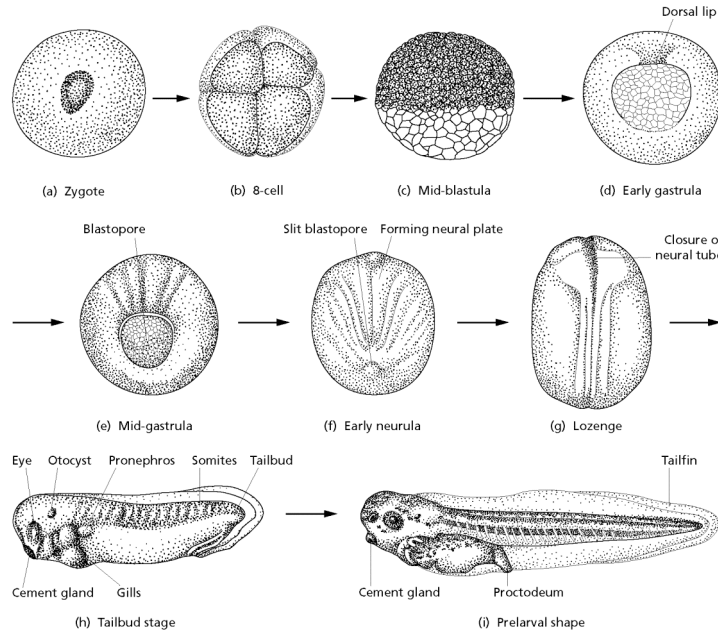# *XENOPUS* DEVELOPMENT

1.1, Introduction

The *Xenopus* egg is clearly polarized into a darkly pigmented animal hemisphere and a lightly

(a) Zygote (b) 8-cell (c) Mid-blastula (d) Early gastrula

(e) Mid-gastrula (f) Early neurula (g) Lozenge

(h) Tailbud stage (i) Prelarval shape

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pigmented vegetal hemisphere. After fertilization the egg undergoes a series of very rapid, and synchronous, cleavage divisions (90 minutes for the first, then every 25-30 minutes – at 21˚C) that over a period of 7 hours will generate the 5,000 cells of the mid-blastula stage embryo. During this period there is no recognisable G1 or G2 phases in the cell cycle and most of the embryonic genome is silent. Development is dependent upon maternal molecules (e.g. mRNAs and proteins) synthesized during oogenesis. After 12 cell divisions the cell-cycle slows considerably becoming 50 minutes for cycle 13, 90 minutes for cycle 14 and 240 minutes for cycle 15. Cell division becomes asynchronous and both G1 and G2 phases are introduced, allowing the embryonic genome to be activated. This is known as the mid-blastula transition (MBT). Timing of the MBT appears to be dependent on the titration of a cytoplasmic factor against increasing DNA content, since the MBT is delayed by one cell cycle in haploid eggs and can be advanced experimentally by injecting DNA.

The blastula stage embryo is a ball of cells surrounding a fluid filled cavity, the blastocoel, which is displaced to the animal hemisphere by the large, yolk rich, vegetal blastomeres. Fate
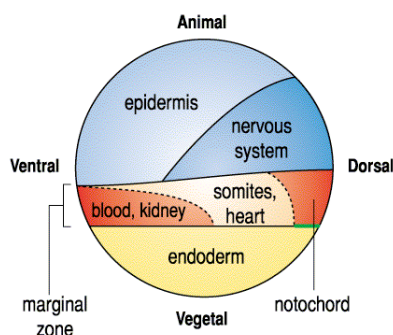

maps have shown that the animal hemisphere will form the ectoderm, while the vegetal hemisphere will form the endoderm. The equatorial region, which is known as the marginal zone, will form the mesoderm. After 10 hours the embryo is composed of ~10,000 cells and undergoes gastrulation, when the germ layers are rearranged such that the endoderm forms the innermost layer, the ectoderm the outermost layer, and the mesoderm the middle layer. Ectoderm will form the skin and nervous system, mesoderm will form the notochord, muscle, cartilage and bone, the urogenital system, connective tissues, and blood, while endoderm will form the digestive system (the lining, not the surrounding smooth muscle and connective tissue), the respiratory system, liver, and pancreas. Gastrulation takes about six hours and is followed by neurulation (15-22 hours after fertilization), when the lateral edges of the neural plate fuse to form the neural tube, the precursor of the central nervous system. Over the next three days the embryo undergoes organogenesis, when all the major tissues and organs are formed and differentiation occurs. After 2-3 months the tadpole will metamorphose into the adult frog, which will take another year to become sexually mature.

1.2, The vertebrate body plan

Following the completion of neurulation the embryo has acquired a body plan that is characteristic of all vertebrate embryos. The brain is already divided into forebrain, midbrain and hindbrain regions. The eyes are forming and an adhesive organ, the cement gland, forms
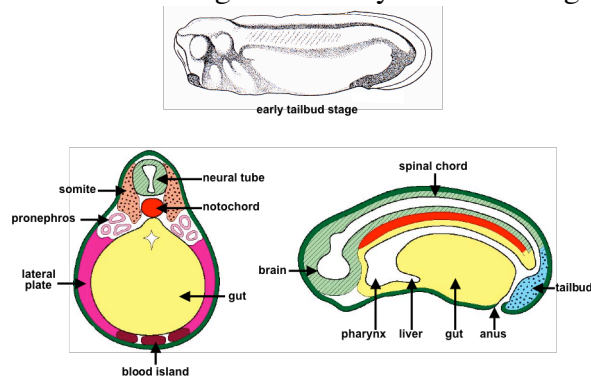

in the "chin" region of the ectoderm. The branchial arches form posterior to the cement gland, in association with the pharynx, and will form the jaws and gills. A cross section through the anterior trunk shows that the mesoderm lies between the epidermis and the endoderm. The neural tube lies along the dorsal midline and beneath this is a mesodermal structure called the notochord, which is composed of large vacuolated cells that add rigidity to the tadpole. The notochord is also the source of signals that are responsible for patterning adjacent structures such as the neural tube and somites. Flanking the notochord are the paired somites, which divide into the dermatome (dermis of the back), myotome (skeletal muscle) and sclerotome (cartilage and bone of the vertebrae). In amphibians, the myotome is easily the largest region of the somite, reflecting the requirement for powerful skeletal muscles for swimming. Somites form in an anterior to posterior sequence, until a total of about 40 are present. Beneath the somites are the pronephros and its associated tubules (a primitive but functional kidney), and the lateral plate mesoderm, which forms the smooth muscle of the digestive tract, the linings of the body cavities, and the dermis of the belly. On the ventral (belly) side of the embryo are the blood islands that differentiate the first population of blood cells. The mesoderm surrounds the yolk rich endoderm that differentiates the epithelial lining of the digestive tract and organs such as the liver and pancreas. Yolk is a source of food for the embryo, sustaining development until the fully differentiated tadpole can feed for itself. At the posterior tip of the embryo is the tailbud, a proliferating population of cells that will form the neural tube, notochord and somites of the tail.

1.3, Animal-vegetal polarity is maternally specified

The *Xenopus* egg possesses a distinct polarity even before it is fertilized, with a darkly

pigmented animal hemisphere and a lightly pigmented vegetal hemisphere. Within the egg the yolk is much more abundant in the vegetal hemisphere, while the nucleus (germinal vesicle) is located in the animal hemisphere. Several maternal mRNAs have been identified that are localized to either the animal or vegetal hemisphere during oogenesis and some of these are involved in early patterning events. Examples include mRNAs encoding the extracellular signalling molecules Vg1 and Wnt11 and the T-box transcription factor VegT. Translation of *Vg1* and *Wnt11* mRNAs begins during oogenesis, while translation of *VegT* mRNA is not initiated until after fertilization.
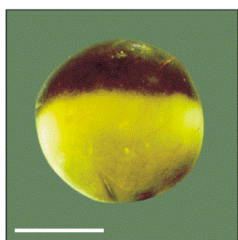
Animal and vegetal explants can be isolated from early blastulae and cultured in a simple salt solution for several days, where they will differentiate into recognizable tissues. Animal explants, called animal caps, will only form epidermis, even though they will also form neural tissue if left in the embryo. Vegetal explants don't differentiate but will express genes that are specific to the endoderm, but not genes that are specific to the ectoderm or mesoderm.

(Differentiation of the endoderm requires it to interact with lateral plate mesoderm, which is absent from vegetal explants). This demonstrates that ectoderm and endoderm are specified by maternal components inherited from the egg, but that neural tissue requires continued interactions with the embryo. Explants from the dorsal marginal zone differentiate dorsal mesoderm (e.g. notochord), while ventral and lateral explants differentiate ventral mesoderm. However, it is impossible to isolate pure mesoderm and marginal zone explants always include ectoderm and endoderm. Therefore, a role for inductive signalling in mesoderm formation, between endoderm and ectoderm, cannot be excluded (see section 2.1). It is notable that lateral marginal zone fragments form blood and very little, if any, muscle, yet their normal fate is to form lots

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of muscle and very little blood (see section 1.1). This suggests that the normal fate of marginal zone fragments is not specified by maternal localized determinants, but requires continued interactions with other regions of the embryo (see section 2.1).
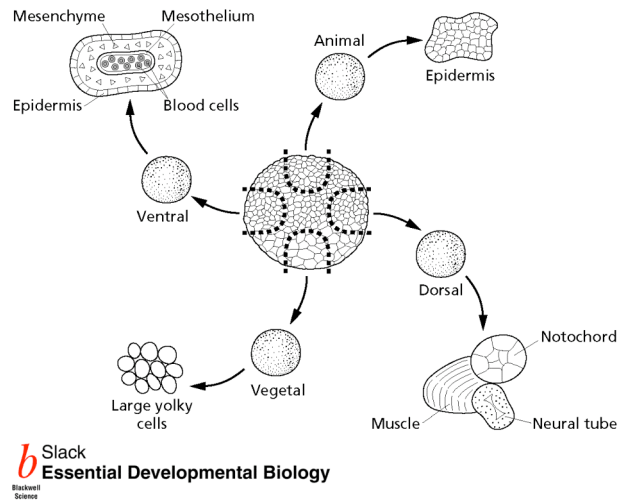
The identity of the maternal components that specify ectoderm is still unknown, but the determinant responsible for endoderm formation is believed to be VegT. This transcription factor induces the expression of endoderm specific genes (e.g. *bix1-4*, *mix1-2*, *sox17*) when injected into the animal hemisphere, showing that it is sufficient for endoderm formation. Moreover, injection of antisense oligonucleotides for *VegT* into *Xenopus* oocytes results in degradation of maternal *VegT* mRNA (before it is translated) and the resulting embryos fail to
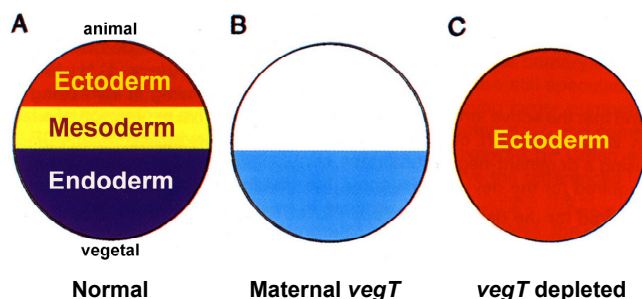
express endoderm-specific genes. Thus VegT is sufficient and necessary for endoderm formation. VegT depleted embryos also form little or no mesoderm, yet maternal VegT is not inherited by this germ layer! The explanation is that VegT activates the expression of vegetal signals (e.g. Xnr1, Derriere) that are required for mesoderm formation (section 2.3). Ectoderm is expanded in VegT depleted embryos, demonstrating that VegT represses ectodermal fates. Indeed, it is possible that ectoderm is a "default state" that forms in the absence of maternal VegT.
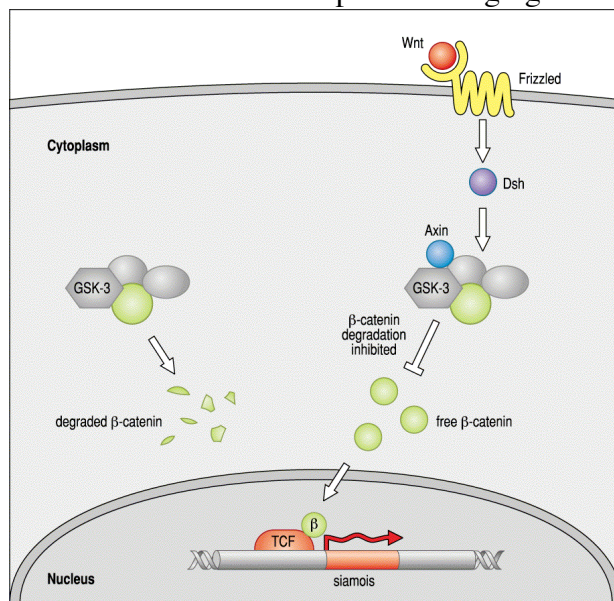
1.4, Cortical rotation

The unfertilized *Xenopus* egg is radially symmetric about the animal-vegetal axis and this symmetry is broken by a series of events set in motion by fertilization. Soon after fertilization (~30 minutes), microtubules have formed between the cortex and inner cytoplasm at the vegetal pole and the egg cortex rotates, by approximately 30°, relative to the inner cytoplasm. At the vegetal pole this movement is away from the sperm entry point and towards the future dorsal side. Cortical rotation causes the vegetal microtubules to form parallel arrays, in a ventral to dorsal direction. Small particles and membrane bound organelles, have been observed moving along these microtubules, from the vegetal pole towards the dorsal marginal zone. Movement of these particles is blocked by treatments that disrupt the formation of

microtubules, including UV-irradiation of the vegetal pole. Development of dorsal structures is severely disrupted by UV-irradiation and in extreme cases the embryos are completely ventralized (they lack notochord, muscle, pronephros, and neural tube, but lots of blood). In contrast, D_2O randomizes the orientation of the vegetal microtubules and treated embryos are hyperdorsalized (they have an enlarged neural tube, notochord, and muscle, but less pronephros, lateral plate mesoderm, and blood). In the unfertilized egg, the vegetal pole contains factors that induce a second dorsal axis when injected into ventral blastomeres (vegetal pole cytoplasm is injected), but this activity moves towards the equator, on the future

dorsal side, following cortical rotation. Movement is blocked by UV-irradiation of the vegetal pole, suggesting that a dorsal determinant is moved along the vegetal array of microtubules. Good candidates for this dorsal determinant are proteins called dishevelled (Dsh) and GSK3 binding protein (GBP). Both proteins associate with the vegetal array of microtubules and become enriched on the dorsal side of the embryo following cortical rotation.
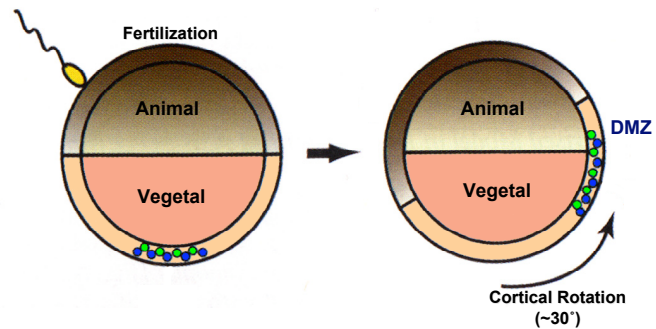
Dsh and GBP are components of the canonical Wnt signalling pathway that regulates gene expression by inducing transfer of the protein ß-catenin from cytoplasm to nucleus. Wnts activate cell surface receptors belonging to the frizzled family, which transduce the signal

across the plasma membrane to Dsh. Activated Dsh, and GBP, interact with a protein complex that includes the enzyme glycogen synthase kinase 3 (GSK3), which phosphorylates ß-catenin and targets it for degradation. Dsh and GBP inactivate GSK3, allowing ß-catenin to accumulate. As expected from this model, ß-catenin is enriched on the dorsal side of the embryo, from early cleavage through to late blastula stages. Injection of ß-catenin mRNA into ventral blastomeres induces a second dorsal axis on the ventral side of the embryo. As a consequence conjoined twins are formed. Depletion of maternal ß-catenin mRNA, by injection of antisense oligon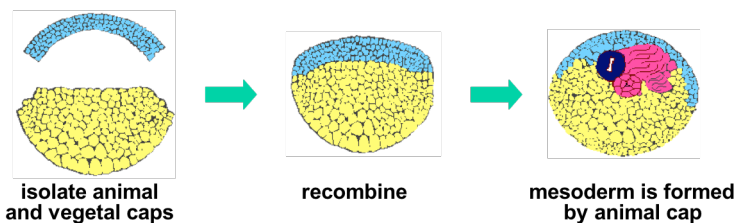ucleotides, blocks the formation of the primary dorsal axis, demonstrating that ß-catenin is both necessary and sufficient for dorsal development. ß-catenin accumulates in nuclei on the dorsal side of the embryo at blastula stages, forming complexes with the transcription factor XTcf3, and activates transcription of dorsally-expressed genes such as *siamois*, *twin*, and *xnr3*. Siamois and Twin are two closely related homeodomain transcription factors that induce secondary dorsal axes when misexpressed in ventral blastomeres (hence the names). Inhibiting Siamois blocks formation of the primary dorsal axis indicating that it is essential for dorsal development. *Siamois* and *twin* are normally expressed in dorsal-vegetal blastomeres, a region of the embryo known as the Nieuwkoop centre (see section 2.1).

For some time, it was thought that Dsh and GBP might be activated on the dorsal side of the embryo independently of Wnt signalling, but recent evidence suggests that this is not correct. Both maternal Wnt11 and a Frizzled 7 are necessary for dorsal specification in *Xenopus* embryos, since deleting maternal mRNA for either gene, by injecting antisense oligonucleotides, causes a loss of dorsal tissues. In both cases, ß-catenin fails to accumulate opposite the site of sperm entry. The mRNA for Wnt11 is localized to the vegetal cortex of *Xenopus* oocytes and moves to

the dorsal side of the embryo following cortical rotation. It is not known if this requires the parallel array of microtubules. Thus we have a picture in which cortical rotation shifts several components of the Wnt signalling pathway to the dorsal side of the embryo, where they prevent degradation of ß-catenin and stimulate transcription of dorsal specific genes.
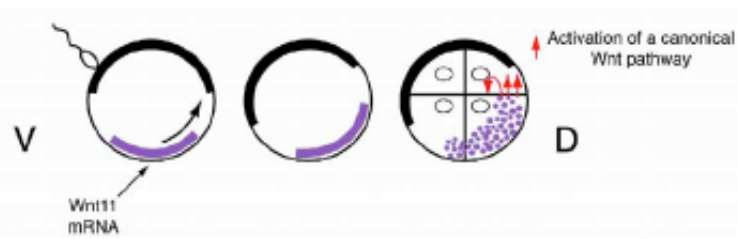
2.1, Mesoderm-induction

Mesoderm is formed in the marginal zone of amphibian blastulae as a result of inductive signals released by vegetal blastomeres, a process known as mesoderm-induction. This was shown in 1969 by the Dutch embryologist Pieter Nieuwkoop (1917-1996), who isolated animal and vegetal explants from axolotl mid-blastulae and found that they formed ectoderm

isolate animal and vegetal caps   recombine   mesoderm is formed by animal cap

and endoderm, respectively, when incubated alone (see section 1.3). However, they also formed mesoderm (notochord, muscle and pronephros) when combined. Nieuwkoop used pigmented and albino embryos as sources for his fragments, enabling him to determine which fragment(s) contributed to the mesoderm. He found that all the mesoderm was derived from the animal cap and concluded that it had been induced by the vegetal hemisphere. Mesoderm was not induced when explants were isolated from early gastrulae, indicating that mesoderm-induction occurs during blastula stages. Nieuwkoop's experiments were subsequently repeated on *Xenopus* and identical results obtained. Not all vegetal cells are able to induce dorsal mesoderm, indeed most will only induce ventral mesoderm. This was demonstrated by dividing the vegetal pole into small fragments along the dorsal-ventral axis and combining them with animal caps. Only the dorsal most fragment induced notochord and muscle, while lateral and ventral fragments induced blood. The small dorsal fragment will also induce a secondary dorsal axis when transplanted to the ventral side of a host blastula, without making any contribution to the mesoderm. Hence it releases a signal that induces dorsal mesoderm in the adjacent ventral marginal zone. This dorsal-vegetal fragment is the Nieuwkoop centre, the location of *siamois* and *twin* expression in late-blastulae (section 1.4), These

results suggest that the vegetal hemisphere releases two inducing signals; a dorsal signal released by the Nieuwkoop centre and a ventral signal released by lateral and ventral blastomeres. Thus only two types of mesoderm should be initially specified in the marginal
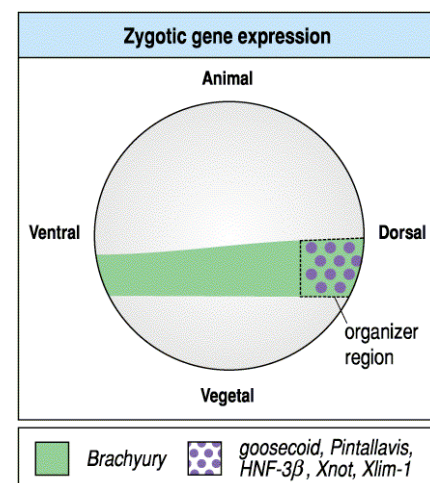
zone and evidence in favour of this was obtained by taking explants from the marginal zone of early gastrulae. Only explants of the dorsal marginal zone differentiated notochord and muscle, while ventral and lateral explants differentiated blood, mesothelium and mesenchyme (see section 1.3). Additional evidence is provided by the initial expression patterns of genes that are directly activated by mesoderm-inducing signals. These genes are first detected in late blastulae and while some (e.g. *brachyury*) are expressed throughout the marginal zone, others (e.g. *goosecoid*, *pintallavis*) are localized to the dorsal sector.

2.2, Mesoderm-Inducing-Factors

The blastula stage animal cap has been used as an assay for identifying molecules with mesoderm inducing activity, by incubating them in a simple salt solution containing the
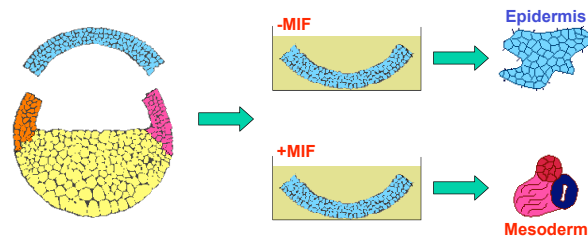
molecule to be tested. Isolated animal caps normally form round balls that differentiate as epidermis, but if a mesoderm inducing factor is present then the animal cap changes its morphology and differentiates mesoderm. The changed morphology is particularly evident if dorsal mesoderm is induced, since the cap undergoes considerable elongation (this is because dorsal mesoderm undergoes convergent-extension, the main mechanism responsible for elongating the anterior-posterior axis during gastrulation and neurulation). Using this assay, a small number of proteins have been identified that have mesoderm inducing activity and most of them are extracellular signalling molecules belonging to either the Fibroblast Growth Factor (FGF) or Transforming Growth Factor-ß (TGF-ß) families. Members of the FGF family usually induce mesenchyme and mesothelium (ventral-type tissue) at low concentrations and muscle at high concentrations, but never notochord. The first member of the TGF-ß family to be identified as a mesoderm inducing factor was Activin, which induces endoderm at high concentrations, notochord and muscle at slightly lower concentrations, and mesenchyme and mesothelium (ventral-type tissues) at the lowest inducing concentrations. Subsequently, Vg1, Derriere, and several Nodal-related (XNr) proteins were shown to have similar inducing activities, while Bone Morphogenetic Proteins (e.g. BMP4) were shown to induce blood, mesenchyme and mesothelium at all concentrations.

The concentration dependent effects of Activin were elegantly demonstrated by John Gurdon (1933-), who sandwiched beads soaked in Activin between two blastula stage animal caps (1994). A few hours later he used *in situ* hybridisation to look at the expression pattern of *goosecoid* (*xgsc*) and *brachyury* (*xbra*) (section 2.1). *Xgsc* was not expressed when low concentrations (1 nM) of Activin were used and *xbra* was localized to cells close to the beads. These cells expressed *xgsc* when high concentrations (4 nM) of Activin were used, while *xbra* was localized to cells further away.
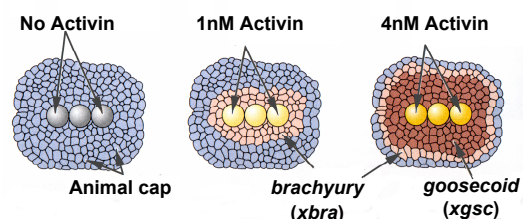
Radiolabelled Activin showed that this signalling molecule diffused away from the beads, forming a gradient with the highest concentration adjacent to the beads. The simplest interpretation is that low concentrations of Activin are sufficient to induce *xbra* expression and that higher concentrations are required to induce *goosecoid* expression. This suggests that the same molecule could be responsible for inducing both dorsal and ventral-type mesoderm: high concentrations secreted by the Nieuwkoop centre inducing dorsal-type mesoderm and low concentrations secreted by the remaining sectors inducing ventral-type mesoderm.

Evidence that members of the TGF-ß family are required for mesoderm-induction was first provided by experiments with a dominant-negative type II Activin receptor (dnActRII). TGF-ß receptors only function as complexes that include both type I and type II receptors, the cytoplasmic kinase domain of the type II receptor phosphorylating and activating the type I receptor. The activated kinase of the type I receptor then activates SMAD2, the major
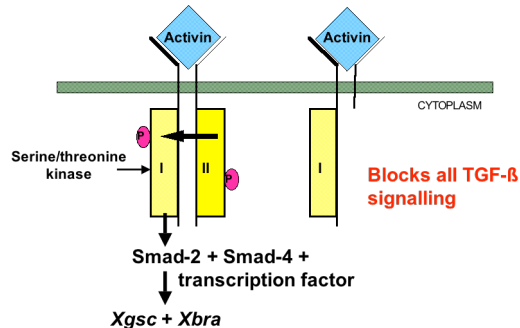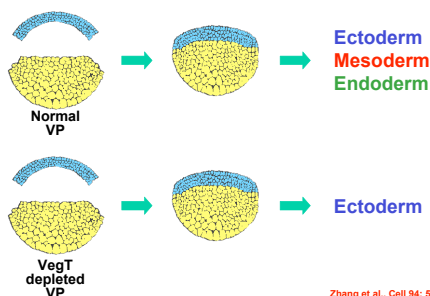

intracellular signal transducer of this pathway. dnActRII lacks the kinase domain but can still form complexes with endogenous type I receptors. However, these complexes are inactivate and cannot phosphorylate SMAD2. Hence this receptor acts as a dominant-inhibitor of Activin signalling and when injected into *Xenopus* embryos it blocks mesoderm formation. Although initially interpreted as evidence that Activin is the natural mesoderm-inducing factor, subsequent studies showed that dnActRII blocks mesoderm-induction by most members of the TGF-ß family (including Vg1, Xnrs, and BMPs). Thus this experiment only shows that the natural signal involves a member of the TGF-ß family. Similar experiments have been performed with an FGF receptor that lacks its intracellular tyrosine kinase domain. This truncated receptor forms complexes with endogenous FGF receptors, but once again they are inactive and cannot stimulate intracellular signalling (via the MAP kinase pathway). Injection of mRNA for this dominant-negative receptor (dnFGFR1) disrupts mesoderm formation in *Xenopus* embryos. The notochord and most of the posterior mesoderm is missing, but anterior mesoderm still forms. Thus FGF signalling is not essential for all the mesoderm. Since dnFGFR1 inhibits signalling by most members of the FGF family, it provides few clues as to the identity of the FGF required. The current view is that FGFs are not involved in the initial inductive event, from the vegetal hemisphere, but are subsequently required in the marginal zone to maintain posterior mesoderm. Consistent with this suggestion, at least three members of the FGF family (FGF3, FGF4 and FGF8) are induced by vegetal signals and localized to the marginal zone. FGF4 is required to maintain the expression of *xbra* and the phenotype of dnFGFR1 injected embryos is identical to the phenotype of embryos lacking functional Xbra.

2.3, Vegetal signalling molecules
A key criteria for a native mesoderm inducing factor is that it must be localized to the vegetal hemisphere of blastulae. The first mRNA found to be localized to the vegetal hemisphere was


vg1, which encodes a member of the TGF-ß family with mesoderm-inducing activity. Mesoderm-induction is impaired in embryos depleted of maternal *vg1* mRNA (by injecting antisense oligonucleotides into oocytes), demonstrating that it has a role in mesoderm-induction. However, it is also clear that maternal signals are not sufficient for mesoderm-induction in *Xenopus* and that zygotic signals are also required. This was demonstrated by studies on *vegT*, which is necessary both for endoderm and mesoderm formation (see section 1.3). VegT depleted vegetal poles have no mesoderm inducing activity when grafted to animal poles, despite containing Vg1. Since VegT is a transcription factor, and not a secreted protein, it cannot be directly responsible for inducing

mesodermal gene expression in the marginal zone. Rather, it must activate zygotic signals that are necessary for mesoderm-induction. (VegT is not translated until after fertilization so can only activate transcription of zygotic genes). Several genes have been identified whose transcription is controlled by maternal VegT and therefore localized to the vegetal pole of blastulae. These include members of the TGF-ß family, such as *derrière*, *xnr1*, *xnr2*, *xnr4*, *xnr5*, and *xnr6* (*Xenopus* has six nodal-related genes but *xnr3* has no mesoderm inducing activity. Mammals have only one *nodal* gene). Expression of all these genes is activated in animal caps injected with *vegT* mRNA and is absent from *vegT* depleted embryos. They are probably directly regulated by VegT, and VegT binding sites have been identified in the promoter of the *xnr1* and *xnr5* genes. These results suggests that Derrière and the Xnrs act downstream of VegT to induce mesoderm in the marginal zone. Consistent with this suggestion, injection of mRNAs for *derrière*, *xnr1*,

VegT activates transcription of nodal-related 1 (*xnr1*)

xnr2 or *xnr4* rescues mesoderm, and endoderm, formation in *vegT* depleted embryos. While Derrière rescues trunk and tail formation, XNr1, XNr2 and XNr4 rescue head and trunk. In conclusion, both maternal (Vg1) and zygotic (Derriere and XNrs) signals are required for mesoderm formation in *Xenopus*.
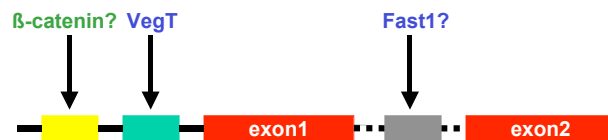
Zygotic transcripts for *xnr5* and *xnr6* are first detected at the 256-cell stage, much earlier than the MBT when transcription of most zygotic genes is activated. They probably collaborate with VegT to activate transcription of *xnr1*, *xnr2*, and *xnr4* at mid-blastula stages, as XNr

nodal-related genes are activated in the vegetal half of late-blastulae
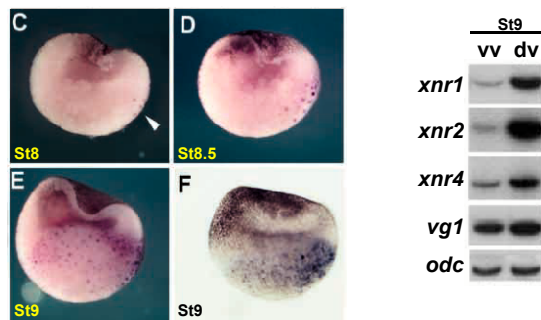
Agius et al., Development 127: 1173-1183 (2000)

responsive elements have been found in the first intron of the *xnr1* gene (bound by the FAST1 transcription factor). Transcripts for *xnr1*, *xnr2*, and *xnr4* are first detected in the dorsal half of the vegetal hemisphere and expression remains higher than in the ventral half throughout blastula stages. Thus high concentrations of Xnrs could account for the ability of dorsal-vegetal blastomeres, the Nieuwkoop centre, to induce dorsal mesoderm (see section 2.1). Expression of *xnr1* is also regulated by the Wnt signalling pathway, which is activated on the dorsal side of *Xenopus* blastulae following cortical rotation (see section 1.3). Wnt responsive elements have been identified in the promoter of the *xnr1* gene and inhibition of ß-catenin blocks transcription of *xnr1*. Moreover, coinjection of ß-catenin and VegT induces significantly higher levels of *xnr1* expression in animal caps than injection of either molecule alone. Thus high levels of *xnr1* expression in the Nieuwkoop centre may result from the combined activities of VegT and ß-catenin.

Compelling evidence that Xnrs have a role in mesoderm-induction was provided by studies with the secreted protein Cerberus (so called because it induces a second head when expressed in ventral blastomeres – it was named after the three headed dog of Greek mythology). Biochemical studies have shown that Cerberus directly binds Wnts, BMPs and Xnrs, thereby preventing these signalling molecules from activating their receptors. Xnrs

binds to a the C-terminal half of Cerberus and a truncated protein containing just this half of
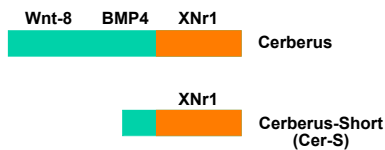
the protein (called Cer-S) inhibits mesoderm-induction by Xnrs, but not Activin, Derrière, or Vg1. Cer-S also blocks the mesoderm-inducing activity of the vegetal pole in animal-vegetal conjugates and blocks mesoderm formation in embryos. Thus XNrs appear to be an essential component of the vegetal mesoderm-inducing signal. In support of this conclusion, genetic studies in both mice and zebrafish have shown that Nodal-related genes are essential for mesoderm

formation. The *nodal* gene (so named because transcripts are first detected in the node, at the anterior end of the primitive streak of mammalian gastrulae) was first identified as an embryonic lethal mutation in the mouse that caused a severe loss of mesoderm and endoderm when homozygous. A similar phenotype is observed in zebrafish embryos that are doubly homozygous for *cyclops* and *squint*, which encode Nodal-related proteins. The single mutations have relatively minor effects on dorsal mesoderm that leads to brain abnormalities,

including the formation of only a single eye (hence the names of these genes). Presumably, the two genes can compensate for each others loss, to a certain extent, and deletion of both genes is required to see the full requirement of Nodal-related genes in mesoderm formation.
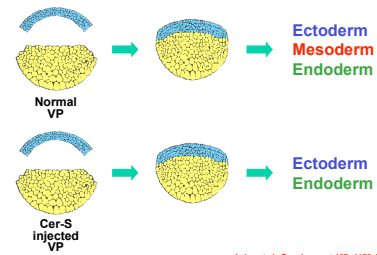
2.4, Mesoderm or endoderm?

TGF-ß signals induce both mesoderm and endoderm, so how do these two germ layers become localized to the marginal zone and vegetal pole respectively? One explanation is that maternal VegT directly activates expression of genes that are not only required for endoderm formation but also repress mesoderm formation. If such genes were transcription factors they would act cell-autonomously to specify endoderm, and inhibit mesoderm, in the vegetal hemisphere. In contrast, Xnrs and Derrière, would diffuse into the adjacent marginal zone where in the absence of VegT they would induce mesoderm. Recent studies have identified several transcription factors as candidates for the endoderm-specific factors. These include members of the Mix family (e.g. Bix1-4, Mix1, Mix2 and Mixer), Gata family (Gata-4, 5, and 6) and Sox family (Sox17), all of which are localized to the vegetal hemisphere of late blastulae. Intriguingly, injection of *bix4* mRNA into *vegT* depleted embryos rescues the expression of endodermal markers, but does not restore the ability of vegetal pole blastomeres to induce mesoderm. Thus Bix4 acts directly downstream of VegT to specify endodermal, but not mesodermal, differentiation.

2.5, A model for mesoderm-induction
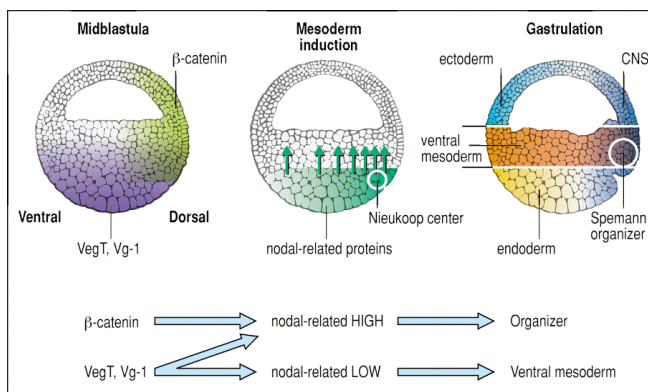
Data presented above suggests a model whereby the maternal transcription factor VegT activates zygotic expression of the TGF-ß family members *derrière*, *xnr1*, *xnr2*, and *xnr4* in the vegetal hemisphere. The maternal TGF-ß family member Vg1 may also have a role in this process. VegT alone is only sufficient to activate low level expression of *xnrs*, the higher levels of expression characteristic of the

Nieuwkoop centre requiring dorsally localized ß-catenin. High levels of Xnrs induce expression of dorsal-specific genes, such as *goosecoid*, in the dorsal marginal zone, while low levels of Xnrs induce expression of ventrally expressed genes such as *xbra*. However, additional factors are also involved, as demonstrated by the presence of Wnt-responsive elements in the *goosecoid* promoter. Both TGF-ß and Wnt responsive elements appear to be necessary for localizing *goosecoid* expression to the dorsal mesoderm. The Wnt-responsive elements are bound by the homeodomain transcription factors Siamois and Twin indicating an additional role for dorsally localized ß-catenin in specifying dorsal mesoderm.
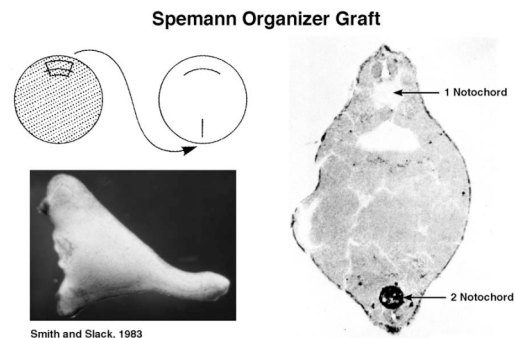
3.1, The Spemann Organizer

As described above (section 2.1), mesoderm inducing signals appear to establish just two territories within the marginal zone. A relatively small dorsal sector (90° of arc) that differentiates dorsal-type mesoderm (notochord) when isolated and a much larger ventral-lateral sector that differentiates ventral-type mesoderm (blood) when isolated. The remaining mesodermal tissues - muscle, pronephros, and lateral plate - are specified by signals that are

Mesoderm Induction

Dorsalization + Neural Induction
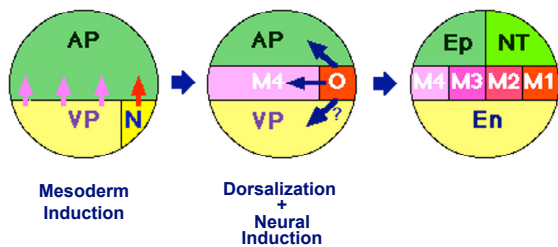
released from the dorsal mesoderm, a process known as dorsalization. The dorsal mesoderm also releases signals that induce the overlying ectoderm to form neural tissue, a process known as neural-induction. Dorsalization and neural-induction were first demonstrated in the experiments of Hans Spemann (1869-1941) and Hilde Mangold (1898-1924), who took the dorsal blastopore lip from an early newt gastrula and implanted it into the ventral side of a second, differently pigmented, host gastrula (1924). The host developed a second dorsal axis, complete with neural tube, notochord, and somites. Within this axis the notochord and a few somite cells were derived from the donor, while the neural tube, and most of the somites, were derived from the host. (Because of the importance of this experiment Spemann

was awarded the 1935 Nobel Prize for Physiology or Medicine. Mangold had died in a domestic accident in 1924) Identical results have been obtained in *Xenopus* and the dorsal marginal zone of the amphibian gastrula is now known as the Spemann organizer. Similar axis inducing organizers have been identified in gastrulae from zebrafish (the shield), chicks (Hensen's node), and mice (the node) – like the amphibian organizer they all give rise to the notochord. Complete secondary axes, including

Spemann Organizer Graft

Smith and Slack, 1983

heads, are only induced if the organizer is grafted at the beginning of gastrulation, at later stages secondary axes lack a head. This has lead to the suggestion that there are two organizers; a head organizer and a trunk organizer. As the mesoderm involutes, the head organizer moves away from the dorsal blastopore lip to be replaced by the trunk organizer.
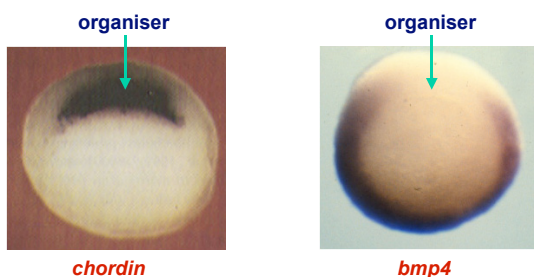
3.2, Organizer signals

Following the discovery of the Spemann organizer, amphibian embryologists spent many years trying to identify the molecules involved. However, the technologies available at the time (1924-1942) were inadequate for the task and no progress was made. The first organizer signal was not identified until 1992, using molecular techniques that were only developed in the previous 10 years. Smith and Harland cloned organizer specific mRNAs and injected them

into UV-irradiated *Xenopus* embryos. As described above (section 1.3), UV-irradiation of the vegetal pole soon after fertilization blocks cortical rotation and the formation of the Nieuwkoop centre, the resulting embryos lack all dorsal mesoderm. An mRNA that rescued dorsal development was identified and found to be localized to the Spemann organizer. It encoded a novel protein that Smith and Harland called Noggin. Injection of *noggin* mRNA into ventral blastomeres induced a partial secondary axis that lack head structures, indicating that it mimicked the trunk organizer. Noggin protein dorsalized isolated ventral mesoderm and induced neural tissue in animal caps.

Two additional proteins, Chordin and Follistatin, with similar properties to Noggin were subsequently identified and their mRNAs were also found to be localized to the Spemann organizer. Chordin induces a secondary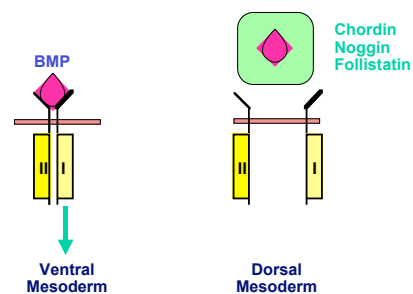 dorsal axis (lacking head structures), while both Chordin and Follistatin neuralize animal caps. Inhibition studies (injecting antisense morpholino oligonucleotides that block translation of target mRNAs) have shown that all three proteins are necessary for the normal function of the organizer. Chordin and then Noggin are probably the most important, since they give the strongest phenotypes when depleted. However, depletion of all three proteins is necessary for full ventralization of the embryo. Follistatin was of special interest because its mode of action was known from mammalian physiology, where it binds to extracellular Activin. Follistatin bound Activin cannot activate its receptors. Subsequent studies showed that it also bound and inhibited BMP7, suggesting that a member of the TGF-ß family promoted ventral development in amphibian embryos and that dorsal development required inhibition of this signal by the organizer. It also suggested that Chordin and Noggin might act in a similar

Expression of *chordin* and *bmp4* in *Xenopus* gastrulae

manner and biochemical studies demonstrated that they do indeed inhibit members of the TGF-ß family, in both cases BMP2 and BMP4 (but not Activin). Transcripts for *bmp4* are uniformly expressed in *Xenopus* blastulae but are lost from the Spemann organizer and neural plate during gastrulation. Injection of *bmp4* mRNA into the organizer causes a complete loss of dorsal structures, the resulting embryos only differentiating ventral mesoderm (blood) and ectoderm (epidermis). Moreover, a dominant-negative BMP receptor (dnBMPR1), which

specifically inhibits BMPs, dorsalizes *Xenopus* embryos. It induces a secondary dorsal axis, lacking head structures, when expressed in ventral blastomeres and neural tissue when expressed in animal caps. BMP4, but not Activin, can also restore epidermal differentiation to dissociated animal caps, which would otherwise differentiate as neural tissue (In contrast to whole animal caps, which form epidermis, dissociated animal caps form neural tissue, presumably because an epidermalizing factor (BMP4) is diluted by dissociation). Taken together, the data suggested that BMP4 specifies ventral fates in both the ectoderm and mesoderm (and probably the endoderm as well) and that dorsal fates are activated in the absence of this signal. (In the nervous system this is known as the neural default model). Genetic studies in zebrafish support this conclusion. Mutations that disturb dorsal-ventral

patterning during gastrulation always affect the BMP signalling pathway and either dorsalize (e.g. mutations in *bmp2* and *bmp7*) or ventralize (e.g. mutations in *chordin*) embryos.

3.3, Head-induction

Although BMP antagonists induce a secondary dorsal axis when expressed in ventral blastomeres, the axis always lacks the anterior regions of the head (although they frequently contain a hindbrain and otic vesicles). BMP antagonists therefore mimic the trunk organizer. How then does an embryo get its full head? Clearly the organizer must release additional signals. The hunt for genes specifically expressed in the Organizer identified *frzb*, *cerberus*, and *dickkopf1* (*dkk1*), all of which appear to be required for head development. Transcripts for *cerberus* are localized to the anterior endoderm, while transcripts for *frzb* and *dkk1* are localized to the prechordal plate (dorsal mesoderm anterior to the notochord). Thus all three genes are specifically expressed in tissues that contribute to the head. They have only weak dorsalizing activity but they neuralize animal caps, cooperating with BMP inhibitors to promote strong neuronal differentiation. Coinjecting *chordin* and *frzb* mRNA gives secondary axes that are much more complete that than injecting *chordin* alone, with eyes usually being formed. However, the eyes are always cyclopic (fused across the midline), indicating that the most anterior regions (the forebrain) are still missing. Almost identical results are obtained

Frzb induces a secondary head when coexpressed with Chordin
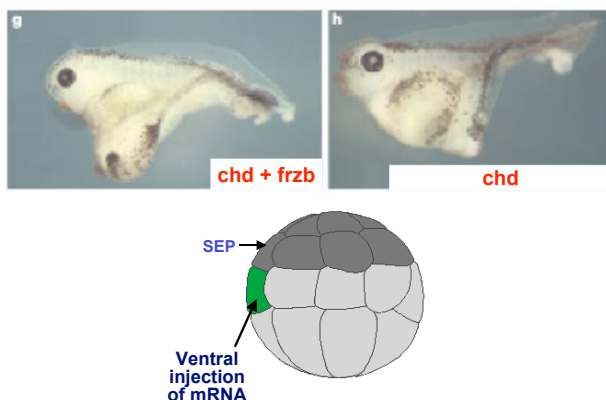
after coinjecting *chordin* and *dkk1* mRNAs, although the heads are often fully formed. Moreover, inhibition of Dkk1 function results in embryos that lack anterior head structures. Cerberus, on the other hand, will induce a fully formed head when injected alone. As described in section 2.3, Cerberus is a protein that binds, and inhibits, members of the Wnt, BMP, and Nodal families of extracellular signalling molecules, indicating that inhibition of Wnt and/or Nodal signalling, as well as BMP signalling, may be necessary for head induction. FrzB has a very similar amino acid sequence to the N-terminal domain of Frizzled receptors, which is known to be responsible for binding Wnt signalling molecules. This suggested that FrzB might be an inhibitor of Wnt signalling, binding and preventing Wnts from activating their receptors. Biochemical experiments proved that this was indeed the case and subsequent studies showed that Dkk1 also inhibits Wnt signalling. Thus inhibition of Wnt signalling appears to be the key event in head induction. Inhibition of Nodal signalling may also be required for full head development, as suggested by experiments with the Nodal-binding domain of Cerberus (Cer-S). Embryos injected with Chordin, FrzB, and Cer-S have fully formed heads. The ability to inhibit BMP, Wnt, and Nodal signalling may explain the formation of a fully formed second head when *Cerberus* mRNA is injected into ventral blastomeres. These experiments suggest that inhibition of BMP, Nodal and Wnt signalling pathways are required for head induction in amphibians.

Although inhibiting BMPs induces only trunk tissues in axis duplication assays, it induces anterior (head) neural tissue in isolated animal caps. This paradox can be explained if the embryo produces posteriorizing signals that inhibit the development of anterior structures, transforming anterior into posterior fates. One candidate for a posteriorizing signal is Wnt3a,

which blocks head development when over-expressed in *Xenopus* embryos. Wnt3a will also transform anterior neural tissue, induced in animal caps by Chordin, into posterior neural tissue. The role of Wnt inhibitors in head induction may be to prevent this transformation, allowing anterior neural tissue to differentiate. Other candidate molecules for posteriorizing signals include FGF4 and Retinoic Acid (RA). Both molecules transform anterior neural tissue into posterior neural tissue in animal caps lacking BMP signalling. *Fgf4* expression is localized to posterior and tailbud mesoderm, where it activates expression of the T-box transcription factor *xbra*. This gene has an essential role in the development of posterior structures, as evident from the "no tail" phenotype of mouse and zebrafish embryos mutant for the *brachyury* gene. A similar phenotype is seen in *Xenopus* embryos expressing a

Neural Induction

head (Frzb, Dkk)
trunk (Chd, Nog)
posterior (FGF, Wnt)

archenteron
dorsal blastopore lip
blastocoel

anterior ──────────→ posterior

dominant-negative mutation for *xbra*. An almost identical phenotype is obtained with the dominant-negative FGF receptor, which blocks FGF4 signalling and induction of *xbra* expression. (*xbra* is a direct target of FGF signalling in *Xenopus* blastulae and early gastrulae) FGF also activates expression of *Xenopus* caudal genes that in turn activate expression of posterior *hox* genes, such as *hoxb-9*. As a consequence, overexpression of FGF4 leads to more posterior specification of the nervous system and a reduction of anterior fates. A similar phenotype is also observed following addition of RA to embryo culture media during gastrulation. A posterior (high) to anterior (low) gradient of RA has been detected in the dorsal mesoderm of *Xenopus* neurulae, and high concentrations of RA have been shown to activate the expression of posterior *hox* genes.
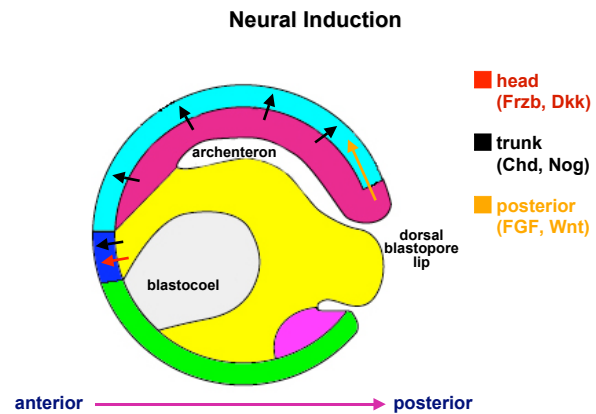
Reading:
The following sections from Principles of Development (2nd Edition) by Lewis Wolpert
1) Chapter 2. Model organisms: vertebrates. Pages 30-35. (Section 2.1)
2) Chapter 3. Setting up body axes. Pages 67-76 (Sections 3.1-3.4).
3) Chapter 3. Specification of germ layers. Pages 83-105 (Sections 3.9-3.21).
4) Chapter 4. The role of the organizer & neural induction. Pages 125-133 (Sections 4.6-4.9).
or
the following sections from Developmental Biology (7th edition) by Scott Gilbert
Chapter 10. Early development and axis formation in amphibians. pages 305-343
　　　　Also see pages 210-213