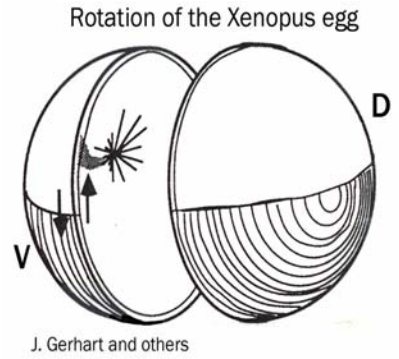
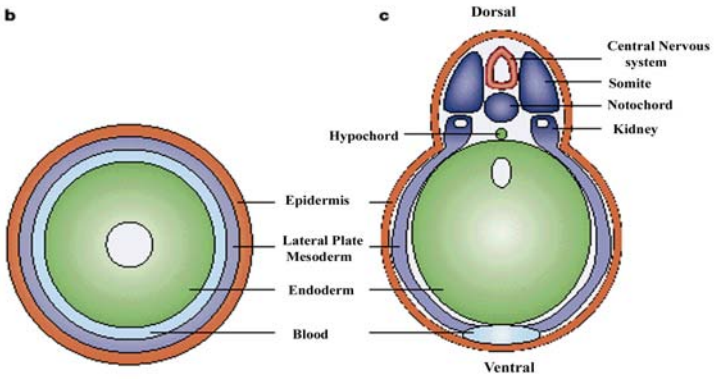
THE CORTICAL ROTATION, THE WNT PATHWAY AND MESODERM INDUCTION – Lectures 2 and 3

1. The cortical rotation

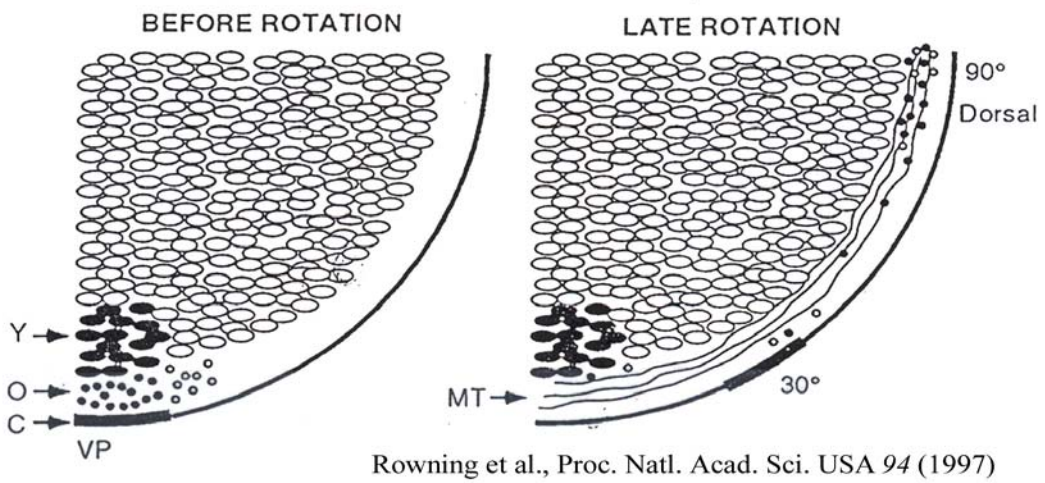


Let us return to *Xenopus* fertilization. The future dorsal lip in amphibians usually forms opposite to the sperm entry point (Roux's sperm-imbibed silk thread experiment). The sperm brings in a large centriole. Microtubules form arrays under the egg cortex and drive a 30° rotation of the cortex with respect to the internal yolk. The rotation can be blocked by UV-irradiation of the vegetal (bottom) pole of the egg, or by nocodazole or other treatments that interfere with microtubule polymerization.



UV treatment results in embryos lacking all dorsal structures and consisting only of ventral tissues, called a "belly piece" by Spemann (these UV-treated embryos still have the three germ layers, although all are of a ventral character). If after UV treatment the embryo is placed upside down, or centrifuged, a dorsal axis is formed in embryos. The cortical rotation sets the polarity of the egg, in particular the dorsal side. This early event results in induction of dorsal mesoderm at the blastula stage. The opposite effect to UV treatment, a radially dorsalized embryo, can be obtained by culturing embryos in LiCl (see below).

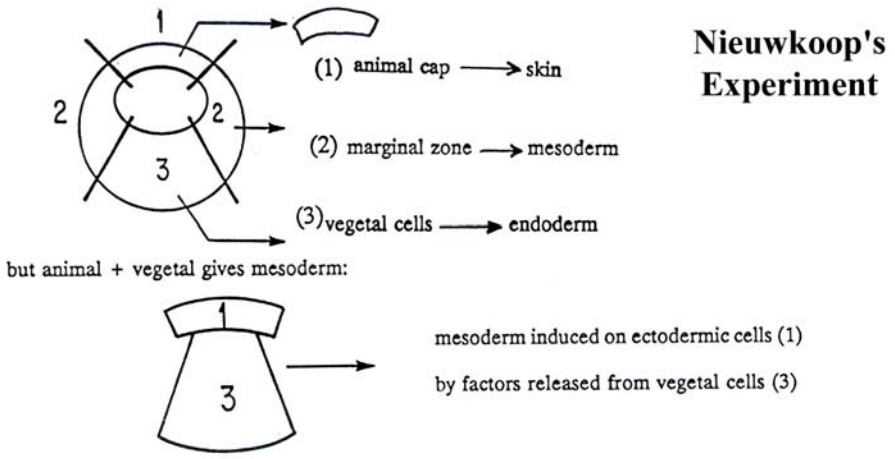
It has been documented that dorsal membrane vesicles exist that can move much more than the 30º of the cortical rotation. The initial rotation serves to align parallel arrays of radial microtubules, but then membrane vesicles can be transported along these microtubules for 90º or more, all the way from the vegetal pole to the dorsal side. These microtubule driven movements would transport dorsal-axis inducing material to the dorsal side. If the yolk platelets (Y) are labeled with Nile red (vital dye) and the cell membrane (C) and the membranous intracellular organelles (O) with the lipofilic dye DiO (that inserts in membranes), the confocal microscope shows that organelles can undergo rapid transport of up to 50 μm/min along the microtubule tracks toward the dorsal side. It is thought that these membrane vesicles contain the initial dorsalizing signal.



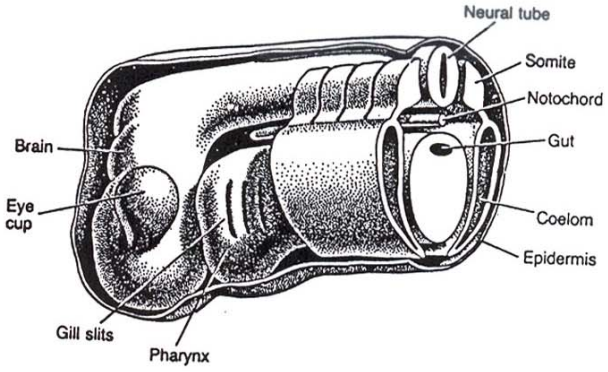
Rowning et al., Proc. Natl. Acad. Sci. USA *94* (1997)

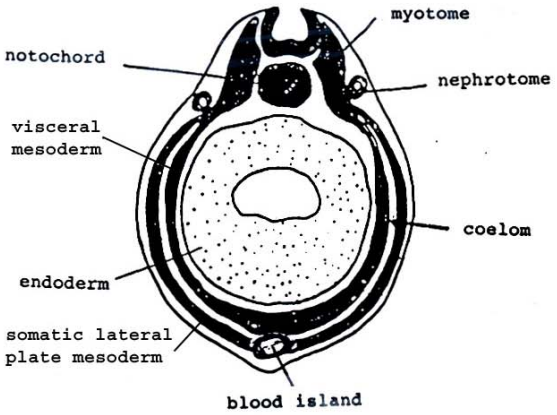
2. Nieuwkoop's blastula experiment. Mesoderm formation is induced by cell-cell interactions.

The use of explanted embryo fragments demonstrated that mesoderm is formed by induction. The animal cap (top) fragment of a blastula if cultured gives rise to skin ectoderm. The vegetal (bottom) fragment gives rise to endoderm. If a conjugate is made from an animal cap and a vegetal fragment (Nieuwkoop, 1969), mesoderm is induced in animal caps cells by soluble factors secreted by vegetal cells.

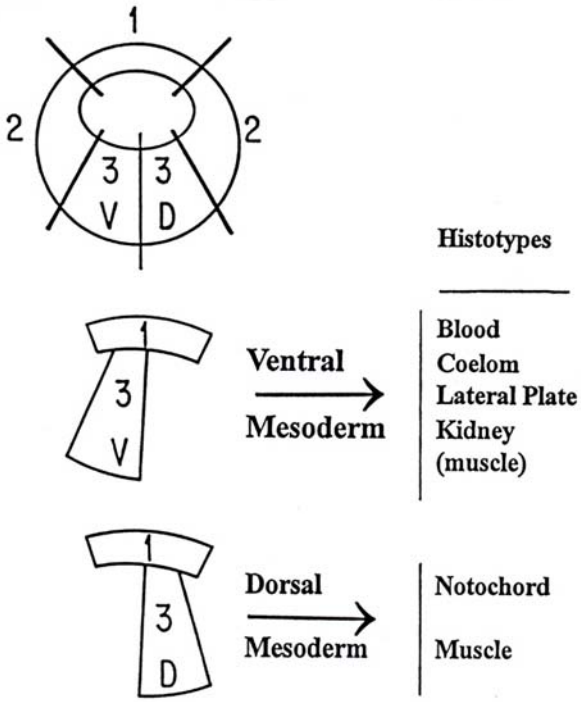


The mesodermal layer has multiple components. Chordates are defined by having A) a dorsal central nervous system, B) notochord, C) gill slits in the pharynx and D) a postanal tail. The mesoderm of chordates consists of, from dorsal to ventral: 1) notochord, 2) somites (which form mostly muscle in *Xenopus*), 3) nephrotome or future kidney, 4) lateral plate mesoderm which is divided by a coelomic cavity (peritoneum) covered by mesothelium into an external layer (somatic mesoderm or somatopleure) and an internal layer (visceral mesoderm or splachnopleure) and finally 5) blood islands in the ventral side. These tissue allocations are established by the end of gastrulation..

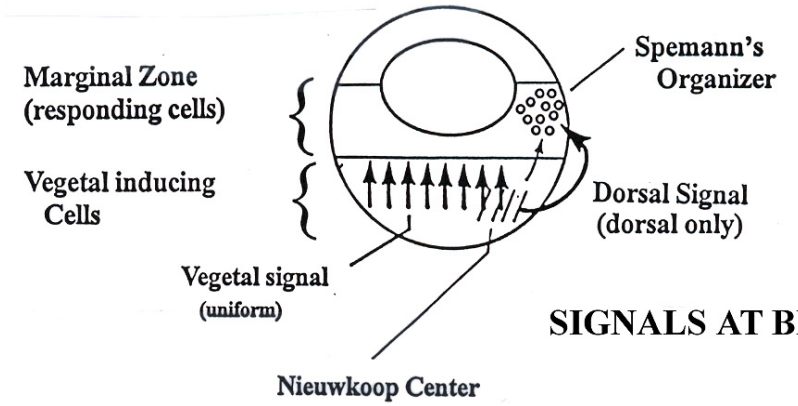


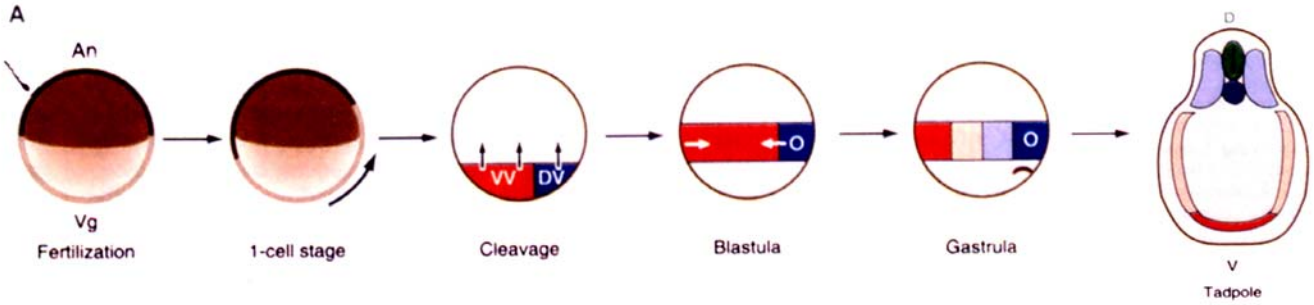


If the vegetal fragment is subdivided into a dorsal and a ventral fragment, different tissues will be induced after conjugation with ectoderm. The dorsal side can be distinguished because it has a dorsal crescent of lighter pigmentation caused by the cortical rotation.



In the three-signal model, the first two signals postulated were for the induction of mesoderm. 1) The ventral signal would be released uniformly throughout the vegetal region. This signal forms mesoderm in a ring of cells comprising the entire marginal zone. 2) A dorsal signal would be released by a group of vegetal cells designated the Nieuwkoop center at the blastula stage; this signal would induce a group of overlying mesodermal cells, called "Spemann's organizer". 3) The third signal arises from the organizer, a new signaling center that at the gastrula stage dorsalizes the mesoderm and induces neural tissue. In this lecture we will discuss the early signals, in particular their molecular nature.





3. The Nieuwkoop center is a region of β-catenin signaling.

S. Schneider et al. / Mechanisms of Development 57 (1996) 191–198

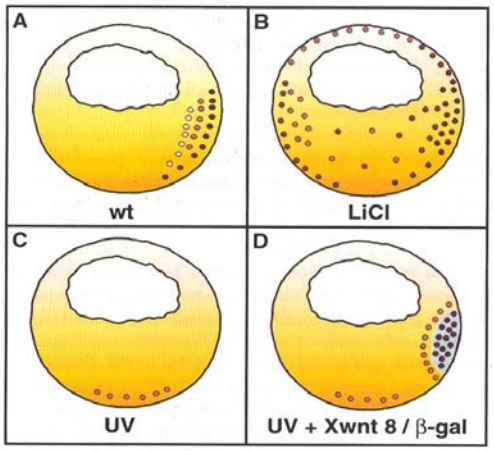
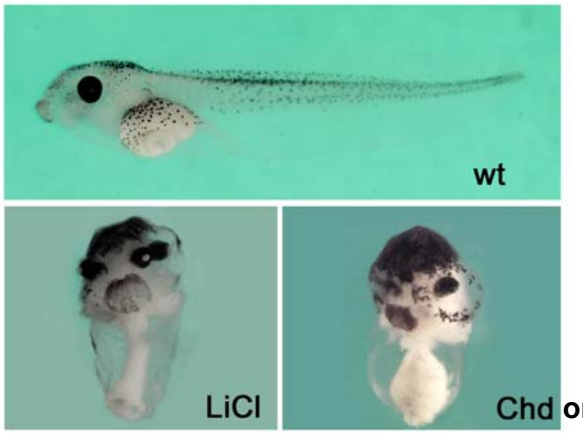


Fig. 4. Schematic distribution and staining intensity of β-catenin-positive nuclei in wildtype (wt), and axis manipulated embryos at the blastula-stage. (A) wt-embryo with the domain of β-catenin-positive nuclei in the dorsomarginal and dorsovegetal region. (B) Li-treated embryo with an expanded domain of β-catenin-positive nuclei. (C) UV-ventralized embryo lacking β-catenin-positive nuclei in the marginal zone and showing nuclear staining at the vegetal pole. (D) UV-ventralized embryo with induced β-catenin translocation into nuclei at the site of *Xwnt8/β-gal* mRNA injection in the marginal region. Dark red indicates strong nuclear β-catenin staining, lighter red indicates weaker staining, and blue indicates β-gal staining.

A very important advance on the nature of the Nieuwkoop center signal has been the discovery that β-catenin provides the earliest asymmetry found in the *Xenopus* egg. The dorsal signal is mediated by the Wnt/β-Catenin signal.

β-catenin protein is found uniformly in the cytoplasm of the unfertilized egg. Already by the 2-cell stage cytoplasmic expression is more intense in the dorsal cytoplasm. By the 16-cell stage, or better still at midblastula, it can be seen that β-catenin translocates into nuclei in a large part of the dorsal side. β-catenin is also seen in the inner side of cell membranes over the entire embryo. Nuclear translocation of β-catenin depends on the cortical rotation; in UV embryos it is found only in the vegetal pole nuclei. The microtubule arrays seen during cortical rotation transport vegetal cytoplasmic components into the marginal zone. Treatment

with LiCl at the 32-cell stage results in all mesoderm in the marginal zone becoming organizer (how this works to be explained in a moment). Since there is no transcription until MBT, the β-Catenin mRNA is of maternal origin.



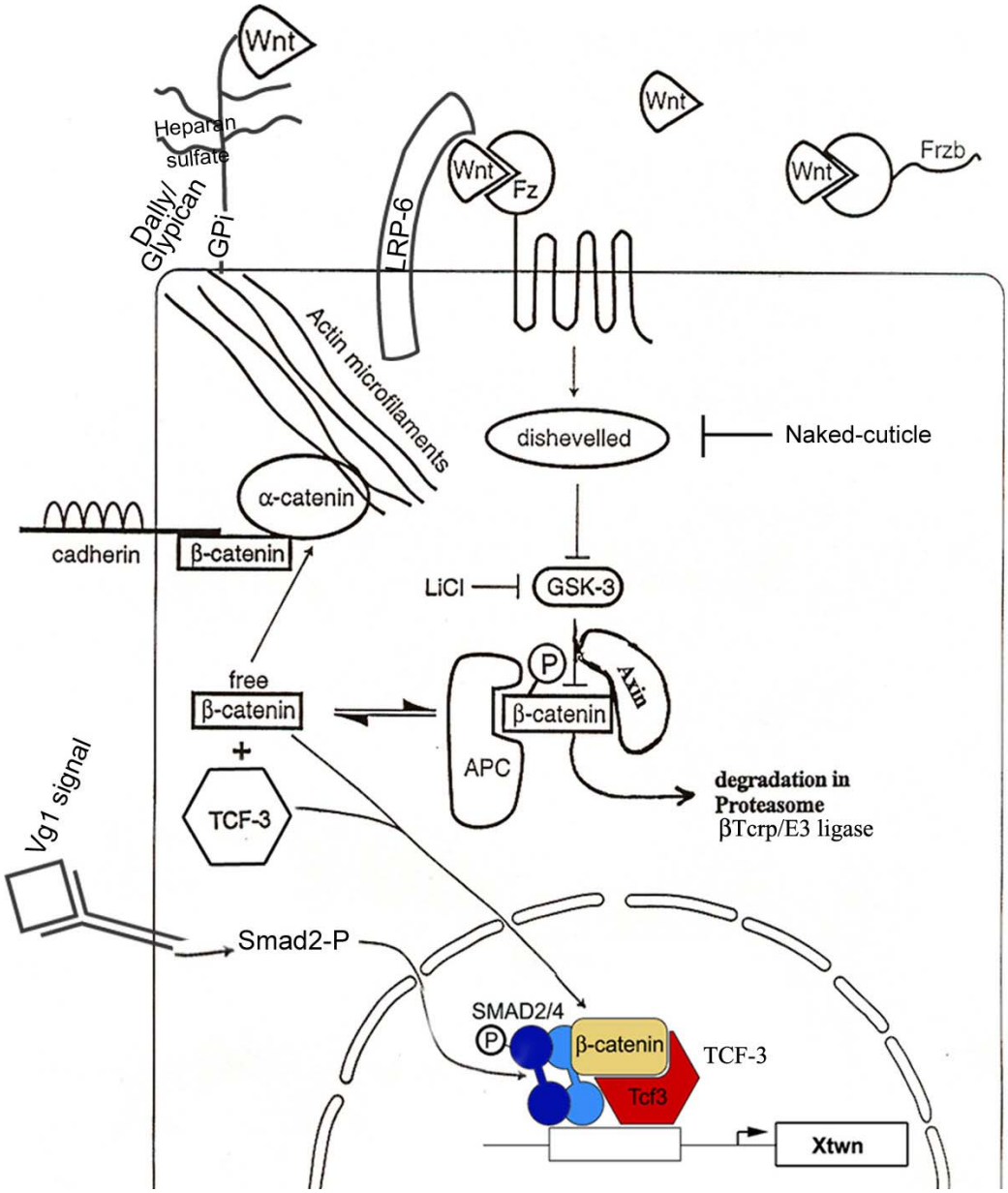
In these LiCl treated embryos β-catenin is found in nuclei all over the embryo. β-catenin is a central player of the Wnt pathway; ectopic expression of *Xwnt-8* mRNA results in β-catenin stabilization and nuclear translocation near the injected side. *Xwnt-8* mRNA is thought to mimic an endogenous signal, but it is not expressed in the egg (at the gastrula stage it is expressed ventrolaterally and functions to promote development of this region). The amount of free β-catenin plays a central role in dorso-ventral polarity; β-catenin translocates into nuclei where protein its levels are high. When oocytes are depleted of β-catenin mRNA using DNA oligonucleotides and eggs are fertilized, there is no dorsal axis and the embryos develop exactly as UV-treated embryos. When β-catenin mRNA is depleted, vegetal embryonic fragments cannot induce organizer tissue in the animal caps of Nieuwkoop recombinants at midblastula. Therefore β-catenin is required to generate the dorsal mesoderm-inducing signal of the Nieuwkoop center.

3. The Wnt/β-Catenin Pathway

Wnt signaling pathway includes many components. Wnt pathway signals result in the inhibition of β-Catenin degradation. In the absence of Wnt β-Catenin is destroyed, a wasteful but effective strategy.

1) Wnts were discovered by Varmus and Nusse as oncogenes activated by MMTV (mouse mammary tumor virus) integration sites and encode secreted signaling factors. There are about 20 Wnts in mammals. Wnts induce twinning in embryos.

2) Frizzled and Frzbs. Family of seven transmembrane serpentine receptors that transduce the signal. They contain an extracellular cysteine-rich domain (CRD) that is sufficient to bind Wnt. Frzbs are Wnt antagonists consisting of a secreted CRD lacking the transmembrane portion.



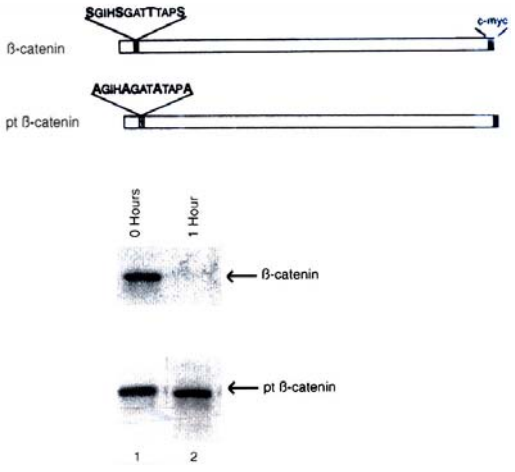
3) LRP-6 (Low-density lipoprotein receptor (LDLR)-related protein six, a homologue of the *Drosophila* gene *arrow*). LRP-6 has recently emerged as co-receptor that is required together with frizzled for Wnt signaling. In *Drosophila arrow* loss of function leads to a lack of wingless signaling. LRP-6 is a transmembrane domain protein with 4 EGF repeats and 3 LDLR repeats in the extracellular domain. In *Xenopus*, overexpression of LRP-6 mRNA leads to twinning. Deletion of the LRP-5/6 extracellular domain increases β-catenin signaling. The extracellular domain serves to inhibit LRP-5/6 signaling. Conversely, when the cytoplasmic domain of LRP-6 is deleted, it acts as a dominant-negative construct, blocking signaling by Wnts. Presumably this sequesters Wnt ligands. In biochemical studies the extracellular domain of LRP-6 can bind Wnt-1, and that in the presence of Frizzled CRD a tight ternary complex of LRP-6, Wnt and Fz is formed. Thus, Wnt signaling through the canonical pathway requires both Fz and the LRP-6 co-

receptor (another pathway of Wnt signaling, called Planar Cell Polarity, does not require LRP-6/*arrow*).

4) Glypicans/dally are heparan sulphate proteoglycans (HSPG) with a core protein and long chains of sulphated sugars. The cell surface is covered in HSPGs that act as an "extracellular fly paper" that concentrates ligands at the cell surface. Glypicans are HSPGs attached to the external cell membrane via a GPI (glycosyl phosphatidylinositol) linkage. *Drosophila* mutants lacking *dally* have a wingless$^{-/-}$ phenotype (lack of smooth cuticle, all ventral epidermis converted into denticles). However, these defects can be ameliorated by overexpression of the wingless ligand. *Dally* facilitates binding of Wnt to the Fz/LRP-6 co-receptor by recruiting Wnt ligand.

5) dishevelled, a protein containing Dlx and PDZ protein interaction domains, that in some way inhibits GSK-3 function. After Wnt signaling Dishevelled becomes phosphorylated (probably by casein kinase I) and becomes an inhibitor of GSK-3. The activity of Dsh is regulated in a negative feed-back loop by *naked-cuticle (nkd)*. *nkd* loss-of-function mutations in *Drosophila* result in an increased Wg signaling (lack of denticle belts). *nkd* is induced by Wnt signaling and therefore is an intracellular antagonist of the pathway. It does this, both in flies and vertebrates, by binding and blocking *dsh*. *nkd* functions as a feedback inhibitor to limit the duration and intensity of the Wnt signal.

6) GSK-3 (glycogen synthase kinase-3, a homologue of ZW-3/shaggy of Drosophila). This is a serine-threonine kinase that is active in many cells. In the embryo it is more active on the ventral side than on the dorsal side. A dominant-negative point mutation that abolishes catalytic activity induces twinning (i.e., dorsalization). LiCl is a potent dorsalizing agent, causing all mesoderm to become organizer, because it inhibits the activity of GSK-3.



7) β-catenin, the key player in all this, is phosphorylated in the amino terminus in four ser/threonines by GSK-3 and this leads to β-catenin degradation. If these residues are mutated, S^{35} β-catenin made in rabbit reticulocyte in vitro system is much more stable when injected into *Xenopus* embryos:

Wild-type β-catenin is more stable when injected on the dorsal side than on the ventral side. β-catenin is the homologue of *Drosophila armadillo.* It links the intracellular domain of the cadherin cell adhesion molecules (which form adherens junctions) to α-catenin and actin microfilaments, and also has a nuclear function. Microinjection of β-catenin mRNA leads to the formation of secondary (twinned) axes and depletion of β-catenin mRNA by

antisense oligonucleotides leads to embryos lacking dorsal axes. The amount of free β-catenin in the cytoplasm regulates the early dorsal-ventral decisions.

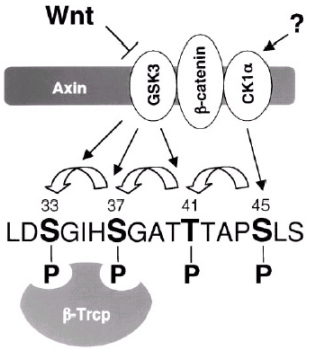
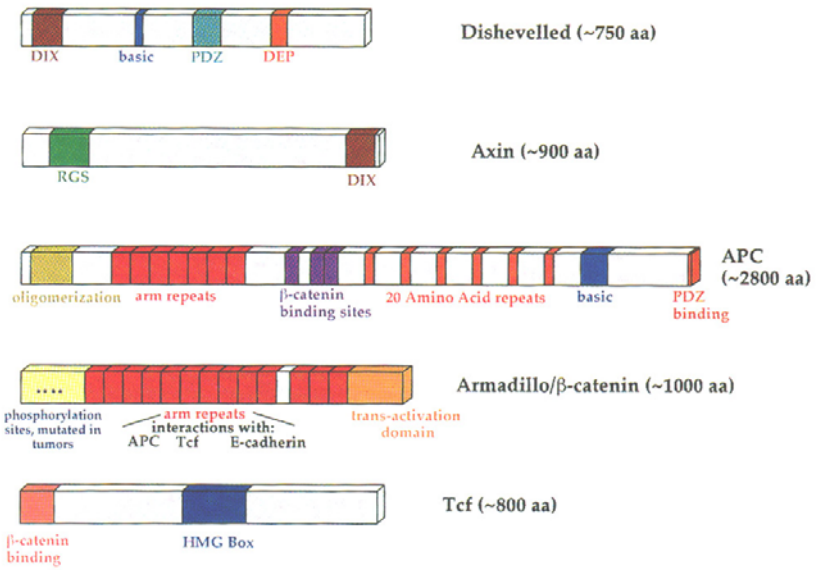


Figure 8. A Model for β-Catenin Phosphorylation and Recognition by β-Trcp

CKIα, GSK-3, and β-catenin each bind a different domain of Axin such that CKIα and GSK-3 sandwich β-catenin in the Axin complex. CKIα phosphorylation of S45 allows GSK-3 to phosphorylate T41, then S37 and S33. Phosphorylation of S37 and S33 creates the recognition site for β-Trcp. Wnt signaling inhibits GSK-3 phosphorylation of T41, S37, and S33. How CKIα phosphorylation of S45 is regulated remains unknown.

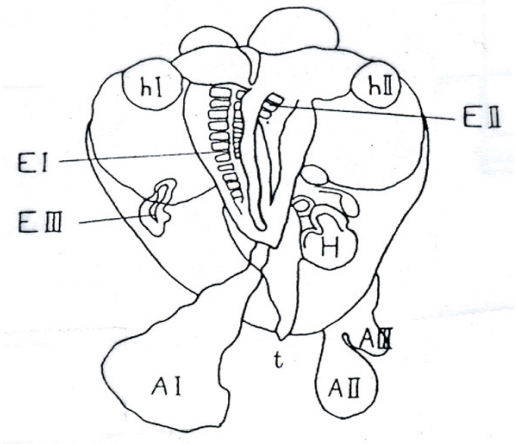
Mutations in the 4 ser/Thr phosphorylation sites lead to β-catenin stabilization and cancer. Why do single mutations stabilize the protein? Phosphorylation is by a dual-kinase mechanism. First Casein Kinase 1α (one of the first protein kinases to be discovered) phosphorylates a serine, and only then GSK-3 can phosphorylate the other Ser/Thr residurs. Once the two most amino-terminal Ser are phosphorylated, the protein is recognized by β-Trcp (Slimb in *Drosophila*). β-Trcp is an E3 ubiquitin ligase that targets bound proteins for ubiquitinylation via its F-box domain (jackknife mechanism). β-catenin is sandwiched between GSK-3 and CKIα when all three proteins bind to Axin, a very important component of the destruction complex (Liu et al., Cell *108*, 837m 2002). Dual-kinase mechanisms provide specificity in many biochemical reactions.

8) APC is a protein that binds to β-catenin and permits its phosphorylation by GSK-3. APC means adenopolyposis coli, a gene mutated in most familial, and in 60% of spontaneous colon carcinomas. These tumors have increased β-catenin levels because the β-catenin protein is degraded more slowly in the absence of APC. Of the colon carcinomas that have a normal APC gene, 50% have mutations in the phosphorylation sites of β-catenin instead. Others have mutations in Axin. APC has 7 "armadillo" repeats and β-catenin has 13; they are responsible for protein-protein interactions. APC is also found in ruffling membranes of motile cells, where it and to microtubules.



Cadigan, K.M. and Nusse, R. Genes Dev. 11, 3286-3305 (1997)

9) Axin is part of the β-catenin destruction complex

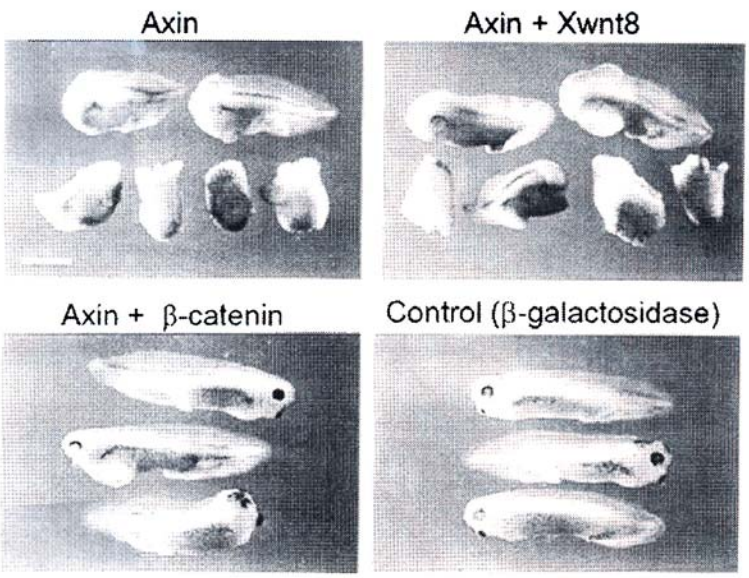


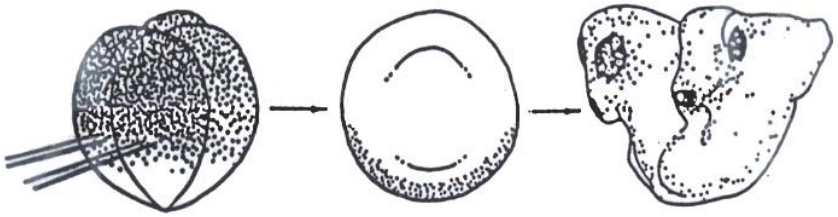
EMBRYONIC DUPLICATIONS IN MICE

S. GLUECKSOHN

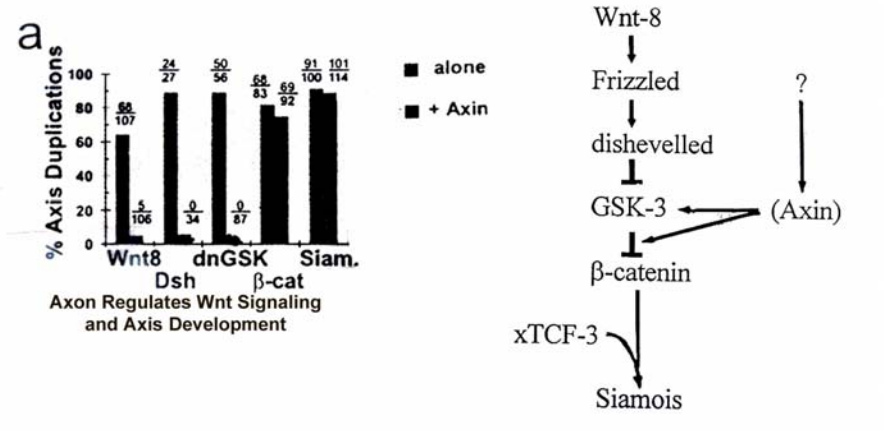
Do these pathways described in *Xenopus* function in mouse as well? Forty years ago, Salome Glückshon-Welsch (an ex-student of Spemann) described a mutation that produced multiple embryonic axes in homozygotes.

The 10-day embryo shown here has elements from three embryonic axes (EI-EIII, two hearts (hI, hII) and three allantois (AI-AIII). Loss of function of the Axin gene produces multiple axes. In heterozygotes fused or kinky tail vertebrae are seen. In 1997 the gene was cloned using a transgene accidentally inserted in Axin (Zeng et al., Cell *90*, 181-192, 1997, a wonderful paper). Axin mRNA is expressed in all cells and encoded a novel protein whose function is to inhibit axis formation. Using *Xenopus* embryos Constantini and colleagues showed that Axin inhibits Wnt signaling. Injection of Axin synthetic mRNA into the dorsal side of the embryo ventralizes the dorsal axis. Co-injection with *Xwnt-8* mRNA (a potent dorsalizer) did not change this, whereas co-injection of β-catenin and Axin prevented ventralization by Axin. As a control β-gal was injected and had no effect. (An antisense morpholino for Axin causes dorsalization). This analysis indicated that Axin acted downstream of *Xwnt-8* but upstream of β-catenin. To determine the level in the pathway at which this acts they made use of mRNA injections into the ventral side of the embryo, which in case of the components of the Wnt pathway can cause the induction of a second dorsal lip and extensive axial duplication. This is a very convenient assay:

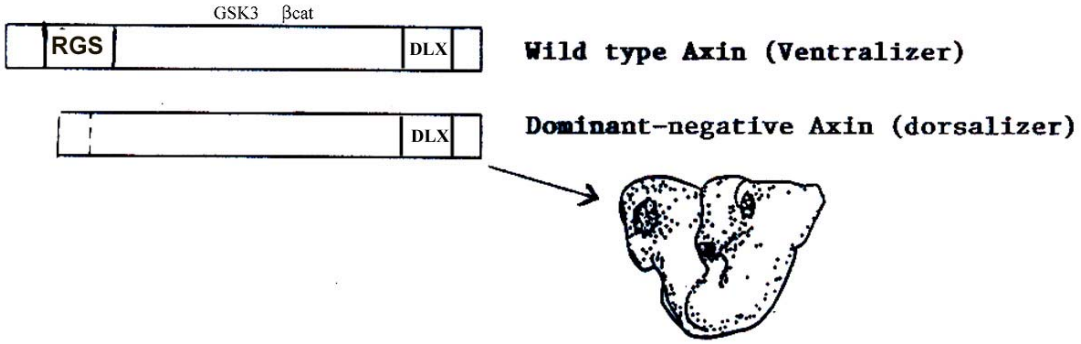




Axin by itself has no effect on the ventral side, but if co-injected with *Xwnt-8*, *dishevelled* or dominant-negative GSK-3 (kinase activity inactivated by a point mutation) mRNAs, it abolished their axis-duplicating activity. The axis duplicating activity of β-catenin or *Siamois* was not affected. Thus, Axin appears to negatively regulate Wnt signaling either at the level of GSK-3 or downstream, but upstream of β-catenin.

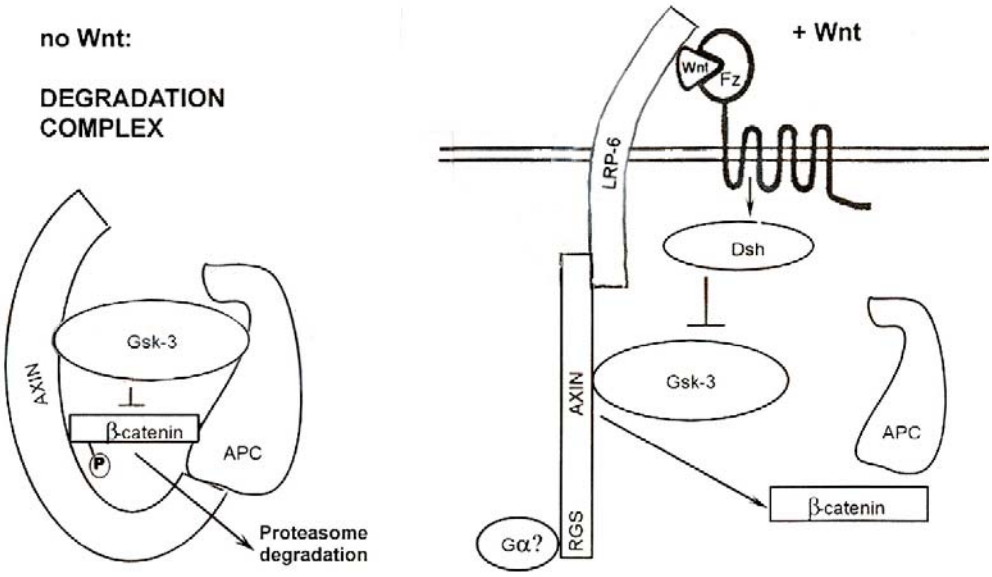


Axin contains an RGS (Regulation of G protein signaling) domain, which is conserved in many proteins that bind Gα subunits of G proteins. These binding domains act as GTPase-activating proteins. Deletion of the RGS domain from Axin causes it to become a dominant-negative protein, which instead of ventralizing the embryo, causes dorsalization and axial duplication (Wnt activator).



This implies that in the *Xenopus* embryo the RGS domain is required for Axin to limit the function of the Wnt pathway. Axin functions in the embryo to inhibit formation of ectopic axes, as indicated by the original mouse mutant). Axin has been recently been shown to form a part of a macromolecular degradation complex composed of APC, β-catenin, GSK-3 and axin, among other proteins.

The degradation complex facilitates phosphorylation of β-catenin. When Wnt signals through its two co-receptors, the carboxy-terminal domain of LRP-5/6 binds to the COOH terminus of Axin (via the DLX domain). β-catenin is no longer phosphorylated, becomes stable and translocates into the nucleus. LRP-6 channels the signal to the canonical β-catenin Wnt pathway. Thus, LRP signals through direct binding to axin (Mao et al., Mol. Cell *7*, 801-809, 2001):



10) Dickkopf inhibits LRP-6 function

Dickkopf is a secreted BMP inhibitor that blocks all β-Catenin canonical Wnt signaling discovered by Christof Niehrs. Dkk was found to bind to LRP-6. Dkk-1 also binds to a single-pass transmembrane protein called Kremen. When ternary complexes are formed between Dkk, Lrp-6 and Kremen, it is rapidly translocated to intracellular vesicles by endocytosis. In this way, the co-receptor LRP-6 is depleted from the cell surface, and canonical β-Catenin signaling is blocked. Since frizzled can still function, other aspects of Wnt signals (called planar cell polarity signaling, in which Dsh signals through the small GTPase RhoA, and the serine-threonine kinas JNK) are unaffected when Dkk removes the LRP-6 co-receptor (see Mao et al., Nature *417*, 664-667, 2002).

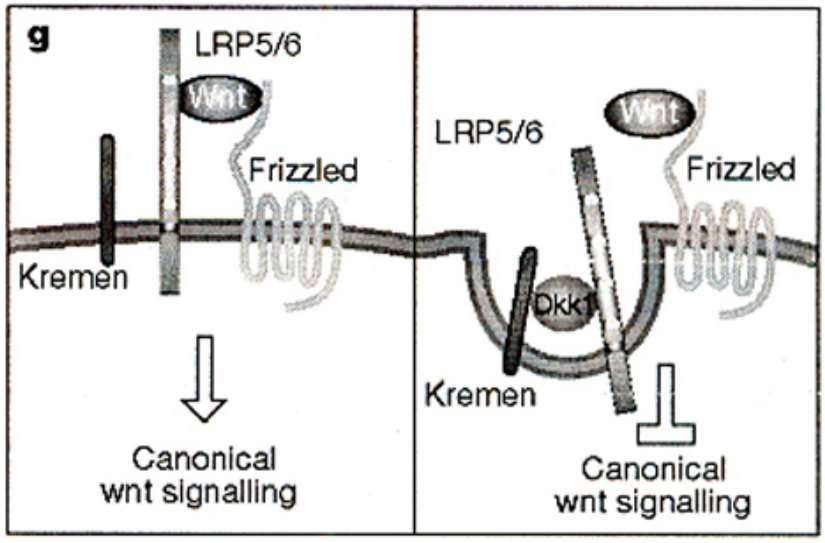
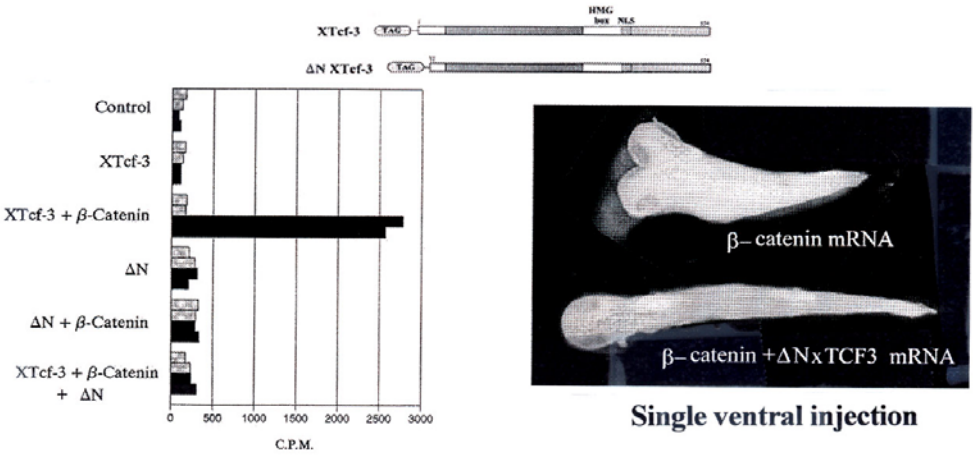


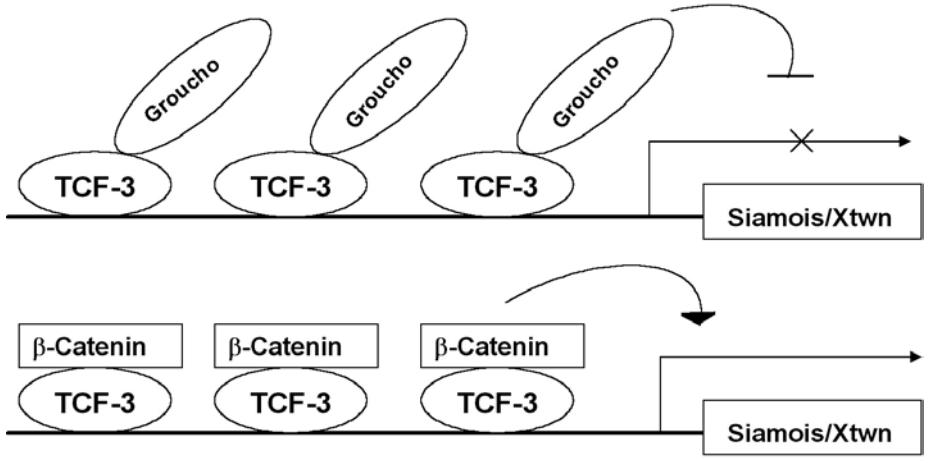
Figure 5 Dkk1 and Kremen trigger removal of LRP6 from the cell surface.
Dkk1, LRP5/6 and Kremen form a ternary complex that triggers rapid internalization and depletion of cell-surface LRP5/6 and thus blockage of canonical Wnt signalling (right).

11) TCF-3/Lef-1 for "T-Cell" or Lymphocyte Enhancer Factors are DNA-binding proteins that were isolated because they bind to the T-cell receptor enhancer. However, they were considered "architectural" HMG chromatin proteins because they could not activate transcription of reporter genes. A yeast 2-hybrid screen found that the amino-terminal end of TCF bound β-catenin (very nice paper by Molenaar et al., 1996). In co-transfection assays with a reporter construct, both β-catenin and TCF-3 are required for activation. β-catenin is therefore a co-activator of the xTCF-3 transcription factor (a homologue of *Drosophila* Pangolin). Deletion of the amino terminus of TCF-3 (ΔN) generates a potent dominant-negative (DN-xTCF-3).



ΔN is a potent dominant negative (DN-xTCF-3) in co-transfection experiments because it binds to DNA but not to β-catenin. The DN-xTCF-3 was able to block axis formation by β-catenin mRNA injected in the ventral side (figure), as well as Goosecoid transcription and the endogenous dorsal axis when injected in the dorsal side.

Wild-type TCF-3 is on its own potent repressor of transcription. It recruits the adaptor protein Groucho, which in turn recruits Histone cleacetylaes (HDACs).

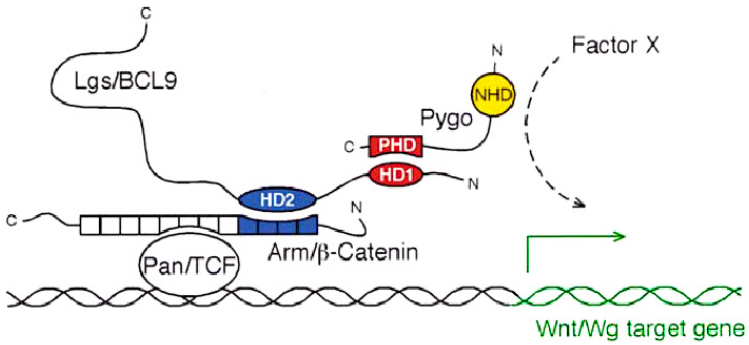


Binding of β-Catenin would displace Groucho and bring in a transcription activation domain located in its carboxy-terminal domain, converting TCF-3 into an

activator. In general, LEF-1 is a better activator of transcription and therefore the multiple transcription factors to which β-Catenin binds are not equivalent.

12) *Legless* and *Pygopus* connect β-Catenin to the transcriptional machinery

By screening for loss-of-function *Drosophila* mutants that suppressed the rough eye phenotype caused by wingless overexpression (under the control of the sevenless promoter), K. Basler identified two new genes required for the transmission of the Wnt signal.



Model for the activation of Wnt target genes by Lgs/BCL9 and Pygo. Lgs/BCL9 tethers Pygo to β-catenin, which is bound to regulatory regions for Wnt targets by Pan/TCF. Protein-protein interaction domains are highlighted in blue and red.

Kramps et al., Cell *109*, 47-60 (2002)

Legless shares three homology domains (HD1-3) with Human B Cell Lymphoma gene 9 (BCL0), a gene overexpressed in certain chromosome translocations. BCL9/Legless binds to B-Catenin and to a PHD (plant homology domain) in Pygopus. (Pygopus is named after a legless lizard). Its function is to recruit Pygopus to β-Catenin. If the PDH domain of Pygopus is replaced by HD2 of Legless, this activates transcription and rescues both Legless and Pygopus mutant flies.

13) Wnt target genes. Siamois and Xtwn

Two very related homeobox gens have been cloned in *Xenopus*. *Siamois* and *Xtwn* induce secondary axes and their promoters contain multiple xTCF-3 binding sites. Thus, increased free β-Catenin results in the activation of transcription by xTCF-3/LEF-1, which in turn transcribes *Siamois* and *Xtwn*. The *Siamois* promoter has three xTCF-3/LEF-1 binding sites.

14) The Struhl hypothesis

In *Drosophila*, the β-Catenin homologue Armadillo has been proposed to export repressive forms of TCF/Pangolin out of the nucleus. This is a very interesting development and we will discuss it in class. Please have a look at Chan and Struhl, Cell

111, 265, 2002 and bring your own copy to class. I will discuss some of its figures. The work is exceptional for its boldness.

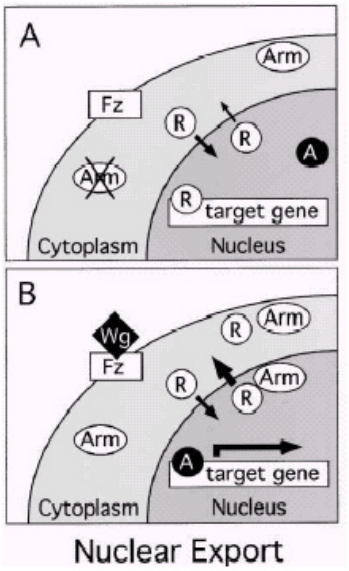


Figure 8. Nuclear Export and Cytosolic Activation Models for Wg Signal Transduction by Arm

(A and B) Nuclear export model.
(A) In the absence of Wg, cytosolic Arm is targeted for degradation by the Axin/APC/GSK3 complex (X) and both repressor (R) and activator (A) forms of Pan accumulate in the nucleus, where the repressor form predominates and blocks transcription of Wg target genes.
(B) In the presence of Wg, activation of Fz receptors allows Arm to escape targeting by the Axin/APC/GSK3 complex and Arm accumulates in the cytoplasm and nucleus. In the nucleus, Arm binds selectively to the repressor form of Pan and mediates its export from the nucleus at a rate that exceeds that of its import. As a consequence, the repressor form is depleted from the nucleus, and the remaining activator form activates transcription of Wg target genes.

Chan, S.K. and Struhl, G.
Cell *111*, 265-280 (2002)

15) The cortical rotation stabilizes β-Catenin, but a Wnt signal has not been identified in *Xenopus*

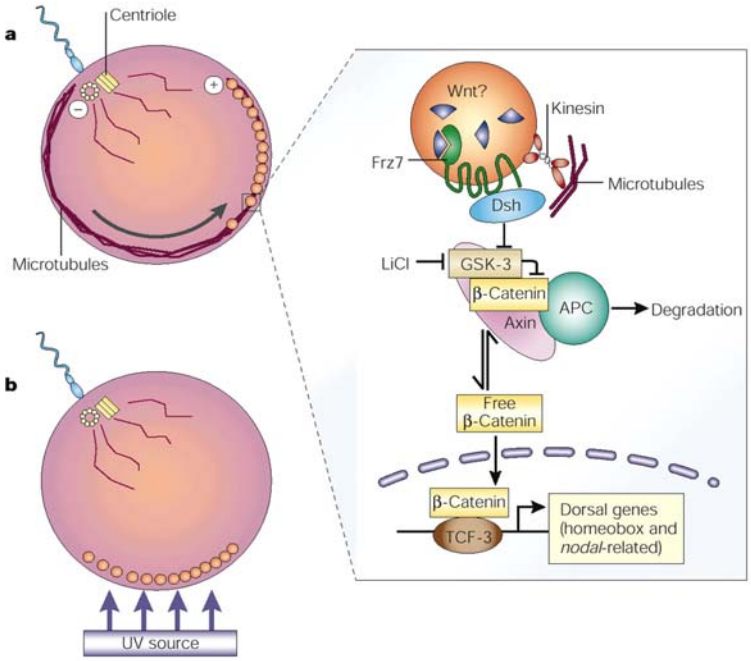
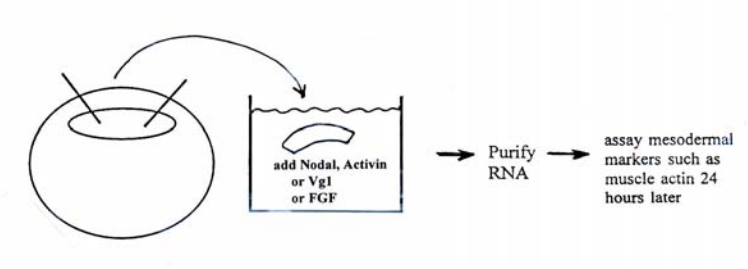


Figure 3 | **Dorsal determinants and the transport of membrane vesicles to the dorsal side. a** | After fertilization, parallel arrays of cortical microtubules extend from the centriole and the large aster at the sperm entry point towards the dorsal side (where the plus end lies), and transport small 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.

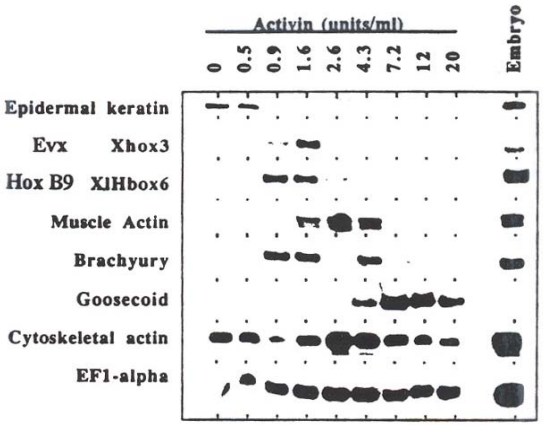
Although it seems likely that a maternal Wnt-signal might be transported from the vegetal pole to the dorsal side by the cortical rotation, an endogenous Wnt protein in the egg has not been identified. Conceivably GSK-3 could be inhibited on the dorsal side by Wnt signals from the membrane vesicles that travel along microtubules. The Nieuwkoop center is formed in the region in which Vg1 (Smad2) and VegT intersect with nuclear β-catenin.

16) Peptide growth factors can induce mesoderm

Let us return to the Nieuwkoop experiment. The *Xenopus* animal cap explant provides an excellent tool to study which growth factors can induce mesoderm:



A number of factors of the TGF-β (transforming growth factor-β) family are able to induce mesoderm. Activin, Vg-1, derrière, and Nodal (a gene that when mutated in mice results in the absence of mesoderm) all induce dorsal mesoderm. BMPs (Bone Morphogenetic Proteins) and FGF can induce only ventral mesoderm.



Dose response thresholds for activin. (Green and Smith, 1992)

Different doses of nodal, activin or Vg-1 result in very distinct threshold effects in *Xenopus* animal caps, acting as a morphogen.

Nodals, Vg-1 and Activin signal by binding to a type II serine-threonine kinase receptor that in turn phosphorylates a type I receptor which then phosphorylates the COOH end of Smad2, which then signals by acting as a co-activator of DNA-binding partners (such as for example FAST, Forkhead activin-sensitive transcription factor, a member of the winged-helix family of transcription factors) that bind to activin response elements.

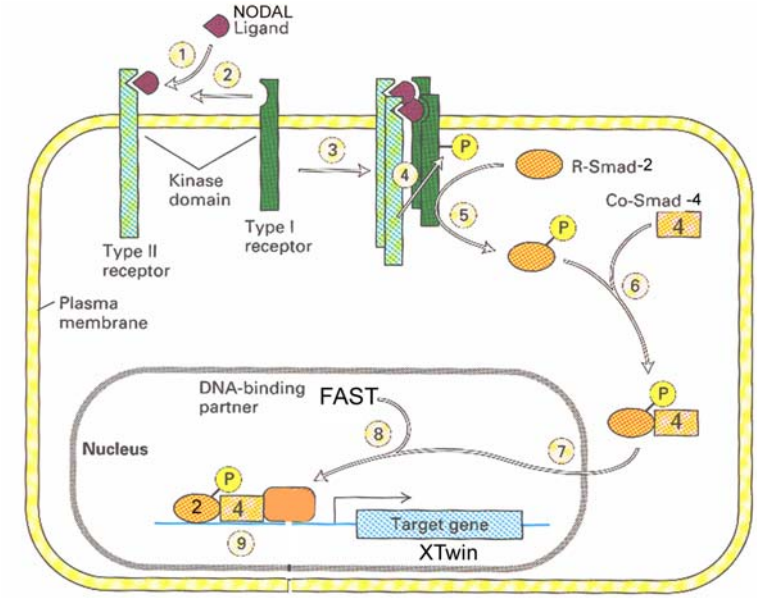
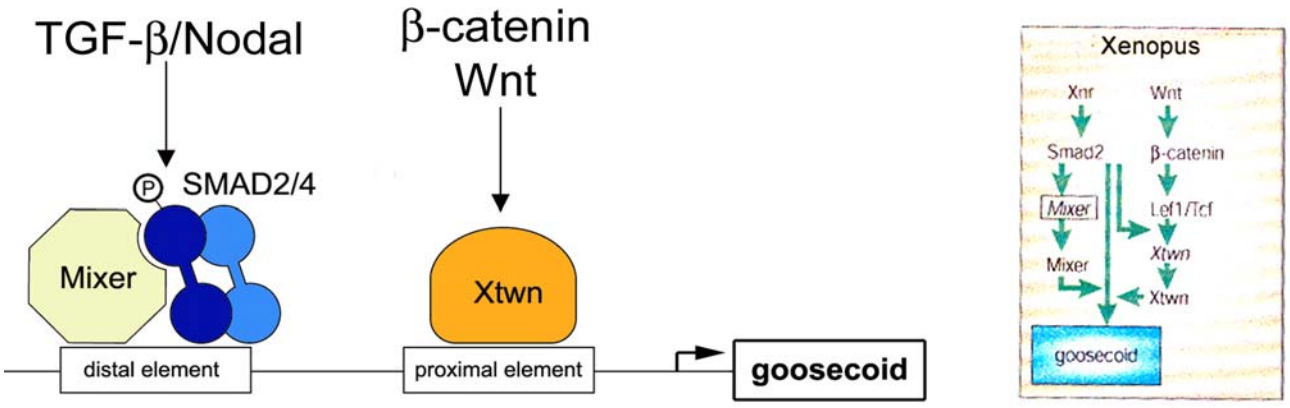


FIGURE 23-3 TGFβ (Nodal, Vg1, Activin) signaling pathway.

Binding of ligand to the type I and type II receptors, which are serine/threonine kinases, induces formation of multimeric receptors. Type II receptors phosphorylate type I receptors in the juxtamembrane region. Activated type I receptors specifically phosphorylate receptor-regulated Smads (R-Smads), which then dimerize with Co-Smads in the cytosol. The R-Smad/Co-Smad complex translocates to the nucleus where it binds to regulatory sequences in combination with specific transcription factors, leading to transcription of specific target genes. [Adapted from J. Massagué, 1998. *Annu. Rev. Biochem.* 67:753.]

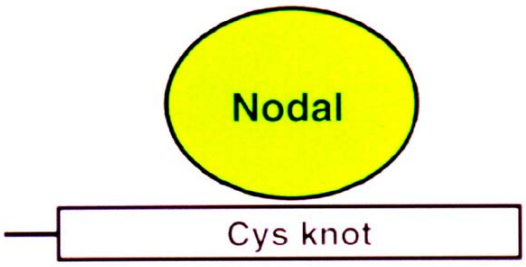
In the case of the organizer-specific gene *goosecoid* we understand in detail of how the Wnt and Activin/Nodal pathway are integrated at separate enhancers.



The goosecoid promoter binds and is synergistically activated by *Xtwn* or *Siamois* and by Nodal/related TGF-β signals. The organizer is formed in the region of the embryo where TGF-β signals (that activate the homeobox mixer and Smad-2/4) intersect with high levels of Xtwn induced by β-Catenin.

6. Mesoderm in *Xenopus* is induced by a gradient of Nodal related signals.

What is the molecular nature of the endogenous mesoderm inducer? The signals provided by maternal β-catenin, Vg1 and VegT converge in the transcriptional regulation of Nodal-related signals. A fragment of Cerberus, a secreted protein, was found to bind specifically to Xnrs (*Xenopus* Nodal-Related factors) but not to other TGFβs of similar activities such as Vg-1 and Activin. We will discuss Cerberus later on. Here its carboxy-terminal fragment, Cerberus-short, which consists of a cystine-knot motif, will be used as an anti-nodal reagent. Cer-S is completely specific for Nodal signals, which it binds to with high affinity preventing binding to the receptor. There are five Nodal-related molecules in *Xenopus*, making the simultaneous inhibition of all of them very difficult. But Cer-S inhibits all five, and in a very specific way for it does not bind to or block Activin, Vg1 or derrière (all are TGF-βs that induce dorsal mesoderm) nor BMP-4 (which induces ventral mesoderm).



Using this anti-Nodal secreted factor Agius et al. (2000) showed that the mesoderm induction discovered by Nieuwkoop is mediated by a gradient consisting of multiple Xnrs, with a maximum on the dorsal side. The main finding was that cer-S, both as injected mRNA or soluble protein, can block mesoderm induction by endodermal explants at the blastula stage.

They first asked whether a TGF-β-like signal secreted by endoderm is required for mesoderm induction. To this end, a truncated activin type 1 receptor (Chang et al., 1997), tALK4, was expressed in the animal cap cells that receive the signal. As shown in Fig. 3B, *tALK4* mRNA blocked induction of the pan-mesodermal marker *Xbra* by the endogenous endodermal signal. This implicated a requirement for TGF-β signaling in Nieuwkoop conjugates after only 2 hours of contact, but did not distinguish which factor was involved, since tALK4 blocks signaling by the mesoderm inducing factors *activin*, *derrière*, *A-Vg1* and *Xnr1*.

They next tested whether the endodermal signal required Nodal-related factors by microinjecting *cer-S* mRNA into the vegetal pole of early embryos. Endodermal explants from these embryos were prepared at stage 8 to 8.5, recombined with uninjected animal caps and analyzed only after 2 hours of contact with vegetal explants; i.e., during the period in which mesoderm induction occurs in vivo. It was found that in these Nieuwkoop recombinants *cer-S* inhibited not only the induction of the organizer markers *goosecoid* and *chd*, but also the ventral marker *Xwnt8* and the pan-mesodermal marker *Xbra* (Fig. 3C, compare lanes 2 and 4). As a negative control they used *follistatin* mRNA (Fig. 3C, lane 3), an inhibitor of Activin and BMPs, which failed to prevent mesoderm induction.

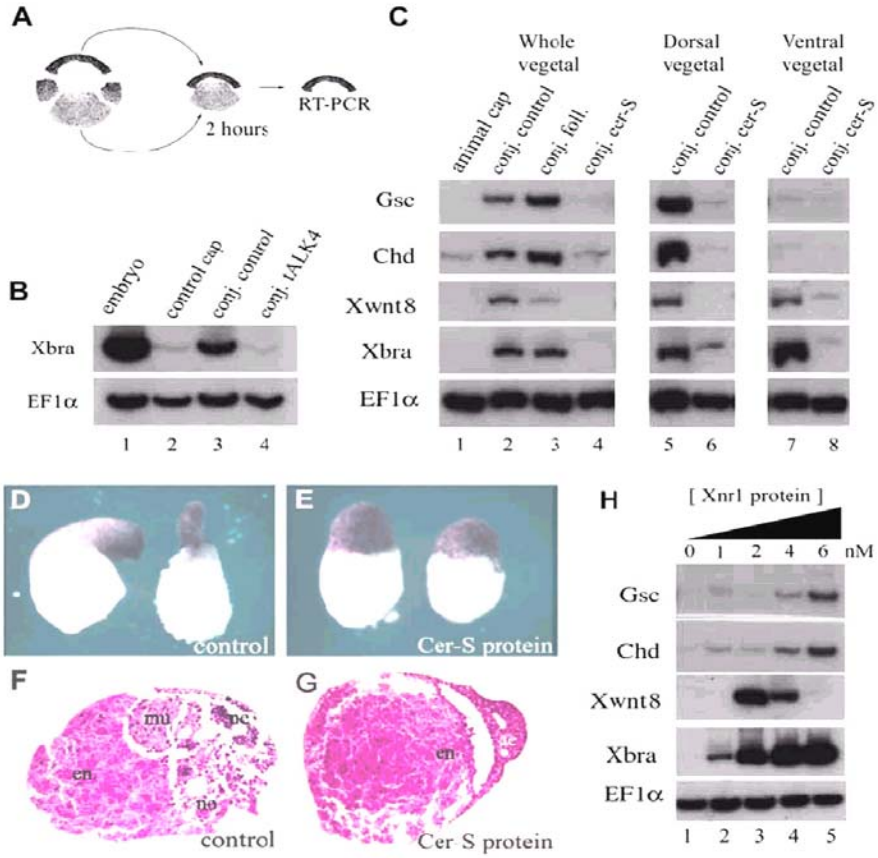


Fig. 3. The endogenous mesoderm-inducing signals are blocked by Cer-S in Nieuwkoop animal-vegetal conjugates. (A) Experimental design. (B) Microinjection of *tALK4* mRNA (*500* pg into each animal blastomere at 8-cell stage) blocks the response of animal caps to endogenous mesoderm-inducing signals (compare lanes 3 and 4). Caps were in contact with endoderm for 2 hours and are compared to control animal caps incubated without endoderm (lane 2). *EF1α* is a control for RNA recovery. (C) Lanes 1-4, Nieuwkoop recombinants of uninjected animal caps with vegetal pole explants injected with *follistatin* (2 ng) or *cer-S* (600 pg) mRNA. Note in lane 4 that *cer-S* blocks dorsal (*gsc*, *chd*), ventral (*Xwnt-8*) and pan-mesodermal (*Xbra*) markers, whereas in lane 3 *follistatin* mRNA has only a slight dorsalizing effect (total conjugates n=45, two experiments). This amount of *follistatin* mRNA was sufficient to abolish the activity of activin mRNA in co-injection assays (not shown). Lane 5, dorsal endoderm (Nieuwkoop center) induces preferentially the organizer markers *gsc* and *chd* (n=16, three independent experiments). Lane 7, ventral endoderm induces ventral markers *Xwnt-8* and the pan-mesodermal marker *Xbra* (n=17). Lanes 6 and 8, *cer-S* mRNA in the endodermal fragment prevents both dorsal and ventral mesoderm inductions (n=15 each). Conjugates were prepared between stage 8 and 8.5 and harvested for RNA after two hours. (D-G) External and histological morphology of vegetal fragments conjugated in the presence of control conditioned medium or of 20 nM Cer-S (Piccolo et al., 1999) protein and cultured until stage 36. Note that sections of the control contain muscle (mu), notochord (no) and some neural tissue (ne), whereas in the protein-treated sample the animal cap remains as atypical epidermis (ae) and endoderm (en) (n=26, three independent experiments). (H) Animal caps treated for two hours with control oocyte conditioned medium (lane 1) or with increasing doses (lanes 2-5) of Xnr1 protein (Piccolo et al., 1999). Increasing concentrations of Xnr1 protein induce first ventral and then dorsal mesodermal markers, producing thresholds after 2 hours in culture.

Agius et al. Development 127, 1173-1183 (2000)

In the reciprocal gain-of-function experiment, Xnr1 protein was added to stage 8 animal caps and incubated for 2 hours. At low concentrations (2 nM) Xnr1 protein induced ventral mesoderm and at higher doses (6 nM) dorsal mesoderm, producing sharp thresholds after only two hours of incubation (Fig. 3H, lanes 3-5). These loss- and gain-of-function experiments indicate that Nodal-related signals are necessary and sufficient for the induction of both dorsal and ventral mesoderm at the blastula stage.

What was surprising about this result is that two different signals were expected, one dorsal and one ventral, but nodals were necessary and sufficient to induce the mesoderm.

Endogenous Xnrs are expressed during blastula in a graded fashion in endoderm. Xnr1 expression starts on the dorsal side at midblastula and from there expands to the rest of the endoderm (Fig. 4C-E). Thus, dorsal endoderm (also known as the Nieuwkoop center) expresses *Xnr1* not only at higher levels but also for a longer period of time than ventral endoderm during the blastula stage. Microinjection of *cer-S* mRNA into embryos, causes a dose-dependent inhibition of *Xbra* expression in embryos (Fig.5). Since ventral *Xbra* is blocked at low doses and dorsal *Xbra* expression at high doses of *cer-S* mRNA, this result is consistent with the requirement of an endogenous Xnr gradient of activity for induction of mesoderm.

Fig. 4. Endogenous Xnrs are expressed at the right time and place to function as mesoderm inducers. (C) Stage 8 embryo showing a few nuclei stained in the dorsal vegetal mass (arrowhead). (D) Stage 8.5 blastula embryo in which *Xnr1* expression has expanded into neighboring vegetal cells. (E) Stage 9 blastula embryo displaying graded *Xnr1* expression throughout the embryonic endoderm. Note that *Xnr1* expression on the dorsal side is of longer duration, in addition to reaching higher levels than in ventral endoderm.

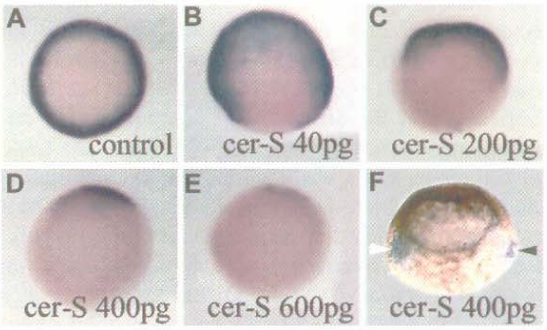


Fig. 5. Injections of *cer-S* mRNA dose-dependently reduce Xbra expression in gastrula embryos. Embryos are injected radially in the vegetal pole at the 4 cell stage, then processed for *Xbra* in situ staining at stage 10.5. (A) Control uninjected embryo, *Xbra* is expressed as a mesodermal ring. (B-E) Embryos injected with increasing amounts of *cer-S* mRNA, showing graded reduction of the *Xbra* expression domain. (F) Embryos injected vegetally with 400 pg of *cer-S* mRNA at the 4-cell stage and with *lacZ* lineage tracer mRNA into blastomere C4 at the 32-cell stage. In this lateral view the white arrowhead indicates *lacZ* in the ventral side (note that the pigment in the animal cap also marks the ventral side) and the black arrowhead points to the expression of *Xbra* transcripts on the dorsal side (n=51).

These results suggest that the classical 3-signal model for mesoderm induction in *Xenopus* can be modified in the way shown in the 2-signal model below. Maternal activities such as dorsal β-catenin and vegetal VegT and Vg1 cooperate to set up a zygotic dorsal to ventral gradient in the endoderm composed of multiple Xnrs at the blastula stage, when mesoderm induction takes place. At

high Nodal-related concentrations, which require a functional β-catenin pathway in the dorsal side of the embryo, the Spemann organizer (expressing genes such as *chordin*, *noggin* and *Frzb-1*) is induced in overlying cells by early gastrula. In the ventral side, VegT and Vg1 would lead to the production of lower levels of Nodal-related signals, and ventral mesoderm (expressing genes such as *Xwnt8* and *BMP-4*) is induced. Similarly, in ΔN-XTcf-3 injected embryos, the uniformly distributed VegT and Vg1 products would produce low levels of Xnrs sufficient to induce ventral mesoderm at the
gastrula stage.

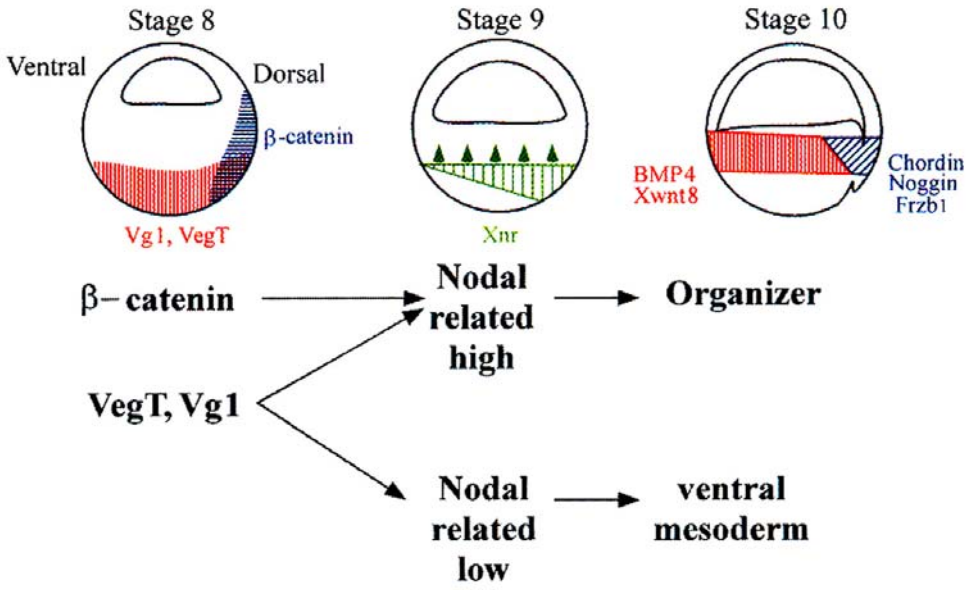


Fig. 7. Model of mesoderm induction at the blastula stage by a dorsal to ventral gradient composed of multiple Nodal-related genes expressed in endoderm.

A particularly attractive aspect of this model is that it may help explain a long-standing puzzle in *Xenopus* embryology. A surprisingly large number of microinjected molecules are able to rescue, often completely, the UV ventralized phenotype that results from interfering with cortical rotation of the fertilized egg. The UV-rescuing gene products include such diverse molecules as *β-catenin* (and

d Molecules that rescue the UV phenotype

Wnt pathway	TGF-β pathway	BMP antagonists
Wnts	Nodal-related	Chordin
β-Catenin	proteins	Noggin

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).

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.

other members of this signaling pathway), *Vg1*, *Xnr1*, *Xnr2*, *noggin* and *chordin*. Although one can argue that each of these diverse genes acts via different redundant pathways, their common UV-rescue activity is easier to understand if considered as part of a cascade of sequential gene activations. In this view, overexpression of *β-catenin* or *Vg1* would lead to high levels of Xnr expression in blastula endoderm, which in turn would mediate the induction of Spemann organizer in overlying cells, activating genes such as *noggin* and *chordin* that execute dorsal patterning at the gastrula stage.

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