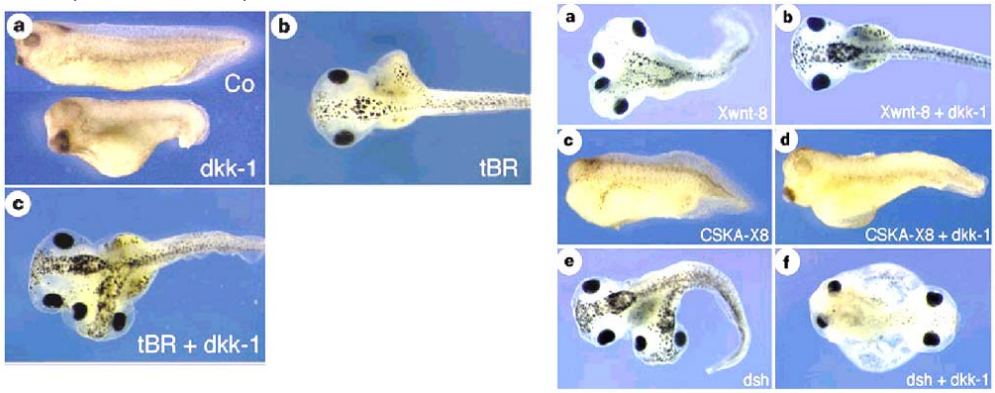


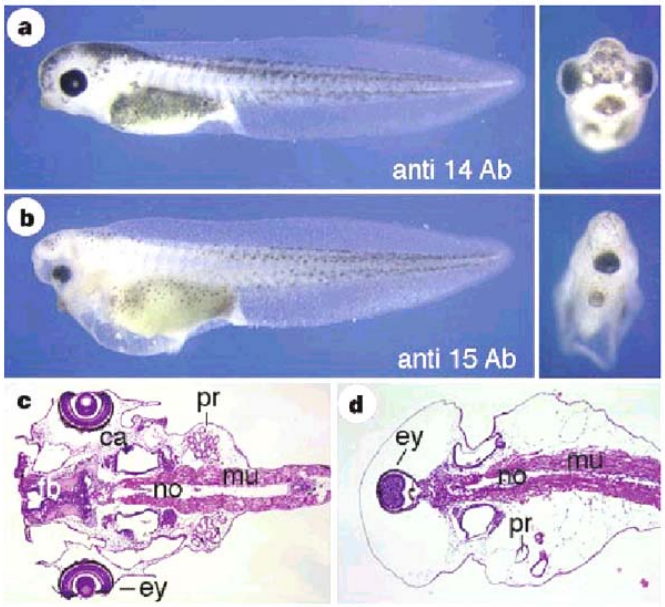
Fig. 1. Two-inhibitor model for organizer regionalization. Trunk induction requires inhibition of BMP signalling while head induction requires dual inhibition of Wnt and BMP signalling. The organizer produces factors that inhibit both types of signals (anti-wnt, Dkk1, Cerberus and Frzb; anti-BMP, Cerberus, Noggin, Chordin and Follistatin). Regional specificity of induction results from differential expression of Wnt and BMP inhibitors in endomesoderm and chordamesoderm. Note that Cerberus also inhibits Nodal and Activin signalling, which may be important to maintain anterior endomesodermal fate (adapted from Glinka *et al.*, 1997).

The new gene was expressed in prechordal mesoderm and induced large heads or complete axes (trunk + head). Cerberus does not induce trunks.



Dkk-1 mRNA inhibited early *Xwnt-8* mRNA effects and also late effects caused by injection of plasmid DNA of cytoskeletal actin promoter-Xwnt-8 (pCSKA-Xwnt-8) at the gastrula stage. This effect occurred upstream of Dsh. Since Dkk encodes a secreted protein it should function as a secreted Wnt antagonist.

In *Xenopus* antibody injections into the blastocoel caused cyclopia.

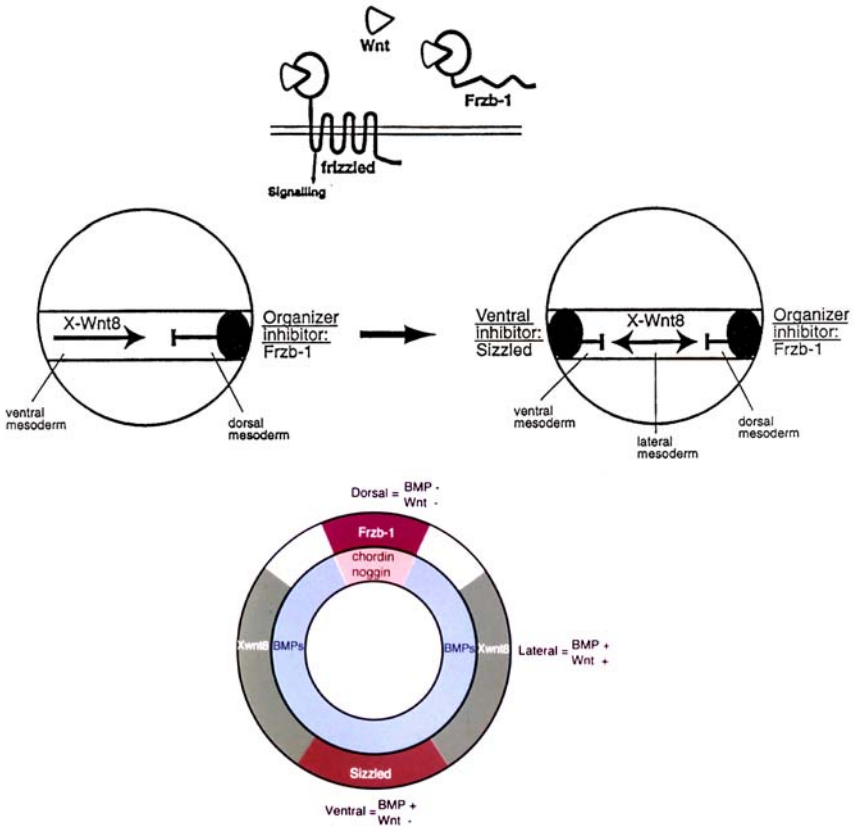


How does this head inducer work? By inhibiting canonical Wnt signaling, as described in lecture 2.

11. Head induction II: Frzb-1 antagonizes *Xwnt-8*.

Another factor secreted by the organizer is Frzb-1, which antagonizes the ventralizing effects of *Xwnt-8*, a gene normally expressed in the lateral and ventral marginal zone. *Xwnt-8* DNA constructs that drive expression of *Xwnt-8* at gastrula turn head and notochord structures into muscle, i.e., into more ventral structures. Frzb-1 is a secreted protein containing a domain similar to the putative Wnt-binding region of the frizzled family of transmembrane receptors. *Frzb-1* is widely expressed in adult mammalian tissues. In the *Xenopus* gastrula, it is expressed and regulated as a typical Spemann organizer component. Injection of *Frzb-1* mRNA blocks expression of *XMyoD* mRNA and leads to embryos with enlarged heads and shortened trunks. An anti-BMP co-injected with Frzb-1 induces heads. Cultured cells transfected with a membrane-tethered form of Wnt-1 bind epitope-tagged Frzb-1 in the 10^{-10} M range. The organizer secretes many other Wnt antagonists of the Frzb.

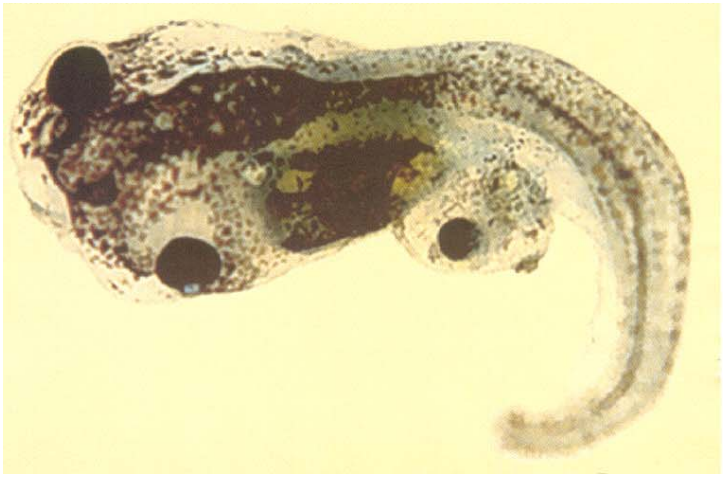
At a later stage during gastrulation a second secreted frizzled-like protein is secreted by the ventral marginal zone. This gene, called *sizzled*, was discovered by M. Kirschner at Harvard and serves to restrict *Xwnt-8* activity to the somite forming region. The marginal zone becomes subdivided by multiple inhibitory factors into regions of distinct cell fates



The inibition of BMP and Wnt signaling can be sufficient for the formation of head organizer, whereas BMP inhibition is required for trunk organizer. The work on Cerberus suggests that inhibiting Nodal is also an important step for the formation of the head field.

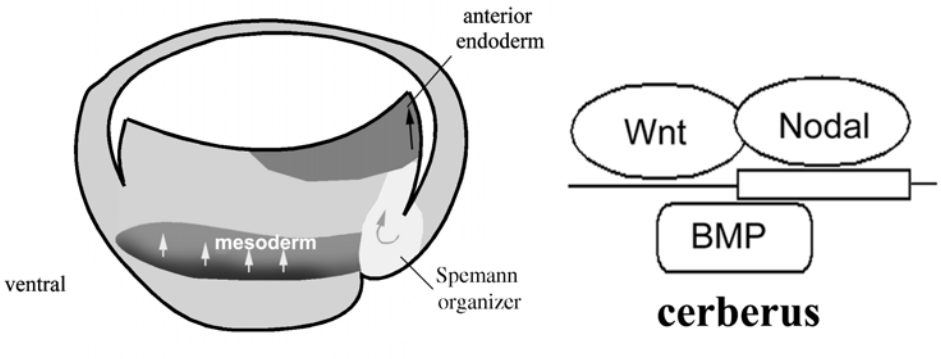
12. Head induction III: Cerberus and head development

Cerberus, a factor with head-inducing activities is a secreted inhibitor that antagonizes simultaenously Nodal, BMP-4 and Xwnt-8.



Cerberus mRNA injected into a single D4 (ventral vegetal) blastomere of a 32-cell *Xenopus* embryo induces head structures as well as a duplicated heart and liver. The secondary eye (a single cyclopic eye) and olfactory placode can be readily seen. (From Bouwmeester et al., 1996; photograph courtesy of E. M. De Robertis.)

Cerberus is expressed in the anterior-most endoderm, and provided the first suggestion that anterior endoderm is involved in head induction.



Cerberus is activated by early signals in Endoderm (Vg-1, nodal) and by organizer factors (such as Chordin and Frzb-1). The secretion of Cerberus into the extracellular space at the mid gastrula stage would be an important downstream event that locks the head program in place by simultaneously blocking three signaling pathways – Nodal, BMP-4 and Xwnt-8 – that are involved in trunk formation. Cerberus generates a trunk-free territory in the anterior of the embryo. Studies in mouse embryos strongly suggest an important role for anterior endoderm in head formation.

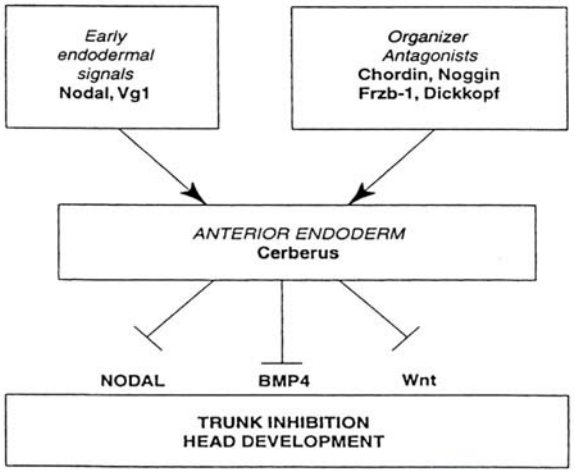


Figure 4 Model of the formation and function of anterior endoderm in *Xenopus* head development.

References Lecture 5

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