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THE ESTABLISHMENT OF SPEMANN'S ORGANIZER AND PATTERNING OF THE VERTEBRATE EMBRYO

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Abstract

Molecular studies have begun to unravel the sequential cell-cell signalling events that establish the dorsal-ventral, or 'back-to-belly', axis of vertebrate animals. In *Xenopus* and zebrafish, these events start with the movement of membrane vesicles associated with dorsal determinants. This mediates the induction of mesoderm by generating gradients of growth factors. Dorsal mesoderm then becomes a signalling centre, the Spemann's organizer, which secretes several antagonists of growth-factor signalling. Recent studies have led to new models for the regulation of cell-cell signalling during development, which may also apply to the homeostasis of adult tissues.

With its rapid embryonic development, large egg size (1-2 mm in diameter) and high numbers of embryos (1,500 per female), *Xenopus* provides a favourable model system for the study of vertebrate development, and it has been used extensively to probe the events in early embryogenesis. The dorsal side of the amphibian embryo contains the information for the differentiation of many different cell types. At the gastrula stage, the dorsal side of the embryo can be recognized by the presence of the dorsal blastopore lip (FIG. 1). When the gastrula embryo is bisected by ligature with a hair loop into dorsal and ventral fragments, the half that contains the dorsal blastopore lip develops into an entire embryo, whereas the ventral half remains as a 'belly piece' (*Bauchstück*) that is devoid of all axial organs[1]. In a complementary experiment, the dorsal lip transplanted to the ventral side of a host embryo induces the formation of a twinned embryo that contains axial structures and organs[2]. This dorsal-ventral difference can be traced back to the fertilization stage, with the dorsal side always forming opposite to the sperm entry point.

DORSAL CRESCENT

Region of reduced pigmentation that marks the future dorsal side of the fertilized egg.

The first external sign of asymmetry in the *Xenopus* egg is the appearance of an unpigmented DORSAL CRESCENT (called the grey crescent in some amphibians)[3], which is caused by a rotation of the egg cortical cytoplasm that is driven by microtubules[4] (FIG. 1). Dorsal determination seems to be associated with the cytoplasm that surrounds the heavy yolk platelets in the vegetal pole. When the heavy yolk and associated cytoplasm is made to flow towards the animal pole of an amphibian egg, for example by inverting the egg by 180° or by centrifugation, a twinned dorsal axis is formed[5]. Isolating the molecules that mediate the phenomena behind these experimental observations has been the Holy Grail of amphibian embryology. Remarkably, the general outlines of a molecular pathway that regulates dorsal development from fertilization to gastrulation are starting to emerge.

Here we review how dorsal determinants located in membrane vesicles in the vegetal pole of the embryo are transported to the dorsal side by cortical microtubules. This event correlates with the activation of the canonical Wnt signalling pathway on the dorsal side, causing the

stabilization and nuclear localization of the β-Catenin protein. In turn, this leads to the generation of a gradient of signalling molecules related to Nodal in the endodermal region at the blastula stage, resulting in the induction and patterning of the mesodermal germ layer. During gastrulation, a signalling centre (Spemann's organizer) becomes established in the dorsal mesoderm and expresses numerous organizer-specific genes, notably secreted proteins that bind to growth factors in the extracellular space and prevent them from signalling. These antagonists include molecules such as Noggin, Chordin, Cerberus, Frzb-1, Crescent and Dickkopf (DKK). One of the main conclusions from this research is that cell differentiation in the gastrula embryo is regulated by inhibitory secreted molecules. We also address in some detail the Chordin pathway, in which signalling by BONE MORPHOGENETIC PROTEINS (BMPs) bound to Chordin is regulated by the combined action of the protease Xolloid and of another BMP-binding protein called Twisted-gastrulation (xTsg).

BONE MORPHOGENETIC PROTEINS

(BMPs). Molecules of the TGF-β family that can induce bone formation and ventralize the vertebrate embryo. In zebrafish, a mutation in either *BMP-2b* or *BMP-7* has a similar effect, suggesting that they work as heterodimers; either mutation also inhibits transcription of *BMP-4*.

Rescue of the effects of UV irradiation

The critical function of the dorsal determinants stored in the *Xenopus* egg is best illustrated by the experiments shown in FIG. 2. When fertilized eggs are irradiated at the vegetal pole with ultraviolet light, which impairs microtubule function by crosslinking GTP to tubulin, the embryo develops as a belly piece, lacking all dorsal axial organs (FIG. 2a, left). Other treatments that disrupt microtubules, such as nocodazole and low temperature, have similar outcomes[4]. The ventralized belly pieces develop all three germ layers, but the mesoderm is of the ventral type (lateral plate mesoderm and blood) and the ectoderm consists exclusively of epidermis[6-8] (FIG. 2b). Remarkably, these ventralized embryos can be completely rescued by the microinjection of certain synthetic messenger RNAs. For example, upon a single injection of *chordin* mRNA into 4- to 32-cell embryos, the formation of head, trunk and tail is restored (FIG. 2a, right), and the rescued embryos contain a variety of dorsal tissues, such as central nervous system (CNS) in ectoderm, and somite, notochord and kidney in mesoderm (FIG. 2c). The most extraordinary aspect of the ultraviolet-rescue experiment is that the same result can be obtained with a large variety of mRNAs that function in very different signalling pathways (FIG. 2d). Complete rescue can be caused by the microinjection of mRNAs that encode Wnts and several downstream effectors of its pathway, such as β-Catenin and proteins that regulate β-Catenin degradation[9-11], and by microinjection of a number of Nodal-related factors[12] or BMP antagonists, such as Noggin and Chordin[13,14]. How can such diverse molecules all have the same properties in such a simple ultraviolet-rescue assay? The thesis that we develop in the following pages is that Wnts, Nodals and anti-BMP molecules represent sequential steps in a common pathway leading to dorsal axis development. The ultimate consequence of activating this pathway in the embryo would be to generate a region of low BMP signalling that is required for dorsal differentiation.

Dorsal determinants stabilize β-Catenin

A breakthrough in the study of early dorsal axis establishment was the discovery that β-Catenin is translocated into the nuclei of cells on the dorsal side of both *Xenopus* and zebrafish embryos at the early blastula stage[15,16]. *Xenopus* β-Catenin has been shown to be required for dorsal axis formation by the inhibition of maternal mRNAs using antisense oligonucleotides[17,18]. β-Catenin protein starts to accumulate in the dorsal cytoplasm as early as the 2-cell stage and

in dorsal nuclei by the 16-cell stage[16]. By the midblastula stage, nuclei stain positively for β-Catenin in the entire dorsal side, including regions that later give rise to endoderm, mesoderm and ectoderm; this shows that dorsal signals are present in a much wider area than was previously thought[15].

Another crucial discovery has been that the cortical microtubules, which extend in parallel arrays from the sperm entry point to the dorsal side of the embryo, not only mediate the 30° rotation of the egg cortex with respect to the underlying cytoplasm, but also transport membrane vesicles located at the vegetal pole to the dorsal side[19] (FIG. 3a). Importantly, these membrane vesicles are transported dorsally 90° or more towards the animal pole, at the extraordinary speed of 25-40 μm min^{-1} (REF. 19). The membrane vesicles are able to bind the fusion protein Dishevelled-green fluorescent protein (Dsh-GFP) and transport it to the dorsal side[20]. Dsh is a component of the Wnt signalling pathway and it associates with the cytoplasmic side of membranes on activation by Wnt[11]. When the mRNA that encodes the Frizzled-7 Wnt receptor is ablated in oocytes that have been microinjected with antisense oligonucleotides, the formation of the dorsal axis is abolished[21]. It is therefore attractive to speculate that Frizzled-7 may be present in the membrane vesicles that function as dorsal determinants, becoming activated by an as yet unidentified Wnt protein present inside the membrane vesicles and leading to the stabilization and nuclear localization of β-Catenin (FIG. 3a).

HYPOCHORD

Transient rod-like structure derived from the endoderm, which is located beneath the notochord in vertebrate embryos.

In ultraviolet-treated embryos, the parallel microtubular arrays do not form, and nuclear β-Catenin is found only in vegetal pole nuclei[15] (FIG. 3b). The membrane vesicles marked by Dsh-GFP remain in the vegetal pole[20], and the expression of β-Catenin target genes, such as homeobox (*siamois* and *xtwin*) and secreted factor genes (*nodal-related-3* and *cerberus*), remain confined to the vegetal pole[8,22,23]. In this vegetal location, the β-Catenin signal does not reach the marginal zone and animal cap, and is unable to trigger the differentiation of dorsal cells into mesoderm and ectoderm. When vegetal cytoplasm is injected into marginal or animal regions, however, the differentiation of dorsal tissues is restored[23].

In the zebrafish, arrays of parallel microtubules are formed in the vegetal pole of the zygote that, when disrupted by ultraviolet irradiation, result in ventralization of the embryo[24]. Partial removal of the vegetal yolk cell mass results in severely ventralized embryos[25,26]. It is therefore conceivable that zebrafish vegetal cytoplasm contains vesicles similar to those that function as dorsal determinants in *Xenopus*. In some freshwater snails (for example, *Bithynia*), a small protrusion of cytoplasm of less than 1% of the egg volume, called a polar lobe, is formed during early cleavage, and its removal results in the loss of many mesodermal structures. These polar lobes are filled with intriguing small membrane vesicles of about 3 μm in diameter, which are located at the vegetal pole cortex during oogenesis[27]. In the mouse, genetic evidence supports the proposed role of the Wnt pathway in axis formation. Mutation of *axin*, an inhibitor of β-Catenin, results in duplicated axes[10], and targeted gene inactivation of *Wnt-3* or *β-catenin* results in the absence of axis formation[28,29].

In conclusion, recent studies indicate that the initial dorsal asymmetry in the vertebrate embryo may be triggered by the transport of dorsal membrane vesicles capable of activating the β-Catenin pathway.

Mesoderm induction by Nodals

The next step in dorsal-ventral axis formation is the induction of mesoderm by the endoderm. The established view from embryological studies was that the endoderm releases two signals, one from the ventral endoderm, which induces ventral mesoderm, and a second from the dorsal endoderm (a region called the Nieuwkoop centre), which induces dorsal mesoderm (or Spemann's organizer tissue). A third signal subsequently emanates from the Spemann's organizer in the plane of the mesoderm to refine the initial dorsal-ventral pattern[7,30] (FIG. 4). The mesoderm-inducing signal is released by endoderm after midblastula[31] and can be investigated experimentally by combining explants of vegetal and animal tissue, as done initially by Nieuwkoop.

Nodal-related proteins, which belong to the transforming growth factor (TGF)-β family of growth factors, have important functions in mesoderm formation in different species. In the mouse, only one *nodal* gene has been identified, and the effects of its inactivation suggest a central function in the formation or maintenance of mesoderm[32,33]. In zebrafish, two *nodal*-related genes, *cyclops* (*cyc*) and *squint* (*sqt*), have been found[34]. In the absence of each individual gene, cyclopic embryos develop, but when both gene products are removed, embryos lack head and trunk dorsal mesoderm, as well as endoderm, and fail to express the organizer-specific homeobox gene *goosecoid*[35].

We mentioned above that ultraviolet-induced ventralization of *Xenopus* embryos is completely rescued by microinjection of mRNAs encoding proteins related to Nodal[12]. In *Xenopus*, there are five mesoderm-inducing *Nodal*-related (*Xnrs*) genes (REFS 12,[36]; and M. Asashima, personal communication), making loss-of-function studies particularly difficult. However, the induction of mesoderm by endoderm was discovered initially in amphibians, and has been analysed in considerable detail in these animals[30].

A specific inhibitor of *Xnrs* provided a way of testing the role of these molecules in *Xenopus* mesoderm induction. It was found that one of the inhibitory proteins secreted by the organizer, Cerberus[37], was an antagonist of Xnrs, and that the Xnr-binding activity resided in the carboxy-terminal domain[38]. A construct comprising only this domain, called Cer-short, provides a valuable tool to test the role of the several Xnrs in development, as it specifically inhibits mesoderm-inducing Xnrs but not other TGF-β molecules, such as Activin, Vg1, BMP-4 and Derrière[39].

When Nieuwkoop's original mesoderm-induction experiments of combining animal and vegetal explants were repeated using Cer-short as a reagent to inhibit Xnrs, the induction of both dorsal and ventral mesoderm was blocked[39]. At the blastula stage, Xnrs are expressed in a dorsal to ventral gradient in endodermal cells[39], which is accompanied by the preferential phosphorylation of Smad2 (a downstream effector of TGF-β signalling) on the dorsal side[40]. Dose-response experiments using increasing amounts of *cer-short* mRNA confirmed the existence of an endogenous Xnr activity gradient in endoderm[39]. A modified model of *Xenopus* early development, in which the induction of both dorsal and ventral mesoderm is mediated by a gradient of several Nodal-related signals released by the endoderm at the blastula stage, is shown in FIG. 4. In this view, the three-signal model mentioned above may be considered a two-signal one[30,39].

The gradient of *Xnr* expression in the endoderm is thought to be activated by three maternally provided molecules: Vg1,VegT and β-Catenin (FIG. 4).Vg1, a TGF-β factor, and VegT, a T-box transcription factor, are both localized to the vegetal pole of the *Xenopus* oocyte and are potent inducers of endoderm[41,42]. Depletion of maternal VegT leads to the absence of endoderm[42]. In VegT-depleted embryos, *Xnr* transcription and mesoderm formation are severely inhibited and can be rescued by injection of *Xnr* mRNA[43]. Wild-type embryos

microinjected with VegT and Vg1 have only low levels of *Xnr* transcription; however, when β-Catenin is also provided, it cooperates with VegT and Vg1 to achieve the high levels of Xnr expression that cause organizer induction[39] (FIG. 4). A plausible explanation for the ultraviolet-induced phenotype, in which all three germ layers are present (FIG. 2b), is that, even though β-Catenin is lacking on the dorsal side, endogenous VegT and Vg1 levels in endoderm are sufficient to generate low levels of Xnr signalling, thereby mediating the induction of ventral mesoderm (FIG. 4).

In conclusion, after midblastula, the β-Catenin signal, in combination with other maternal genes, activates a dorsal-ventral gradient of several Nodal-related signals in the endoderm that, in turn, mediate the induction and patterning of the mesodermal layer.

β-Catenin dorsalizes the three germ layers

As discussed above, nuclear β-Catenin accumulates in the entire dorsal side of the *Xenopus* blastula[15]. This stabilization of β-Catenin protein has profound effects in all three germ layers. It has been known for a long time that the dorsal ectoderm of the embryo is predisposed, when compared with ventral ectoderm, to respond to neural-inducing signals[44]. Ectopic expression of β-Catenin in animal-cap ectoderm is able to repress *BMP-4* transcription, thus promoting neural development[45]. Similarly, signalling through β-Catenin represses transcription of the epidermal homeobox gene *Dlx3*,a *Xenopus* anti-neural factor[46]. In zebrafish, β-Catenin both induces transcription of the homeobox gene *bozozok*, which is related to *goosecoid*, and is required for the dorsal repression of *BMP-2b/4* transcription in the dorsal marginal zone[47, 48].

In the mesoderm, even after the initial induction has taken place, β-Catenin and TGF-β signals (probably Nodal-related) are integrated at the level of promoter DNA to trigger transcription of the organizer-specific gene *goosecoid*[49-51]. In *Xenopus*, β-Catenin can also directly activate transcription of the homeobox genes *siamois* and *xtwin*[8]. These two closely related homeobox genes are transcriptional activators and therefore are different from *bozozok* and *goosecoid* in zebrafish, which are considered to be transcriptional repressors[52].The *goosecoid* promoter in *Xenopus* contains two distinct DNA regulatory elements (see supplementary information online, FIG. S1). Xtwin/Siamois binds to the proximal element, thereby relaying the β-Catenin signal. The distal element contains binding sites for a mesendodermal homeodomain protein, Mixer, and for a heterodimer of Smad2 and Smad4. Transcription of *mixer* is activated, and nuclear translocation of the Smad2/Smad4 heterodimer is stimulated by the TGF-β/Xnr signal that, in this way, is transduced to the *goosecoid* promoter[51]. In the promoters of other TGF-β-inducible genes, the function of Mixer is carried out by the Forkhead-related protein FAST-1 (REF. 53). The Wnt and TGF-β pathways might even be integrated in the absence of separate promoter elements, as in the case of the x*twin* promoter[54,55] (see supplementary information online, FIG. S1).

In the endoderm, inhibition of β-Catenin signalling by ultraviolet irradiation blocks the induction ofXlHbox-8, a marker of dorsal endoderm, which can be restored by microinjection of TGF-β factors[56]. Cell-dissociation experiments have shown that the secretion of TGF-β factors, such as Xnrs, is required for endodermal differentiation[57]. In addition, the initial expression of *Xnr-1*, *Xnr-2* and *Xnr-4* in the endoderm requires the β-Catenin pathway[39].

In conclusion, signals triggered by the dorsal determinants pattern all three germ layers. β-Catenin activates genes such as *siamois* and *bozozok*, and the expression of Nodal-related factors, which subsequently cooperate in several parallel pathways as effectors of dorsal development[52,58,59]. Detailed studies on promoters, such as that of *goosecoid*, are gradually providing an understanding of how these many pathways are brought together at the level of transcriptional regulation of organizer-specific genes.

The organizer secretes antagonists

At the gastrula stage, the main dorsalizing centre of the embryo is Spemann's organizer, which is located in the dorsal mesoderm (FIG. 4). Its molecular exploration proved a productive fishing ground for the discovery of new genes (see supplementary information online, FIG. S2) and produced unexpected findings. The main surprise was that the organizer is a source of secreted antagonists that bind to growth factors in the extracellular space and prevent them from binding to their cognate receptors. These novel antagonists can be classified according to the growth factors that they inhibit (FIG. 5).

BMP antagonists

The BMP antagonists Chordin, Noggin and Follistatin do not share any common structural elements[13,14,60]; however, a single ventral injection of mRNAs of any of these proteins leads to the formation of twinned axes, recapitulating the effects of grafting Spemann's organizer. When BMP antagonists are overexpressed ubiquitously, embryos become radially dorsalized, mimicking the phenotype caused by lithium chloride (LiCl) treatment during early cleavage. LiCl transforms the entire mesoderm into Spemann's organizer and acts through the inhibition of GSK-3, a serine/threonine kinase that phosphorylates β-Catenin and targets it for degradation[61,62] (FIG. 3a). So these anti-BMP activities mimic those caused by increased β-Catenin signalling. Transcription of *noggin* and *chordin* is induced radially by treatment with LiCl and both genes were cloned during the course of screens involving LiCl treatment of *Xenopus* embryos[13,14]. Conversely, *chordin* and *noggin* transcripts are greatly reduced in ultraviolet-treated embryos and, as mentioned above, when microinjected, they completely rescue the ultraviolet-induced ventralized phenotype (FIG. 2a). The expression of Chordin is negatively regulated by signals from the ventral side of the embryo that activate ventralizing homeobox genes, such as *vent* and *vox*, which repress *chordin* expression in ventral-lateral regions of the embryo[63-65]. Chordin and Noggin bind BMPs directly in the extracellular space, preventing BMPs from binding to and signalling through its cognate BMP receptor[66,67]. Follistatin also binds BMPs, although, unlike the other BMP antagonists, the Follistatin-BMP complex can bind to the BMP receptor but is unable to signal[68].

Wnt inhibitors

Wnt inhibitors secreted by the organizer are of two types, Frzbs and Dkks (FIG. 5). Frzb-1 is a secreted protein that contains a domain similar to the Wnt-binding region of the Frizzled Wnt receptors, and functions by binding to Wnts and antagonizing their activity[69,70]. Frzb-1 is a member of a large family of related Wnt inhibitors that have been renamed secreted Frizzled-related proteins (sFRPs)[11]. Other members of this family are also expressed in Spemann's organizer, namely *sFRP-2* and *crescent*[71]. Another sFRP, *sizzled*, is expressed in the ventral side of the gastrula[72]. Dkk-1 was the founding member of a new class of Wnt antagonists, and contains two new cysteine-rich domains[73]. Dkk-1 and Frzb-1 are expressed in the deep layers of Spemann's organizer, including the future head mesoderm and anterior endoderm. When ubiquitously over-expressed, Dkk-1 and Frzb-1 lead to embryos with enlarged heads and a shortened trunk. This phenotype is caused by an expansion of Spemann's organizer, thought to be caused by inhibition of ventralizing Xwnt-8 signals present in the ventral-lateral marginal zone[69,73,74]. In combination with inhibitors of BMP signalling, Dkk-1 and Frzb-1 cooperate in the formation of head structures, and microinjection of neutralizing Dkk antibodies causes microcephaly[73,75]. The many Wnt antagonists expressed by Spemann's organizer differ in biological activities. For example, overexpression of *crescent* mRNA causes cyclopia, whereas overexpression of *frzb-1* mRNA leads to enlarged eyes[71]. This indicates that different Wnt antagonists bind to overlapping, but distinct sets of Wnt signals.

Cerberus

Cerberus, a secreted protein of 260 amino acids expressed in the anterior-most region of involuting endoderm[37], is the founding member of a large family of cell-cell signalling regulators[76]. Microinjection of *cerberus* mRNA into the ventral side of the embryo leads to induction of ectopic head structures in the absence of trunk formation[37]. Cerberus protein is a multivalent antagonist that binds to Xnrs, Xwnt-8 and BMP-4 in the extracellular space[38]. These three signalling pathways are required for trunk development, and secretion of Cerberus by anterior endoderm serves to maintain a trunk-free region in the anterior of the embryo so that the head territory can develop.

TGF-β/Nodal receptor antagonists

Antivin/Lefty is an Activin/Nodal antagonist that has been isolated in frog, fish and mouse. It is a divergent member of the TGF-β superfamily that lacks the α-helix required for dimerization[77,78]. Mouse mutants lacking *lefty-2* form excess mesoderm, a phenotype that is partially suppressed by heterozygosity for *nodal*, suggesting that the main function of Lefty-2 is to downregulate Nodal signalling[79]. Because transcription of *antivin/lefty* is induced by Nodal, it acts as a feedback inhibitor that limits the Nodal signal in time and space[79,80]. Interestingly, the phenotypic effects of Antivin/Lefty in zebrafish can be suppressed by overexpression of the extracellular domain of the Activin/Nodal receptor type IIB (REF. 79). This result indicates that Antivin/Lefty may block Nodal signalling by binding to TGF-β/Nodal receptors and inhibiting their activity (FIG. 5). In *Xenopus*, Xnr-3 (a divergent Xnr) lacks mesoderm-inducing activity but can induce neuralization instead[81]. Although its mechanism of action has not been determined, it may be worthwhile in future studies to explore whether Xnr-3 can also function as a competitive inhibitor of TGF-β receptors.

In conclusion, studies of Spemann's organizer have led to the discovery of new mechanisms of regulating cell-cell signalling, in which secreted antagonists of growth factors are important in patterning the vertebrate embryo.

Chordin and dorsal-ventral patterning

We now discuss in more depth the protein Chordin, as studies on this molecule have uncovered a finely regulated pathway of cell-cell signalling. Chordin is a secreted protein that contains four internal cysteine-rich domains of about 70 amino acids (designated CRs, see supplementary information online, FIG. S3) that bind BMP molecules and prevent their binding to BMP receptors[66]. All four CRs bind BMPs at a detectable level, but CR1 and CR3 bind more effectively than do CR2 and CR4. The affinity of the binding of BMPs to CR1 or CR3 is ten times lower than that of full-length Chordin for BMPs[82]. In agreement with these biochemical properties, injection of mRNA encoding CR1 or CR3 causes dorsalization in *Xenopus* embryos[82]. Although less effective than full-length Chordin, the CR1 and CR3 modules retain significant anti-BMP activity.

Genetic analyses in zebrafish, mouse and *Drosophila* support an important function of Chordin in dorsal-ventral patterning. In zebrafish, the strongest ventralizing mutation isolated in extensive genetic screens corresponded to the inactivation of the Chordin homologue Chordino[83,84]. In *chordino* mutants, neural plate and dorsal mesoderm are reduced, whereas epidermis and ventral mesoderm are expanded at the gastrula stage[85] (FIG. 6a). The crucial role of BMP signalling in early embryonic patterning is illustrated by the fact that the dorsal-ventral mutants identified so far in zebrafish encode components of the BMP signalling pathway. Mutants in *swirl/BMP-2b*, *somitabun/Smad5* and *snail house/BMP-7* have strong dorsalized phenotypes[86-88]. The *swirl* dorsalized phenotype is epistatic to *chordino* in *chordino;swirl* double mutants[85], suggesting that Chordin is a specific antagonist of BMP

ventralizing activities. In mouse, *chordin;noggin* double mutants lack the forebrain and anterior notochord, and have a greatly reduced pharyngeal endoderm, as well as a randomized left-right axis[89]. These genetic studies in zebrafish and mouse show that secreted Chordin protein is required for correct patterning of the three germ layers.

In *Drosophila*, the product of the *short-gastrulation* (*sog*) gene[90] is the functional homologue of Chordin[91] and antagonizes the BMP homologues *Dpp* and *screw*, which are the zygotic dorsal-ventral morphogens of the fly embryo[92-94]. Loss-of-function mutations in *sog* result in an expansion of dorsal ectoderm at the expense of neurogenic ectoderm[90] (FIG. 6b). As in the case of *swirl* and *chordino*, in double-mutant studies, *dpp* and *screw* are epistatic to *sog*[93,95], suggesting that *sog*/*chordin* is a specific antagonist of BMP signalling.

Proteolytic control of Chordin activity

In *Drosophila*, a protease called Tolloid is an integral component of the dorsal-ventral patterning system, and functions as an enhancer of DPP/BMP signals[92,96]. In zebrafish, the most frequently isolated dorsalized mutant, *mini-fin*, is a loss of function of the zebrafish *tolloid* gene. This mutant shows a reduction of ventral and an expansion of dorsal markers at late gastrula, and eventually develops into a viable fish that lacks the ventral tail fin[97].

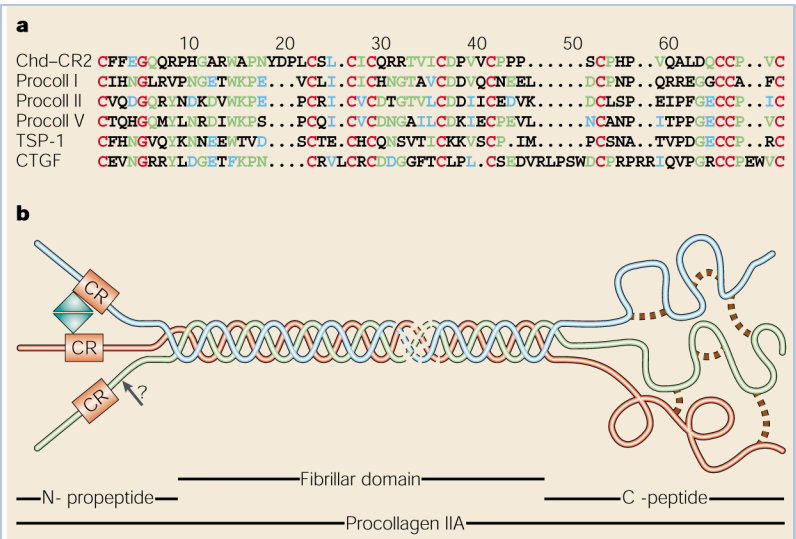
Using a direct biochemical approach, it was found that Tolloid and its *Xenopus* homologue, Xolloid, function by cleaving inactive SOG-DPP or Chordin-BMP complexes[98,99] (FIG. 7a). Cleavage of these complexes permits the re-activation of BMPs, which are then able to signal again, ventralizing *Xenopus* explants[98].Recently, the precise cleavage sites of Xolloid in Chordin have been identified[100] and found to be located at conserved aspartate residues 30 amino acids downstream of CR1 and 16 amino acids downstream of CR3. Therefore, the cleavage of Chordin by Xolloid releases intact CR modules in a complex with BMPs, and this cleavage may allow previously inactive BMP molecules to signal once again.

A new player: Twisted-gastrulation

Recent results from *Drosophila* and *Xenopus* indicate that a further molecule may participate in the Chordin/Sog, Xolloid/Tolloid, BMP/Dpp pathway. The *Drosophila* gene *twisted-gastrulation* (*dtsg*) encodes a secreted protein necessary for the formation of the amnioserosa, the tissue in the *Drosophila* embryo that requires the highest levels of Dpp/Screw activity[101,102] (FIG. 6b). Therefore, dTsg is a molecule that is required to attain maximal BMP signalling activity. Careful inspection of the amino-acid sequence of dTsg revealed some sequence similarity with the CRs of Chordin, which was validated biochemically by the demonstration that dTsg can bind BMPs[103].The *Xenopus twisted-gastrulation* homologue (*xtsg*) is expressed in the ventral pole of the embryo as part of the *BMP-4* SYNEXPRESSION group, suggesting that this gene may function in the BMP pathway[103,104].

Box 1

Conservation of cysteine-rich modules



Individual cysteine-rich (CR) domains similar to those of Chordin are present in fibrillar procollagen I, procollagen II and procollagen V, Thrombospondin-1 (TSP-1), and connective tissue growth factor (CTGF) (a). Other proteins contain several CR domains, such as Nel-1, Crossveinless-2, CRIM-1 and Kielin, which contain 4, 5, 6 and 27 CR domains, respectively[107-110]. In the case of procollagen IIA, the CR domain is located in the N-propeptide region and directly binds to BMP-4 and to TGF-β1 (REFS 82, [106]). In *Xenopus* assays, full-length procollagen IIA mRNA has anti-BMP activity, which requires the CR domain[82]; however, the monomeric collagen IIA CR module does not have detectable biological activity. After secretion, procollagen IIA forms a homotrimer and the N propeptide containing the CR remains attached to collagen fibrils in the extracellular matrix (b)[106]. This structure brings together three CR domains and, as in the case of the multiple CR repeats of Chordin, may result in the stabilization of the interaction between the CRs and BMP. The cleavage closest to the CR, which would release bound growth factors, is hypothetical and is indicated by a question mark. The CRIM-1 homologue of *Caenorhabditis elegans* also has anti-BMP activity in *Xenopus* embryos[82]. Kielin has dorsalizing activity that is consistent with moderate anti-BMP activity[110]. The Crossveinless-2 protein of *Drosophila* contains five CR repeats but, unlike the other CR-containing proteins, is required genetically to increase levels of DPP/BMP activity during formation of the cross veins of the fruit fly wing[108]. Thus, it seems that many CR modules are involved in growth-factor regulation in the extracellular matrix.

A combination of co-injection experiments in *Xenopus* embryos and protein crosslinking[103] support the biochemical pathway depicted in FIG. 7b. xTsg is expressed in the same cells that synthesize BMP-4, and it is therefore possible that a complex of both proteins is secreted by ventral cells. In addition to binding BMPs, the xTsg protein is able to bind to Chordin itself. In the presence of xTsg, Chordin is a more efficient BMP-binding protein, forming a stable ternary complex[103]. As Chordin/BMP complexes do not bind to BMP receptors, in this first aspect of its function xTsg would convert Chordin into a better antagonist of BMP signalling (FIG. 7b).

SYNEXPRESSION GROUP

A group of genes that have similar expression domains in the embryo, which usually correlate with function in a common biochemical pathway.

After Xolloid cleavage, however, xTsg-BMP binary complexes are formed preferentially. These binary complexes do not interfere with binding of BMP to its receptor and replace inhibitory CR1-BMP complexes. In agreement with these biochemical experiments, dorsalization by CR1 is readily competed by *xtsg* mRNA, and reduction of endogenous xTsg levels by a dominantnegative form of xTsg increases the anti-BMP effects of CR1 mRNA in *Xenopus* embryos[103]. In this second function, xTsg would provide a permissive signal to allow peak BMP signalling (FIG. 7b). BMP activity would be maximal in regions that have the highest concentrations of Xolloid, Chordin proteolytic fragments and xTsg. In *Drosophila*, the homologous region would correspond to the amnioserosa. In regions of the embryo in which residual full-length Chordin is still present, xTsg would facilitate the binding of any BMPs that have been released by Xolloid cleavage to fresh molecules of Chordin; this mechanism could facilitate the generation of borders between embryonic territories and ensure that peak BMP signalling occurs only in a specific region of the BMP activity gradient. In future, it will be worthwhile to investigate whether additional factors, such as a co-receptor, exist in this pathway. In addition to the two biochemical functions described above, xTsg also enhances the cleavage of Chordin by Xolloid in microinjected embryos (E.M.D.R., J.L. and M.O., unpublished observations). This third function suggests that the Chordin-BMP-xTsg ternary complex may be a better substrate for Xolloid cleavage.

Studies in *Drosophila* add a further layer of complexity to the function of Tsg. Constructs of Sog, termed Supersogs, which contain CR1 and further amino acids downstream of the first Tolloid cleavage site show new signalling properties. When overexpressed in the wing imaginal disc, full-length SOG will inhibit the BMP factor Glass bottom boat/60A (GBB), but not DPP. However, Supersog will inhibit DPP as well[105]. Because Supersog is insensitive to cleavage by Tolloid[105], this inhibitory specificity may be explained by the formation of trimolecular complexes of Supersog, dTSG and DPP. Supersog-like fragments have been detected in *Drosophila* embryos overexpressing SOG. Furthermore, in biochemical studies dTSG binds to SOG and generates Supersog-like fragments in the presence of Tolloid and DPP[105]. In the future, it will be of interest to investigate whether ‘Superchordin’ fragments are generated in vertebrates.

In conclusion, Twisted-gastrulation seems to have many functions in BMP signalling. In vertebrates, it can increase the binding of BMP to full-length Chordin to create a better antagonist, compete for the residual BMP binding activity of CRs, thereby promoting BMP activity, and facilitate cleavage by Xolloid. In *Drosophila*, TSG generates inhibitory specificities by changing the cleavage site of Tolloid on its SOG substrate. Despite the current complexity, these recent studies indicate that xTsg/dTSG are part of an extracellular regulatory pathway involving Chordin/SOG and Xolloid/Tolloid that finely regulates the levels of BMP/DPP signalling during dorsal-ventral patterning.

Chordin-like modules

The CR domains of Chordin are sufficient for BMP binding, and modules of similar sequence are present in many other proteins. The similarities are found in the spacing of the cysteines, as well as in additional amino acids (BOX 1). These Chordin-like CRs may function as binding sites for TGF-β superfamily members in the extracellular matrix[82,106]. Some of these proteins are produced in large amounts and, indeed,procollagen I is the most abundant protein of the human body. Recently deposited extracellular procollagen might serve as a sink for TGF-β factors that could then be released when required for tissue homeostasis. When procollagen is processed by its N-terminal proteinase, the NH_2-propeptide that is released still retains a trimeric structure; it is therefore tempting to propose that a further proteolytic cleavage closer to the CR domain may be required to release individual CR domains and to reactivate the bound growth factors (BOX 1). In addition, proteins related to xTsg may function together with these

other CR modules to further regulate growth-factor signalling. These exciting possibilities will keep researchers occupied in the near future.

Conclusions

The past few years have brought considerable progress in our understanding of how dorsal-ventral patterning is established in *Xenopus* and zebrafish embryos. It appears that the dorsal displacement of membrane vesicles is a key event in stabilizing β-Catenin on the dorsal side of the embryo. This, together with other maternal factors — such as VegT and Vg1 — leads to the generation of a gradient of Nodal-related signals. High levels of Xnrs induce Spemann's organizer in dorsal mesoderm. The organizer secretes a cocktail of growth-factor antagonists that, in turn, further refine the pattern of the three germ layers. The ventralized ultraviolet-induced phenotype can be rescued by injecting vegetal cytoplasm (containing dorsal-determining vesicles), *β-catenin* mRNA, Nodal-related factors, or BMP antagonists, such as Chordin and Noggin. These gene products can all be considered as parts of a continuum that, starting with the prevention of β-Catenin degradation on the dorsal side, eventually leads to the inhibition of BMP signals that would otherwise cause the ventralization of the entire embryo. Although this model is admittedly oversimplified, it is attractive because it provides an explanation for why so many different gene products rescue the ventralization caused by inhibiting the movement of dorsal determinants.

Many questions remain unanswered. Can the elusive dorsal determinant vesicles be purified? Do they contain as yet unknown maternal Wnt ligands? Do additional Nodal-related genes exist in mammals? Can other growth factor/antagonist complexes — such as those of Cerberus, Dkks or Frzbs — also be reactivated by proteolytic regulation? Do all CR modules in extracellular proteins function in the regulation of the TGF-β superfamily? Do extracellular matrix CR modules interact with Twisted-gastrulation homologues? The studies that we have reviewed here identify a plethora of regulatory molecules in the early embryo, and, as we move into the era of the genome, it will be worthwhile to test to what degree these findings will serve as paradigms for understanding homeostasis in adult tissues.

DATABASE LINKS β-Catenin | Nodal | Noggin | Chordin | Cerberus | Frzb-1 | Crescent | Dickkopf | Xolloid | Twisted-gastrulation | Dishevelled | Frizzled-7 Wnt receptor | siamois | Xtwin | Nodal-related-3 | Axin | Cyclops | Squint | Goosecoid | Vg1 | BMP-4 | Derrière | Smad2 | VegT | Dlx-3 | BMP-2 | zebrafish Goosecoid | Mixer | Smad4 | FAST-1 | XIHbox-8 | Xnr-1 | Xnr-2 | Xnr-4 | Follistatin | Vent | Vox | sFRP-2 | Sizzled | Xwnt-8 | lefty-2 | nodal | BMP-2b | Smad5 | BMP-7 | short-gastrulation | Dpp | screw | | Drosophila Tolloid | zebrafish tolloid | Dtsg | GBB | procollagen I | N-terminal proteinase | procollagen II | procollagen V | CTGF | Thrombospondin-1 | Crossveinless-2 | Kielin | APC | GSK-3

FURTHER INFORMATION Xenbase | The zebrafish information network | Axeldb | De Robertis lab homepage

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Supplementary Material

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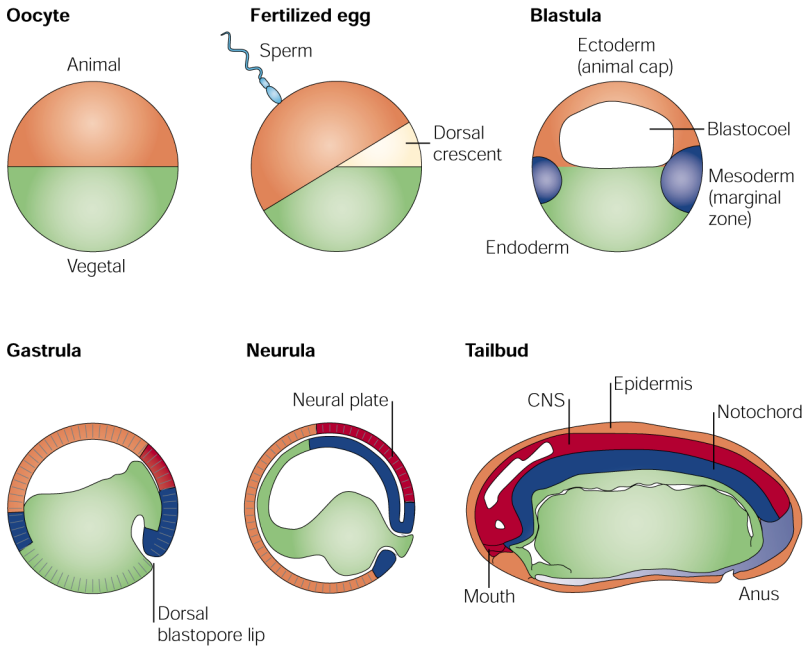

Figure 1. The anatomy of *Xenopus* development

The ovarian oocyte is radially symmetrical and is divided into an animal and a vegetal domain. One hour after fertilization, an unpigmented dorsal crescent is formed in the fertilized egg opposite the sperm entry point. As the embryo rapidly divides into smaller and smaller cells, without intervening growth (cleavage), a cavity called the blastocoel is formed, which defines the blastula stage. By the late blastula stage (9 h of development), the three germ layers become defined. The ectoderm, or animal cap, forms the roof of the blastocoel. The mesoderm is formed in a ring of cells in the marginal zone, located between the ectoderm and endoderm. At the gastrula stage (10 h), involution of the mesoderm towards the inside of the embryo starts at the dorsal blastopore lip. The morphogenetic movements of gastrulation lead to the formation of the vertebrate body plan, patterning the ectoderm, mesoderm and endoderm. At the neurula stage (14 h), the neural plate, or future central nervous system (CNS), becomes visible in dorsal ectoderm. By the tailbud stage (24-42 h), a larva with a neural tube located between the epidermis and the notochord has formed. The blastopore gives rise to the anus, and the mouth is generated by secondary perforation.

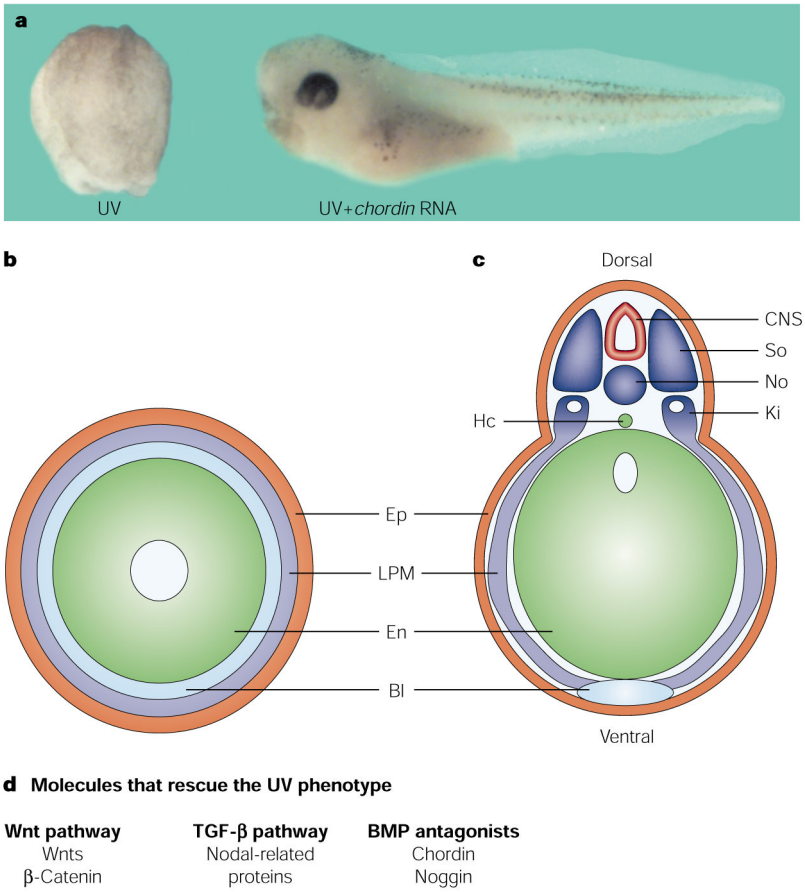

Figure 2. The ultraviolet (UV) phenotype of irradiated embryos can be rescued by many different molecules

a | Ultraviolet-irradiated embryos do not develop axial structures, and differentiate into a belly piece (left). Microinjection of synthetic mRNA for Chordin, an antagonist of bone morphogenetic protein (BMP) signalling, completely rescues the development of axial structures (right). **b** | The belly piece comprises epidermis (Ep), lateral plate mesoderm (LPM), endoderm (En) and blood (Bl), whereas **c** | unirradiated or ultraviolet-treated embryos injected with *chordin* mRNA differentiate dorsal tissues, such as central nervous system (CNS), somites (So), notochord (No), kidney (Ki) and HYPOCHORD (Hc). **d** | A puzzling variety of gene products can rescue the ultraviolet phenotype; the main thesis explored in this review is that these molecules participate in a sequential pathway of biochemical events leading to dorsal development.

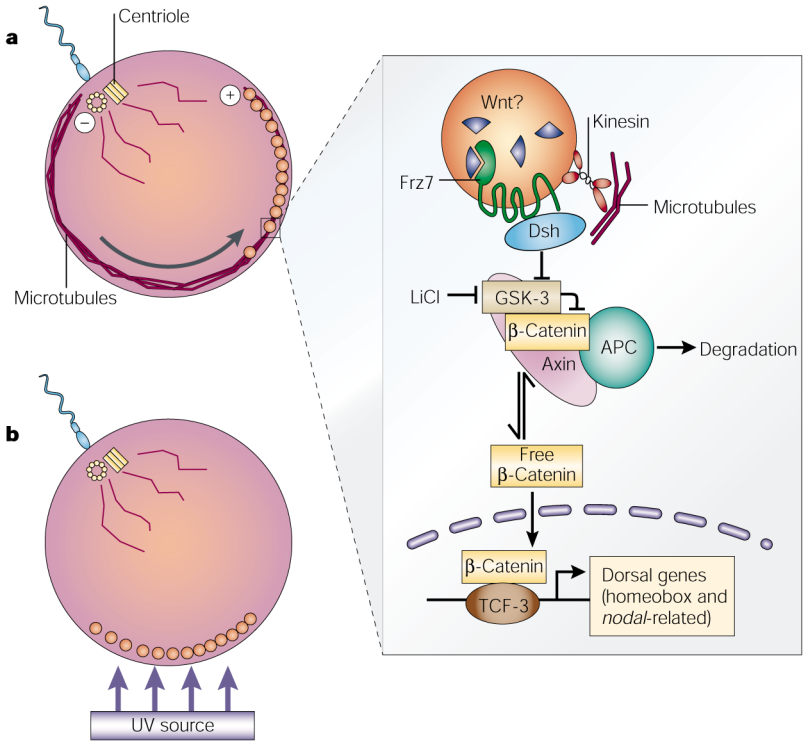

Figure 3. Dorsal determinants and the transport of membrane vesicles to the dorsal side

a | After fertilization, parallel arrays of cortical microtubules extend from the centriole and the large aster at the sperm entry point towards the dorsal side (where the plus end lies), and transport small membrane vesicles from the vegetal towards the dorsal animal pole. The inset shows that the dorsal determinant vesicles are associated with Dishevelled (Dsh), a component of the Wnt signal transduction pathway. The Wnt receptor Frz7 is required for dorsal axis formation, but the Wnt molecules shown inside the vesicles are entirely hypothetical. Kinesins are molecular motors that can transport vesicles towards the plus ends of microtubules. β-Catenin is found in a large cytoplasmic complex that includes Axin, APC (adenomatous polyposis coli) and GSK-3 (glycogen synthase kinase 3). GSK-3 negatively regulates β-Catenin through phosphorylations that target β-Catenin for degradation by the proteasome. GSK-3 can be inhibited by treatment with lithium chloride (LiCl). On stimulation of the Wnt pathway, β-Catenin is stabilized on the dorsal side and can be found in the nucleus, where, together with TCF-3, it activates various target genes, including homeobox and Nodal-related genes (*Xnrs*). Further molecules that participate in this canonical Wnt signalling pathway[111] are not shown in this simplified diagram. **b** | Irradiation of embryos with ultraviolet light disrupts cortical microtubules and prevents the transport of the membrane vesicles to the prospective dorsal side.

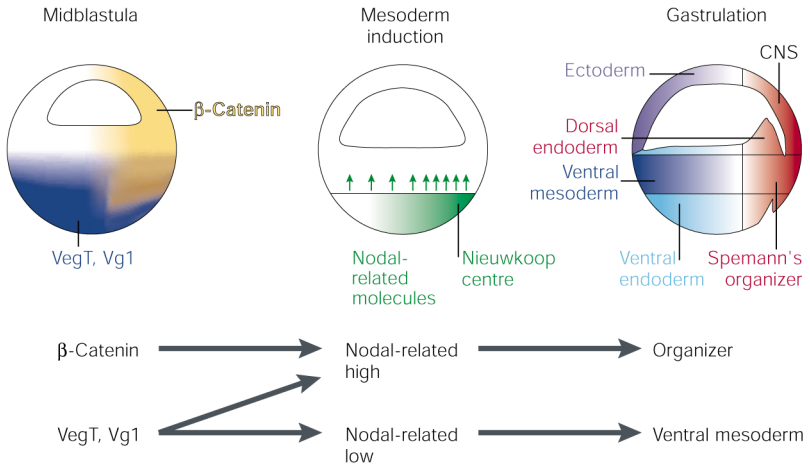

Figure 4. Two-step model of mesoderm induction in *Xenopus*

At the midblastula stage, higher β-Catenin levels on the dorsal side of the embryo, together with the vegetally located transcription factor VegT and the maternal TGF-β-family growth factor Vg1, generate a gradient of Nodal-related molecules expressed in the endoderm. In turn, this gradient induces the formation of overlying mesoderm: low doses of Nodal-related molecules (Xnrs) lead to the formation of ventral mesoderm, whereas high doses lead to the establishment of Spemann's organizer. Nieuwkoop's centre is the region of dorsal endoderm that induces organizer tissue. At the gastrula stage, the organizer secretes a cocktail of factors that refine the initial patterning. Note that β-Catenin is widely distributed on the dorsal side, including in derivatives of the three germ layers. (CNS, central nervous system.)

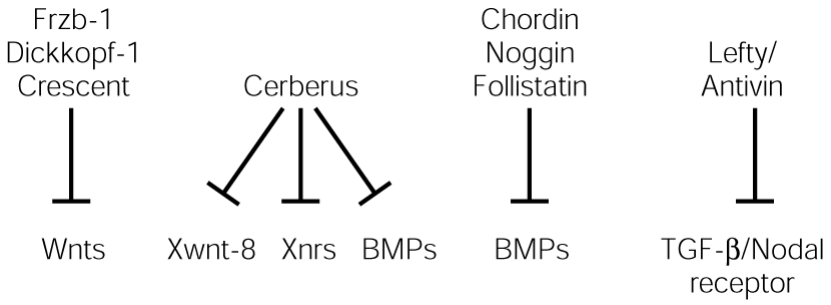

Figure 5. Spemann's organizer is a source of secreted growth factor antagonists

The organizer secretes proteins that bind to different growth factors in the extracellular space and block signalling through their cognate receptors. Crescent, Frzb-1 and Dickkopf-1 are Wnt antagonists. Cerberus is a multivalent inhibitor that antagonizes Xwnt-8, Xnrs and BMPs. Chordin, Noggin and Follistatin bind to and inhibit bone morphogenetic proteins (BMPs). Lefty/Antivin blocks Nodal signalling through a different mechanism, by binding to the TGF-β/Nodal receptor and acting as a competitive inhibitor.

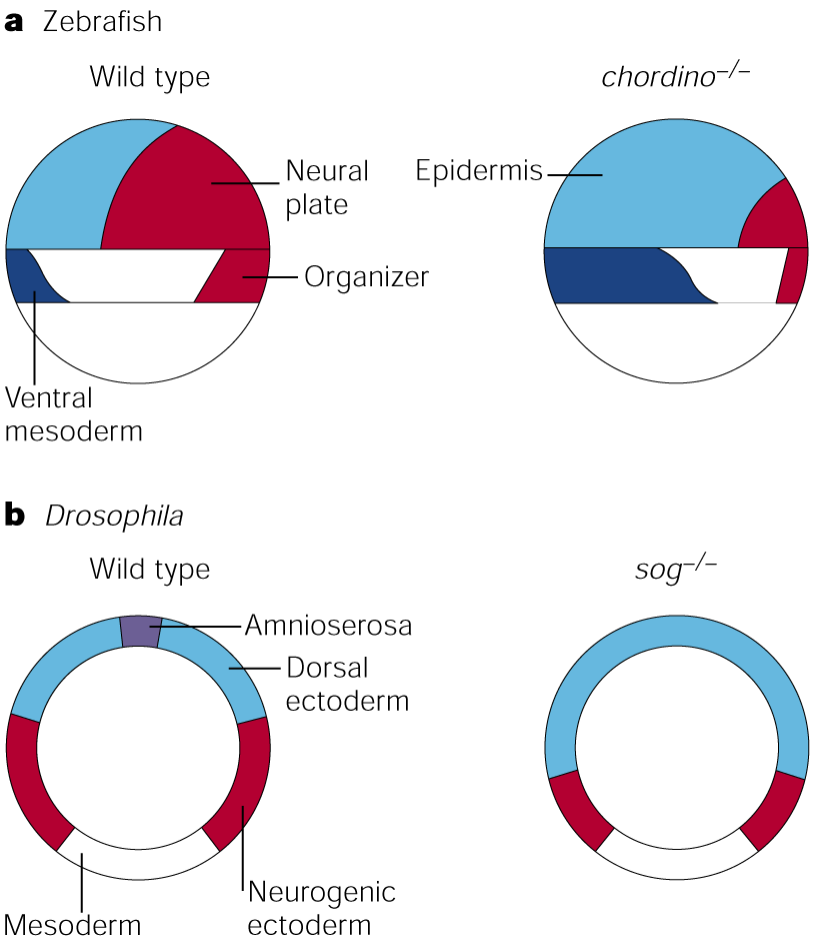

Figure 6. Genetics of *chordin/sog* in zebrafish and *Drosophila*

a | In zebrafish, loss-of-function mutations in *chordino* ventralize the mesoderm and the ectoderm. Organizer tissue and neuroectoderm are reduced, and ventral mesoderm and epidermis are expanded in *chordino*$^{-/-}$ embryos. **b** | In *Drosophila*, mutations in *sog* lead to dorsalized embryos. The neurogenic ectoderm is reduced, the amnioserosa is lost, and the amount of tissue allocated to dorsal ectoderm is expanded. The amnioserosa is the dorsal-most tissue of the fruitfly embryo and forms a thin layer of cells required for proper gastrulation. Formation of the amnioserosa requires the activity of Sog and dTsg. Dorsal-ventral polarity in arthropods has been inverted with respect to that of vertebrates in the course of evolution[112].

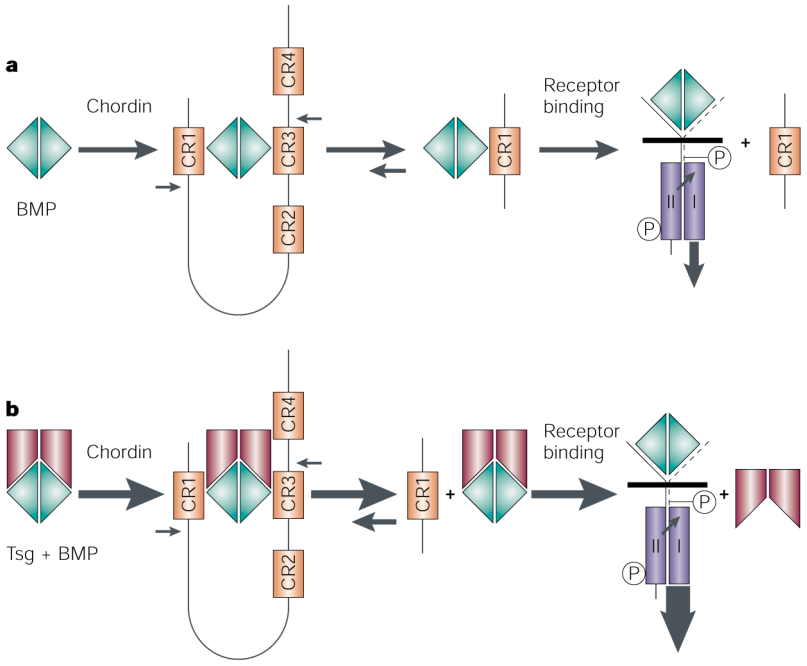

Figure 7. A molecular pathway involving Chordin, Xolloid and Twisted-gastrulation regulates the dorsal-ventral activity gradient of bone morphogenetic protein in *Xenopus*

a | Chordin binds bone morphogenetic protein (BMP) through cysteine-rich domains CR1 and CR3. After cleavage of Chordin by Xolloid (small arrows), the CR modules still bind BMP, but with tenfold reduced affinity. In principle, this lowered affinity might explain why Xolloid cleavage restores BMP signalling from inactive BMP-Chordin complexes. **b** | In the presence of Twisted-gastrulation (xTsg) protein, two new activities are observed. First, xTsg increases the binding of BMP to full-length Chordin, converting the Chordin-xTsg-BMP ternary complex into a better BMP antagonist. Second, after Xolloid cleavage, residual binding of BMP to CR modules is readily competed by xTsg. The binary xTsg-BMP complex does not interfere with BMP receptor binding at physiological concentrations, therefore eliminating inhibition by CR fragments and providing a permissive signal for BMP binding to its cognate receptors. The thicker arrows in **b** indicate the effects of xTsg on this biochemical pathway. Activated BMP receptor is phosphorylated (circled Ps), relaying the signal intracellularly.