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MESODERM INDUCTION IN
AMBYSTOMA MEXICANUM,
A URODELE AMPHIBIAN

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Thesis submitted to the University of Nottingham
for the degree of Doctor of philosophy

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Abstract

Understanding how the germ layers are formed is one of the key questions of developmental biology. Abundant studies in the anuran amphibian *Xenopus laevis* have described that maternal and vegetally localised mRNAs for VegT and Vg1 contribute greatly to the formation of mesoderm and endoderm in the developing embryo. Within *Xenopus* mesendoderm gene-regulatory network (GRN), Wnt/*β-catenin* as well as Nodal and Mix family members have been shown to play important roles. The involvement of several members of the *Nodal* and *Mix* gene families with redundant functions makes the mesendoderm GRN surprisingly complex and difficult to study in *Xenopus laevis*. By contrast, mouse and humans have only single copies of *Nodal* and *Mix*. Since urodeles have an embryology that is basal to amphibians and that has most likely also been conserved during the evolution of amniotes, including mammals, we have investigated the Mix and Nodal genes in the urodele Axolotl in the hope that their gene families contained fewer members. We cloned one *Mix* and two *Nodal* orthologs from the axolotl and showed by Southern blot analysis that there are likely no further copies in the axolotl genome. Morpholino and rescue experiments furthermore showed that *AxNodal-1*, *Mix* and *Brachyury* play essential roles in mesoderm specification in axolotl embryos, suggesting that the urodele Axolotl has a more simplified mesendoderm GRN. In this context, we demonstrate that Mix acts to induce Brachyury expression during mesoderm induction. *Mixl1* shRNA knowdown in mouse embryonic stem cells (mESCs) shows that Mixl1 is involved in the production of mesoderm in mESCs too. Analysis of the localisation of the VegT and Vg1 mRNAs in oocytes revealed that they are neither vegetally

localised in the Axolotl, nor in the basal fish species lungfish and sturgeon. Furthermore, gain and loss of function assays examining the roles of maternal VegT and β-catenin demonstrated that VegT is not required for mesoderm induction, whereas β-catenin is necessary and sufficient for mesoderm induction by activating AxNodal-1 expression in the axolotl. As these results reveal additional similarities to the GRN in mammals they further support our hypothesis that the regulatory network in the axolotl is more closely related to that in amniotes rather than anuran amphibians.

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Common Abbreviations

Ax	Axolotl
cDNA	complementary DNA
CDS	coding sequence
CHX	cycloheximide
CNS	Central nervous system
E	Embryonic day
ES	Embryonic stem
EMS	mesendoderm cells
GRN	Gene regulatory network
LPM	lateral plate mesoderm
MBT	mid-blastula stage
mRNA	messenger RNA
ODC	Ornithine decarboxylase
ORF	open reading frame
PGC	primordial germ cell
qPCR	quantitative PCR
RACE	rapid amplification of cDNA ends
RNA	ribose necleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SIM	Smad interacting motif
UTR	untranslated region
Xl	*Xenopus laevis*
Xt	*Xenopus tropicalis*
YSL	yolk syncitial layer

Chapter 1. Introduction

The transition from protists to metazoans marks the origin of multicellularity and required the specification of distinct cell types. Similarly, the emergence of triploblastic animals from diploblastic organisms marked the origin of the second body axis and the mesoderm, leading to the diversity of body plans in the metazoans. As a result, understanding the evolutionary origins of the germ layers and their molecular regulation has been one of the fundamental questions in developmental biology (Technau and Scholz, 2003).

Since Christian Pander first characterised the germ layers (1817), scientists have continued to define the three germ layers throughout the 19th century (Gilbert, 2006). Haeckel, as a prominent early supporter of Darwin's evolutionary theory, devoted himself to understanding the evolutionary features of developmental processes. Haeckel incorporated the phylogenetic relationships within the animal kingdom and embryonic comparisons of different phyla to generate his fundamental biogenetic law; a developing organism goes through morphological and physiological steps that characterize its ancestors. For over a century, Haeckel's Gastraea theory remained a dominant theory to explain the origin of multicellular animals (Beetschen, 2001).

At the beginning of the 20th century, the amphibian gastrula became a prevailing model for experimental embryologists. These studies led to three key milestones in understanding amphibian gastrulation.

1. Goette first described the existence of the marginal zone in *Bombinator igneus*. Goette also first described the existence of a continuous mesoblastic layer, formed between the ectoblast and hypoblast (Beetschen, 2001).
2. Vogt completed the first comprehensive drawings of the events of amphibian gastrulation, confirming that the mesoderm originates from the marginal zone in an amphibian embryo (Beetschen, 2001).
3. Spemann and Mangold demonstrated that the blastopore lip acted as an organizer, a population of cells that lead to convergent extension, the formation of the notochord, and thus the body axis. The insights of the Spemann-Mangold organizer generate a key concept of primary induction in vertebrate development (Sander and Faessler, 2001).

Germ layers are distinct cell layers that form early during embryonic development, giving rise to all the tissues of the adult. Pieter Nieuwkoop made numerous contributions in the second half of the 20th century, especially in the areas of neural, meso-endoderm, and germ cell induction in chordates (Sander and Faessler, 2001). Prior to this, it was not anticipated that mesoderm would form by induction. In 1969, the now classic experiments by Nieuwkoop revealed that mesoderm can be induced; animal hemisphere explants were induced to form mesoderm by recombining with vegetal hemisphere cells (Nieuwkoop.P.D., 1973; Nieuwkoop.P.D., 1969b; Nieuwkoop.P.D., 1969a).

In 1974, Nieuwkoop made another major contribution, the discovery and analysis of germ cell induction in urodeles. He found that urodele germ cells

are formed by ventral marginal zone cells exposed to an inducer located in ventral meso-endoderm (Sudarwati and Nieuwkoop, 1974). Conversely, in anurans germ cells arise from a cell lineage containing germ plasm (Wylie, 1999). There is no germ cell induction in anurans; therefore, Nieuwkoop's observation came as a surprise to *Xenopus* and *Rana* researchers. Based on this basic difference of urodeles and anurans, Nieuwkoop concluded that amphibia are di-phyletic (Gerhart, 1999).

Vertebrate mesoderm induction and germ cell formation are classical problems in developmental biology and embryos from *Xenopus* and zebrafish have been used to identify key factors involved in these processes. However, key differences in development exist between urodeles and anurans and understanding these might provide us insights into the regulatory mechanisms defining meso-endoderm and germ cell induction. Ultimately, these differences may allow us to probe the evolutionary origins of these inductions.

1.1 Germ layer formation

Gastrulation is a crucial step in early embryogenesis during which morphogenetic movements result in the establishment of the basic body plan and the formation of the primary germ layers. Although understanding the molecular mechanisms controlling complex cell movements and inductive processes remains a challenge, substantial progress has been made to identify and characterize the pathways and molecules implicated in germ layer specification and morphogenesis during vertebrate gastrulation.

The germ layers are the primary tissue layers in an animal. In response to various signals during gastrulation cells develop into one of the three germ layers: (1) the ectoderm or outer skin, (2) the endoderm or inner skin, and (3) the mesoderm or middle skin (Gilbert, 2006). However, reports from *nematodes* and zebrafish indicated that endoderm and mesoderm may derive from a common progenitor, called the mesendoderm (Maduro et al., 2001; Warga and Nusslein-Volhard, 1999).

1.1.1 Ectoderm

The ectoderm is the outermost of the three primary germ layers. It differentiates to give rise to the outer layer of the skin (including the sweat glands, hair, and nails), the teeth, the lens of the eye, parts of the inner ear, the nerves, brain, and spinal cord. In 1924, Spemann and Mangold's grafting experiments demonstrated that prospective ectodermal cells, located on top of the embryo, choose between two fates: epidermal and neural. Organizer signals pattern neural tissues in the dorsal ectoderm. In the absence of these signals, ventral ectoderm differentiates into epidermis (Spemann and Mangold, 1924). The default model for neural induction states that vertebrate ectodermal cells will become nerve cells in the absence of inducing signals. Therefore, in normal intact ectodermal explants (animal caps), BMP4 induces and maintains the epidermal fate; however, inhibiting BMP signalling by BMP antagonists including noggin, follistatin, and chordin, leads to the formation of neural tissue (Hemmati-Brivanlou and Melton, 1997).

1.1.2 Mesoderm

The mesoderm, or middle layer, gives rise to the heart, somites, blood, muscles, skeleton, and other supportive and connective tissues (Gilbert, 2006). Nieuwkoop's experiments on meso-endoderm induction is the basis of the assay used to identify the morphogens involved in mesoderm induction and specification (Nieuwkoop.P.D., 1969b; Nieuwkoop.P.D., 1969a). The ease of this assay has meant that the majority of work on mesoderm induction has been carried out in amphibians. In *Xenopus,* the signals that initiate mesoderm induction emanate from the vegetal pole which contains the future endoderm. In particular VegT and Wnt/β-catenin signals (see Chapter 1.2) activate the transcription of the *Xenopus* nodal related (Xnr) genes, which then initiate mesoderm formation (Figure 1.1A) (Kimelman, 2006) .

In addition to establishing the mesoderm, the dorsal-ventral axis is established along with embryonic patterning prior to gastrulation (Figure 1.1B and C). Recent studies have revealed that the maternal endo-mesodermal determinants VegT and Wnt11 are required to trigger dorsal-ventral axis formation following cortical rotation in *Xenopus* embryos. Moreover, maternal VegT and dorsally stabilized β-catenin act synergistically to create an asymmetry in Nodal expression with higher activity in the dorsal side and lower in the ventral side of the pre-gastrula embryo (Figure 1.1C) (Agius et al., 2000; Katsumoto et al., 2004; Tao et al., 2005).

At the end of the blastula stage, gastrulation begins in the marginal zone, the zone surrounding the equator of the blastula where the animal and vegetal hemispheres meet. In *Xenopus* embryos, the mesoderm is induced in the

marginal zone overlying the vegetal pole. During gastrulation, the marginal cells migrate and reach the dorsal lip then turn inward and travel along the inner surface of the outer animal hemisphere cells (Figure 1.1D).

Fate mapping experiments initiated in the first half of the 20th century identified the location of the mesoderm precursors both prior to and during gastrulation (Beetschen, 2001). Key genes involved in the regulation of mesoderm differentiation such as *Bra*, *Gsc*, *FGF8* and *MyoD* have distinct spatial expression domains. *Bra* is expressed in the marginal zone of the embryo in the late blastula stage. During gastrulation, *Bra* expression iis retained in the margin, but is slightly weaker in itsthe dorsal aspect.marginal zone and sSubsequently, expression is foundmaintained in the entire marginal zone and the prospective notochord. By the tailbud stage, *Bra* is only expressed in the tailbud and notochord (Lerchner et al., 2000). *Gsc* is first expressed at the mid-blastula transition in the organizer (Artinger et al., 1997). In the early gastrula *Gsc* is expressed just above the dorsal lip and appears to overlap with *Bra* expression in the dorsal region. By mid-gastrulation the *Gsc* and *Bra* expression domains are separated into two distinct regions with *Gsc* expression localized to the region of the presumptive prechordal plate mesoderm and *Bra* expressed in the marginal zone and the prospective notochord. *FGF8* mRNA is detectable by RT-PCR at late blastula stages just prior to gastrulation (Fletcher et al., 2006). In situ hybridization to *FGF8* confirmed that the expression of *FGF8* is first seen at early gastrula stage 10 in a narrow ring around the future blastopore and then is restricted dorsally as gastrulation proceeds. By the late gastrula, *FGF8* expression remains in the posterior dorsal mesoderm, and as

neuralization proceeds the expression domains become visible in the anterior; the epidermal crescent of the neural plate territory, in the future midbrain/hindbrain boundary, and anterior neural ridge. Taken together, *FGF8* is a good candidate for mesoderm specification as *FGF8* is expressed in the presumptive mesoderm by gastrulation and in the posterior dorsal mesoderm during early neural development. *MyoD* mRNA is weakly detected at the time of mid-blastula transition (Harvey, 1991). By early gastrulation, the expression of *MyoD* is localised to the lateral marginal zone and ventral mesoderm; however, the expression is excluded in the dorsal region above the blastopore. As gastrulation proceeds, *MyoD* expression becomes more intense in the presumptive mesoderm and in particular the presumptive somites (Frank and Harland, 1991).

1.1.3 Endoderm

The endoderm is the innermost germ layer that gives rise to the epithelia of the digestive and respiratory systems and organs such as the liver, pancreas, lung, gallbladder and thyroid. The timing of endodermal cell fate determination varies relative to gastrulation among diverse species. In *Xenopus*, cells in the vegetal blastomere are already determined to become endoderm by the beginning of gastrulation (Heasman et al., 1985). However, the mesoderm and endoderm cell fates do overlap partially at the 32 cell blastomere stage and fate determination is not completed (Fukuda and Kikuchi, 2005). In *Xenopus*, endoderm fate is segregated before gastrulation; by the mid-blastula stage (stage 8) cells from the vegetal blastomeres become smaller and more confined and then contribute specifically to the endoderm (Grapin-Botton and Constam, 2007). Basal chordates only have

one type of endoderm, which gives rise directly to the lining of the gut; however vertebrates have two types of endoderm, the supra-blastoporal endoderm and sub-blastoporal endoderm. The supra-blastoporal endoderm is thought to be homologous to the definitive endoderm of amniotes and will give rise directly to the gut. The sub-blastoporal endoderm that originates below the blastopore will eventually contribute to the lining of the gut much later in development (Shook and Keller, 2008b). Similar in *Xenopus*, at early gastrulation a thin superficial layer of endoderm precursor extends upward from the blastopore and covers the dorsal and lateral plate mesoderm. Eventually, these superficial cells form as a continuous layer and contribute to the lining of the archenteron. However, cells from the deep layer don't form the archenteron roof but will become intestinal endoderm in tadpoles after the elongation of the gut (Grapin-Botton and Constam, 2007).

The *Xenopus* endoderm originates from the vegetal region where the maternal transcript *VegT* is localized (Xanthos et al., 2001). Sox17, Mix-like homeodomain factors and the nodal-related factors function downstream of VegT and are thought to be involved in endoderm induction (see Chapter 1.2). The expression patterns and ability to induce endoderm suggest that *Sox17* and *Mix-like* genes such as *Mix.1* and *Mixer* are good endodermal markers (Zorn and Wells, 2007). *Sox17* transcripts are first detectable in the late blastula and *Sox17* is expressed at most stages from 9 to 35. By early gastrulation *Sox17* expression is visible in the superficial ring around the blastopore and throughout the vegetal region. However, there is no *Sox17* expression in the presumptive mesoderm where *Bra* is expressed. As grastrulation proceeds *Sox17* expression remains in the endoderm. In the

tailbud embryo, *Sox17* is expressed throughout the entire endoderm and in particular the dorsal wall of the gut. By stage 35, *Sox17* mRNAs become restricted to the posterior endoderm (Hudson et al., 1997). Similar to *Sox17*, *Mix.1* transcripts appear at the mid-blastula stage, however, *Mixer* expression only can be detected during the gastrula stage from 10.25 to 13. Both *Mix.1* and *Mixer* transcripts disappear at the end of gastrulation (stage 13). *In-situ* hybridisation shows that *Mix.1* mRNAs is expressed throughout the vegetal hemisphere and largely overlaps with *Bra* expression in the marginal zone mesoderm. As gastrulation proceeds, the exclusion of *Mix.1* and *Bra* expressions has become more complete (Lemaire et al., 1998). Gastrula embryos hybridised with a *Mixer* probe shows Mixer expression is more specific in the prospective endoderm and strongest at the mesendodermal boundary with no overlap with *Bra* expression (Henry and Melton, 1998). Taken together, the expression patterns for *Sox17*, *Mix.1* and *Mixer* and combined evidence from *Xenopus*, zebrafish and mouse highlight their roles in presumptive endoderm formation (Grapin-Botton and Constam, 2007) (see Chapter 1.2).

Figure 1.1 - Blastula/Gastrula stage of *Xenopus* embryo

The position of mesoderm and endoderm precursor before and during gastrulation in *Xenopus* embryo.

(A) Activation of Nodal singling in *Xenopus* - The maternal vegetally localized transcription factor VegT (blue) activates the transcription of the *Xenopus* nodal-related genes (Xnrs) in the vegetal hemisphere, which then initiate mesoderm formation. **(B)** Fate maps of *Xenopus* embryos at the late blastula/early gastrula stage - Endoderm precursors are in green, mesoderm red and ectoderm yellow. **(C)** The establishment of dorsal-ventral axis – A dorsalising activity (ex: Wnt11) moves from the vegetal pole to one side of the embryo after fertilization after fertilization. At pre-blastula stages this dorsalising activity stabilizes β-catenin on what will be the future dorsal side of the embryo. When zygotic transcription of the Xenopus Nodal genes begins, VegT and β-catenin cooperate to create an asymmetry in Nodal expression, which results in elevated phosphorylated Smad2 levels on the dorsal side of the pre-gastrula embryo. **(D)** In *Xenopus* embryos the endoderm progenitors are derived from the vegetal-most blastomeres and have also been idenitifed from dorsal marginal blastomeres. Moreover, vegetal blastomeres contribute specifically to endoderm, whereas dorsal marginal blastomeres contribute to dorsal mesoderm, including the notochord and somites. Figures are adapted from (Grapin-Botton and Constam, 2007; Kimelman, 2006).

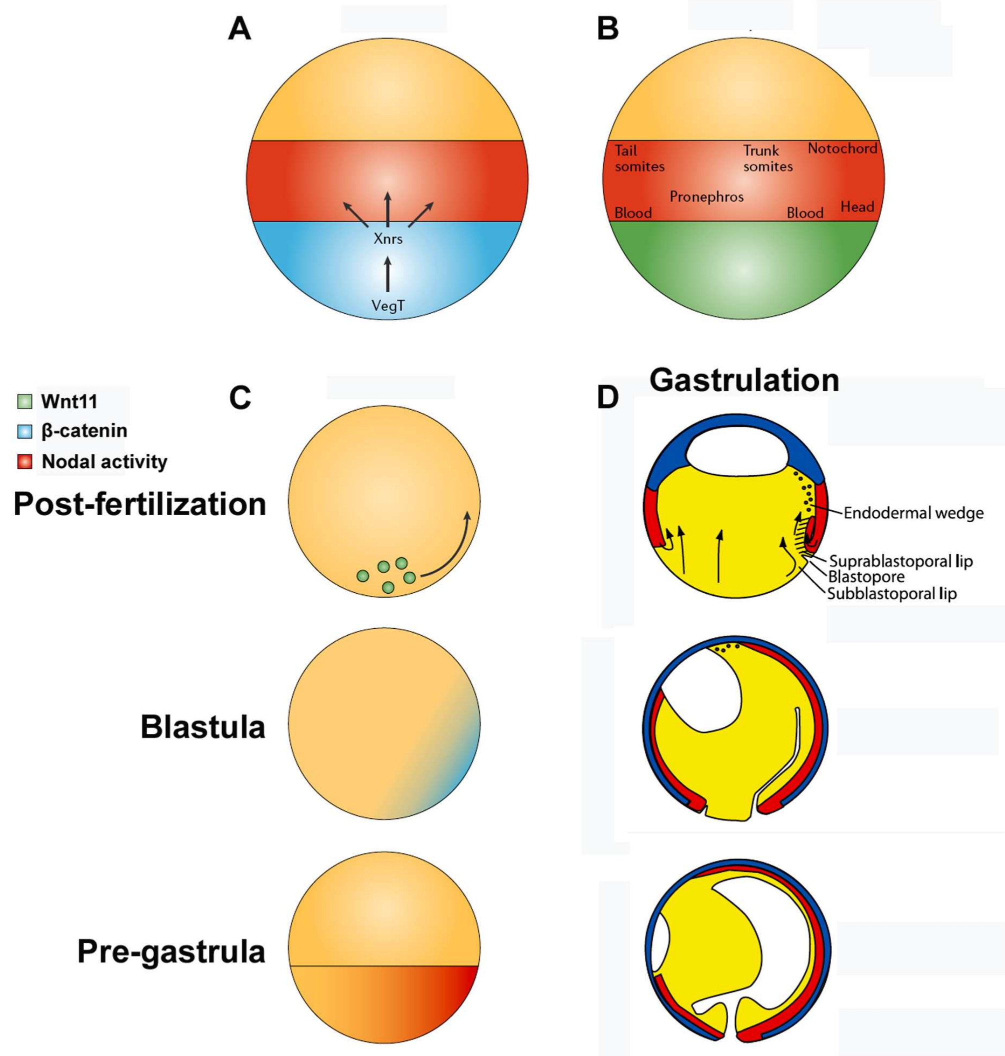


Figure 1.1 - Blastula/Gastrula stage of *Xenopus* embryo

1.1.4 Mesendoderm

In the traditional view of germ layer formation, the three primary germ layers, ectoderm, mesoderm, and endoderm, are clearly distinct and formed early in development. However, there is evidence that a subset of the endoderm and mesoderm share a common progenitor, known as the mesendoderm. For example, reports from *C.elegans*, sea urchin and zebrafish indicate that the endoderm and some of the mesoderm may derive from such a bipotential layer of cells (Rodaway and Patient, 2001). In *Xenopus*, the data indicates that cells of the marginal region contain the precursor cells for future mesoderm and endoderm (Nieuwkoop, 1997). The transcription factors Mix.1 and Brachyury are immediate early markers for endoderm and mesoderm, and are expressed in distinct groups of cells during gastrulation. However, at the start of gastrulation these genes are expressed in the same cells (Lemaire et al., 1998). Thus, the marginal cells and cells which co-express *Mix.1* and *Brachyury* may represent a bi-potent mesendodermal population.

Taken together, the evidence suggests the mesendoderm may represent an ancient germ layer specified by conserved signals. It may give rise to all the mesoderm and endoderm during gastrulation or, more likely, the mesendoderm population may only exist for a limited time and in particular locations in the early gastrula embryo (Rodaway and Patient, 2001).

1.2 Molecular control of the *Xenopus* mesendoderm formation

1.2.1 VegT

VegT is a T-box transcription factor first cloned in *Xenopus laevis* (Zhang and King, 1996). *VegT* orthologs have been found in several other anuran amphibians (Beckham et al., 2003; Nath et al., 2005; Zhang and King, 1996) and the urodele amphibian, the Mexican axolotl (Nath and Elinson, 2007). The *VegT* transcript in *Xenopus* is supplied maternally in the oocyte and is also expressed zygotically within the equatorial zone. *Xenopus VegT* maternal mRNA is localised to the vegetal cortex of the mature oocytes and early embryos (Lustig et al., 1996; Nath and Elinson, 2007; Stennard et al., 1996; Zhang and King, 1996). The anchoring of *VegT* mRNA to the vegetal cortex is required for the correct vegetal localisation of other maternal factors, such as *Vg1* and *Wnt11* as shown by the *in-situ* hybridisation to these mRNAs with undetectable levels at vegetal cortex (Heasman et al., 2001). In addition, VegT is the key mesendodermal determinant responsible for controlling induction of the mesoderm and endoderm. VegT-depletion, carried out by injection of antisense oligonucleotides into *Xenopus* oocytes, results in a failure to form endoderm (as judged by the expression of the *Mix-like* genes, *GATA* factors and *Sox17*) and in a significantly reduced ability to induce mesoderm (as judged by the expression of *Bra*, *MyoD*, *Gsc* and *Wnt8*) from the vegetal mass (Kofron et al., 1999; Xanthos et al., 2001; Zhang et al., 1998).

Maternal VegT has several functions in *Xenopus* development including the

formation of the organizer and the induction of endoderm and mesoderm. After the MBT, vegetaly localised VegT activates the expression of many zygotic genes important for mesendoderm induction within the vegetal hemisphere. Many of its targets are transcription factors which themselves regulate endoderm formation; the expression level of these genes, such as *XlSox17* and the *Mix* and *Gata* gene families, are reduced in *VegT*-depleted embryos (Xanthos et al., 2001). For example, *XlMixer* and *XlGata5* have been shown to be downstream of VegT and TGF-β signalling (discussed below) in separate pathways.

VegT activates mesoderm induction by inducing the expression of TGF-β signalling molecules. Mesoderm is induced at the blastula stage in *Xenopus* in response to a dorsal-ventral gradient composed of multiple Nodal-related genes, including *Xnr1*, *Xnr2*, *Xnr4* and *derriere* expressed in the endoderm alongside *VegT* (Agius et al., 2000; Kofron et al., 1999). *Xnr1*, *Xnr2*, *Xnr4*, or *derriere* mRNA injected into the vegetal masses of *VegT*-depleted embryos can rescue mesoderm formation (Kofron et al., 1999).

VegT is also involved in the establishment of the Spemann organizer in the late blastula. For example, *BMP* and the *Nodal* antagonists expressed in the organizer, such as *chordin*, *cerberus*, *noggin*, and *crescent,* absolutely require both VegT and β-catenin pathways for their expression (Xanthos et al., 2002). Moreover, evidence indicates that VegT acts in synergy with β-catenin to activate these genes before patterning the trunk and inducing head formation in *Xenopus* embryos (Agius et al., 2000; Xanthos et al., 2002).

1.2.2 β-catenin

The use of Wnt ligands for signalling between cells is a conserved feature of metazoan development (Cadigan and Nusse, 1997; Hobmayer et al., 2000; Peifer and Polakis, 2000; Wodarz and Nusse, 1998). Work in *Drosophila* and in other vertebrates have shown that Wnt signals are transduced in at least two distinct ways; a well-established canonical or Wnt/β-catenin pathway, and a non-canonical pathway/s that are β-catenin independent. The canonical Wnt/β-catenin signalling pathway (Figure 1.3) is involved in the regulation of various developmental events, including cell proliferation, migration, polarity, differentiation and axon outgrowth (Eisenmann, 2005). In the early stages of embryogenesis of many organisms, Wnt/β-catenin signalling plays a critical role in establishing the basic body plan. For example, in lower vertebrates like fish and frogs, Wnt/β-catenin signalling is essential for the establishment of the dorsal–ventral (D–V) body axis (Kelly et al., 2000; Moon and Kimelman, 1998). The overexpression of β-catenin in *Xenopus* or zebrafish results in the ectopic formation of a dorsal organizer and a secondary axis (Kelly et al., 2000; Molenaar et al., 1996). Furthermore, inhibition of the maternal canonical Wnt/β-catenin pathway by the overexpression of mutant Tcf3, cadherins or the dominant repressor form of β-catenin, or by the depletion of maternal β-catenin with antisense oligo leads to defects in dorsal axis formation and a reduction in dorsal-specific gene expression in *Xenopus* and zebrafish embryos (Heasman et al., 1994; Montross et al., 2000; Pelegri and Maischein, 1998).

As already discussed, in *Xenopus* the localisation of maternal factors in the vegetal cytoplasm is directly required for endoderm specification and

mesoderm induction (Agius et al., 2000; Zhang et al., 1998). Although vegetally localised maternal *Wnt11* is crucial for the activation of maternal Wnt/β-catenin signalling (Tao et al., 2005), overexpression of Wnt/β-catenin alone does not induce mesoderm or endoderm in *Xenopus* animal caps (Carnac et al., 1996; Sokol, 1993). Rather, dorsal stabilized β-catenin, together with Tcf family members, activates various signalling molecules and transcription factors in the dorsal marginal zone. Thus the key role of Wnt/β-catenin signalling in the pre-gastrula *Xenopus* embryo is to dorsalize the mesoderm and endoderm (Marikawa, 2006).

Whilst activation of the Wnt pathway before the MBT promotes dorsal fates, activation after the MBT leads to the different effect, that is, the ventralization of the dorsal mesoderm (Christian and Moon, 1993). The zygotic Wnt gene, *Wnt8*, is expressed in ventrolateral regions of both *Xenopus* (Christian and Moon, 1993) and zebrafish (Kelly et al., 1995). After the establishment of the dorsal-ventral axis, Wnt/β-catenin activity stimulated by zygotic Wnt8 is required for ventrolateral fates, muscle induction and for repression of dorsal specific genes. Zebrafish wnt8 mutants or *Xenopus* embryos expressing a dominant-negative Xwnt8 have enlarged organizers and lose posterior and ventral tissues (Hoppler et al., 1996; Lekven et al., 2001).

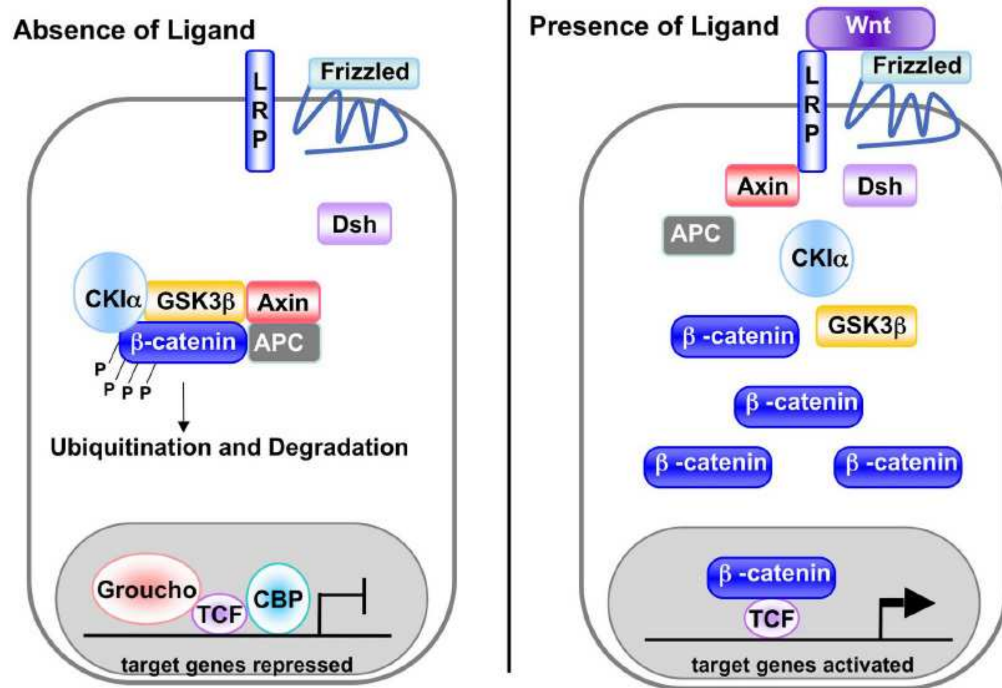


Figure 1.2 - A canonical Wnt/β-catenin signalling pathway

A scheme illustrating canonical Wnt/β-catenin signalling (Eisenmann, 2005) Left, in the absence of Wnt ligand, a complex of Axin, APC, GSK3-β, CK1α and β-catenin located in the cytosol leads to the degradation of β-catenin by phosphorylation and subsequent ubiquitination. Right, with Wnt ligand stimulation, signalling through the Frizzled receptor and LRP5/6 co-receptor complex induces the dual phosphorylation of LRP6 by CK1α and GSK3-β and this allows for the translocation of a protein complex containing Axin from the cytosol to the plasma membrane. Dsh is also recruited to the membrane and binds to Frizzled and Axin binds to phosphorylated LRP5/6. This complex of Frizzled/LRP5/6/Axin/Dsh formed at the membrane induces the stabilization of β-catenin via either sequestration and degradation of Axin leading to the stabilization of β-catenin. Thus β-catenin translocates into the nucleus where it complexes with TCF/LEF family members to mediate transcriptional induction of target genes.

1.2.3 The TGF-β family

The TGF-β family, a large group of over 30 extra-cellular growth factors, is vital for the development and homeostasis of metazoans (Feng and Derynck, 2005). The members consist of many different proteins including TGF-βs, BMP (bone morphogenetic proteins), GDFs (growth differentiation factor), activins, inhibins, MIS (Mullerian inhibiting substance), Nodals and leftys (Dube et al., 1998; Hogan, 1996b; Kingsley, 1994). The ligands and their downstream pathway components are extremely well conserved, controlling many aspects of development including proliferation, adhesion, migration, apoptosis and differentiation (Attisano and Wrana, 2002; Hogan, 1996a; Kingsley, 1994; Massague, 1998). TGF-β family members have to be cleaved to form active ligands. Initially they are translated as prepropeptide precursors with an N-terminal signal peptide, prodomain and the mature domain. The mature ligands are cleaved from the prodomain by furin-like enzymes (Feng and Derynck, 2005). In the mature domain, there are between six and nine conserved cysteine residues which form intra or intermolecular disulfide bonds. For example, Nodal has seven such cysteine residues (Hogan, 1996b; Massague, 1998; Padgett et al., 1997). For the most part, the ligands homodimerize, although heterodimerization also occurs between Nodal and BMP4 or BMP7 (Yeo and Whitman, 2001). In order to activate an intracellular signalling cascade, TGF-β family mature ligands require type I and type II receptors (transmembrane serine and threonine kinase) (Souchelnytskyi et al., 1996; Wrana et al., 1994). However, BMPs and activin/TGF-β typically use different signal transduction pathways. BMPs bind both BMP type I and type II receptors to transmit their signals (Liu et al., 1995; Nishitoh et al., 1996; Nohno et al., 1995). For TGF-β and activins, the

mature ligands must first bind type II receptors and then recruit the type I receptors. After an active ligand-type I/type II complex is formed, the type II receptor will activate type I receptors through phosphorylation, and subsequently the type I receptors phosphorylate downstream SMAD proteins that propagate the signal from the cytoplasm to the nucleus (Heldin et al., 1997; Massague, 1998; Wrana and Pawson, 1997). The original view of TGF-β superfamily signalling pathways suggested there were two distinct pathways; either BMPs or TGF-β/activins, which each phosphorylate different SMADs to transmit their signal responses. For example, BMPs stimulate the phosphorylation of SMAD1/5/8, while TGF-β/activins phosphorylate SMAD2/3 to trigger a signal cascade (Hata et al., 1997; Kretzschmar et al., 1997; Suzuki et al., 1997; Zhang et al., 1996). However, TGF-β also activates both Smad2/3 and Smad1/5/8 in a variety of endothelial, epithelial, fibroblast, and tumor cells (Bharathy et al., 2008; Daly et al., 2008; Liu et al., 2009). Multiple ligands, receptors, binding proteins, and downstream proteins participate in TGF-β super-family signalling cascades and have diverse functions in developmental and physiological regulation. These include early embryonic and extraembryonic development, left-right asymmetry, heart development, vasculogenesis and angiogenesis, skeletal morphogenesis, craniofacial development, body composition and growth, and nervous system development amongst others (Chang et al., 2002; Wu and Hill, 2009). Of particular interest, members of the TGF-β family, particularly those of the activin/Nodal family, are implicated as inducers of the mesoderm and endoderm in early development (Piepenburg et al., 2004; Schier, 2003).

1.2.4 Nodal

The *Nodal* gene was first identified in genetic studies in the mouse. It was named after its expression in the mouse gastrula embryonic organizer, the node (Conlon et al., 1994; Zhou et al., 1993). There are at least seven Nodal-related ligands (Xnr1, Xnr2, Xnr3, Xnr4, Xnr5, Xnr6 and Derriere) in *Xenopus laevis* though numerous tandem duplications of *Xnr5* have been reported (Takahashi et al., 2006). Three *nodal* genes have been reported in zebrafish (*Cyclops, Squint* and *Southpaw*) and this has been shown to be generally true for all teleost fish (Fan and Dougan, 2007).

The Nodal signal transduction pathway is relatively simple, yet is controlled precisely at multiple different levels (Figure 1.4) (Shen, 2007). Nodal ligands are translated as pre-proproteins, usually assembling into dimers when secreted. The subtilisin/kexin family of proprotein convertases process *Nodal* pre-proproteins into active ligands. Like other TGF-β family members, Nodal ligands bind to type I (Alk4 or Alk7) and type II (ActRIIA or ActRIIB) serine-threonine kinase receptors. But unique to the Nodal pathway are co-receptors of the EGF-CFC family, which are small cysteine-rich extracellular proteins attached to the plasma membrane through a glycosyl-phosphatidylinositol (GPI) linkage and are essential for Nodal signalling (Shen and Schier, 2000). On ligand binding, the activated type I receptor phosphorylates the cytoplasmic proteins Smad2 and/or Smad3, leading to their interaction with Smad4. Smad2 and Smad3 have different abilities to regulate target gene transcription (Piek et al., 2001; Yang et al., 2003). The activated Smad complex rapidly translocates to the nucleus where it interacts with other transcription factors to regulate specific gene

expression. The two best characterized transcription factors mediating Nodal signalling are the winged-helix transcription factor FoxH1 and the Mixer subclass of homeodomain proteins (Germain et al., 2000; Whitman, 2001). Moreover genetic analyses in zebrafish demonstrate FoxH1 and Mixer do not account for all Nodal-mediated transcriptional events (Kunwar et al., 2003), additional transcription factors involved in Nodal responses are yet to be identified. Interestingly, recent studies have suggested that additional TGF-β ligands like *Xenopus* Vg1, and its mammalian counterparts Gdf1 and Gdf3 can utilize the core components of this pathway and generate Nodal-like responses *in vivo* and may also have a role in inducing mesoderm and endoderm formation (Andersson et al., 2006; Birsoy et al., 2006; Chen et al., 2006).

Nodal ligands have the properties of a morphogen: a signal that acts at long-range to elicit dose-dependent responses in a developmental field of responsive cells (Ashe and Briscoe, 2006). Previous studies in zebrafish demonstrate that the Nodal ligand, Squint, and its inhibitor, Lefty, can both function as long-range mesoderm regulatory signals in vivo, whereas a second Nodal ligand Cyclops does not (Chen and Schier, 2002; Chen and Schier, 2001). Consistent with this, long-range diffusion and travel of Nodal ligands have been seen in mouse and chick (Meno et al., 2001; Sakuma et al., 2002). In *Xenopus*, similar to the responses to activin signalling, a dose dependent response can be observed in Nodal-mediated mesoderm specification (Gurdon et al., 1994; Gurdon et al., 1999). The current model suggests that a stable Nodal signalling gradient across a developmental field is generated from a source of Nodal signals undergoing positive

auto-regulation and acting at long-range (Norris et al., 2002; Norris and Robertson, 1999). The expression of the Nodal inhibitor, Lefty, is itself induced by the Nodal pathway, and has a greater range than the Nodal ligands (Sakuma et al., 2002). Cells in close proximity to the Nodal source thereby perceive high levels of signalling activity, whereas more distant cells perceive little or no signalling activity, as lateral inhibition by Lefty will prevail over the longer range. Such a regulatory mechanism for Nodal pathway activity may function during mesendoderm specification and left-right patterning (Branford and Yost, 2004).

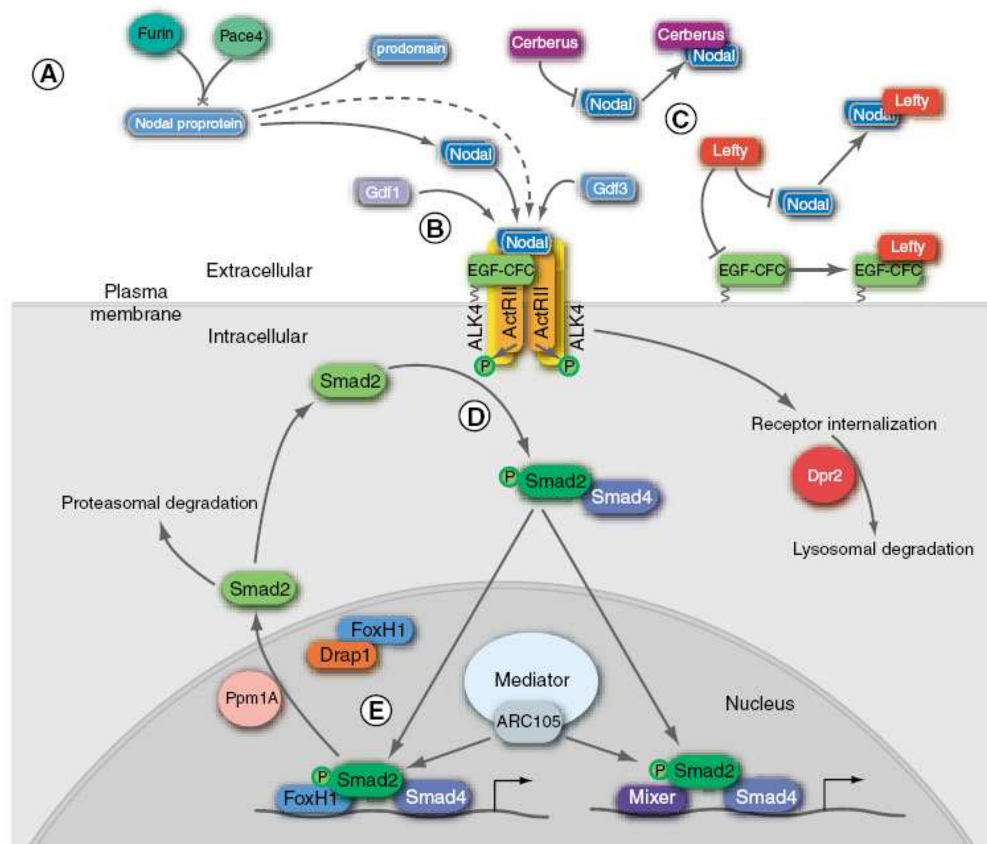


Figure 1.3 - Outline of the Nodal signalling pathway

A. Nodal ligands are usually expressed as homodimeric proproteins, and need to be cleaved by the proprotein convertases Furin and Pace4. **B.** Activated Nodal ligands, as well as Gdf1 and Gdf3, can bind to an EGF-CFC co-receptor and then form a complex with type I receptor (ALK4) and type II receptor (ActRII or ActRIIB) dimers. **C.** Two Nodal signalling antagonists, Cerberus and Lefty, can interact with Nodal ligands; in addition, Lefty proteins can also interact with EGF-CFC co-receptors to inhibit their function. **D.** Receptor activation leads to the phosphorylation of the type I receptor by the type II kinase, as well as phosphorylation of Smad2 (or Smad3). Activated Smad2 or Smad3 associates with Smad4 and translocates to the nucleus, whereas the receptor complex undergoes internalization into endosomes and can be targeted by Dpr2 for lysosomal degradation. **E.** The transcription complex enters into the nucleus; activated Smad2-Smad4 (or Smad3-Smad4) complexes interact with FoxH1 or Mixer on target promoters, and then stimulate transcriptional activation through interactions with ARC105 and the mediator complex. Pathway activity can be inhibited by interaction of Drap1 with FoxH1 or by the Smad phosphatase Ppm1A. Adapted from (Shen, 2007).

Four major roles for Nodal signalling have been described. (Table 1-1) First, the induction of the mesoderm and endoderm. Second, an indirect role in neural induction. Thirdly, the specification of left-right asymmetry and the position of the anterior-posterior axis of the embryo. Finally, Nodal signalling has a role in the maintenance of embryonic stem cell pluripotency (Shen, 2007). The role of Nodal singlling in mesoderm and endoderm formation will be mainly discussed below.

Mesoderm induction- Nodal is of significance not only because of the role it plays in pattern formation, but also its role in differentiation during gastrulation. Previous studies suggest that the Nodals are mesoderm and endoderm inducers in vertebrates such as *Xenopus laevis*, zebrafish and mouse (Shen, 2007). In *Xenopus* six of the seven *Xenopus* nodal-related genes (including *Derriere*) are able to induce mesoderm formation in animal caps (Jones et al., 1995; Joseph and Melton, 1997; Takahashi et al., 2000; White et al., 2002). *Xnr1, 2, 4* and *Derriere* mRNAs can rescue mesoderm formation in *Xenopus* embryos lacking maternal VegT (Kofron et al., 1999). Moreover, inhibition of Nodal activity by cleavage mutants causes defects in mesoderm and endoderm formation in *Xenopus* embryos (Onuma et al., 2002; Osada and Wright, 1999). The zebrafish nodal gene Squint, but not Cyclops, can act as a mesoderm inducer in *Xenopus* animal caps (Rebagliati et al., 1998), whereas overexpression of Squint and Cyclops have overlapping roles in mesendoderm induction in zebrafish embryos (Chen and Schier, 2001). Furthermore, the loss of both zebrafish *nodal* genes *Cyclops* and *Squint* leads to ablation of all trunk and head mesoderm as well as all endoderm (Feldman et al., 1998). Similarly, maternal-zygotic mutants for

the nodal signalling cofactor EGF-CFC gene *one-eyed pinhead (oep)* results in a phenotype resembling that of *Cyc;Sqt* mutants (Gritsman et al., 1999). In the mouse, the loss of Nodal function results in failure to establish the primitive streak, the absence of visceral endoderm and loss of mesoderm (Brennan et al., 2001; Conlon et al., 1994). It is now generally assumed that Nodals serve as mesendoderm inducers in all vertebrates (Schier, 2003).

Although Nodal signalling is essential for mesoderm formation; there appear to be species-specific differences in their interactions with the canonical Wnt signalling pathway. In *Xenopus*, vegetal-localised maternal VegT and Vg1 ligands cooperate with Wnt/β-catenin signalling to activate the transcription of zygotic Nodal related genes, leading to a dorsal-ventral graded Nodal signal that induces dose-dependent mesendoderm formation in the marginal zone (Agius et al., 2000; Kimelman, 2006). In zebrafish, RNase injection eliminating YSL RNAs indicates that ventrolateral Nodal signals and mesendoderm induction require unidentified signals from the YSL, whereas the dorsal Nodal signals and mesoderm induction are YSL-independent and induced by an dorsal stabilized β-catenin dependent signal (Chen and Kimelman, 2000). In *Xenopus* and zebrafish embryos, signals from the early vegetal cytoplasm and the asymmetrical stabilization of β-catenin are essential for the formation of all dorsal and anterior structures (Kimelman, 2006). In the mouse, the unprocessed Nodal from the epiblast can induce the expression of Furin and Pace4 proprotein convertases and BMP4 in the extraembryonic ectoderm. BMP4 signals back to the epiblast to activate Wnt3/β-catenin signalling, which upregulates Nodal and Cripto expression in the epiblast and establishes a Nodal feedback loop that results in the

formation of mesoderm and definitive endoderm (Ben-Haim et al., 2006).

Species	Genes	Genetic manipulations	Major phenotypes
Mouse	*Nodal*	mutation	no mesoderm, more embryonic ectodermal cells with reduced size
Zebrafish	*squint (sqt)*	mutation	cyclopia, defects in the prechordal plate and ventral nervous system
	cyclops (cyc)	mutation	cyclopia, no floor plate, reduced neuron number in ventral midbrain, axonal disturbance in brain and spinal cord; left-right axis defects (only in cycb229 mutants)
	sqt, cyc	double mutation	lack of endoderm and mesoderm, cyclopia, no anterior trunk spinal cord
	southpaw (spaw)	knockdown	abnormal positioning of heart and pancreas, randomized gut looping
Xenopus	*Xnr1*	overexpression	induce mesoderm and endoderm; cause laterality defects
	Xnr2	overexpression	induce mesoderm and endoderm
	Xnr3	overexpression	induce neural tissues
	Xnr4	overexpression	induce mesoderm and endoderm
	Xnr5	overexpression	induce mesoderm and endoderm
	Xnr6	overexpression	induce mesoderm and endoderm
Chick	*cNR-1*	ectopic expression	randomization of heart laterality

Table 1-1 - Phenotypes resulting from mutation/knockdown or misexpression of Nodal members from zebrafish to mouse Adapted from (Tian and Meng, 2006)

Endoderm formation- In *Xenopus* and zebrafish, overexpression of lefty/antivin, an antagonist of Nodal, can cause a complete loss of mesendoderm as measured by the expression of mesoderm and endodermal marker genes (Cheng et al., 2000; Thisse and Thisse, 1999). Misexpression of the Nodals in presumptive *Xenopus* ectoderm can induce cells to become mesoderm or endoderm. Overexpression of Xnr2, Xn5 and Xn6 have the ability to induce endodermal markers such as Mixer, Sox17 and endodermin in animal cap ectoderm (Osada and Wright, 1999; Takahashi et al., 2000). In addition, *Xnr1, 2, 4* and *Derriere* mRNAs can rescue expression of endodermal gene markers such as the *Mix-like* family and *Sox17* in VegT-depleted *Xenopus* embryos (Xanthos et al., 2001). The distinction between mesoderm and endoderm represents a dose-dependent response to Nodal activity, with high doses inducing endoderm and lower doses inducing mesoderm (Agius et al., 2000). Studies in zebrafish suggest that Nodal proteins establish a morphogen gradient to pattern the marginal zone along the animal-vegetal axis, and endoderm specification apparently requires a higher level of Nodal singalling as judged by the expression of *axial/foxa2*, *Sox17* and *Gsc* (Dougan et al., 2003; Gritsman et al., 2000). Endoderm formation requires Nodal signalling, which could be mediated by Mixer homeoproteins and I discuss this further below.

1.2.5 Mix-like factors

Mix-like transcription factors play an important role in mesendoderm, endoderm and mesoderm specification. The founding member of the *Mix-like* family, *Mix.1*, was identified in *Xenopus* embryos as one of the first TGFβ-induced genes in an experiment to investigate mesoderm induction in

early vertebrate development (Rosa, 1989). Although Mix.1 behaves as an immediate early response to mesoderm inducing signals, it is prominently expressed in the prospective endoderm, suggesting a role in patterning the mesoderm and endoderm in *Xenopus* embryos (Rosa, 1989). Studies in *Xenopus* indicate that dimerization of Mix.1 homeodomain proteins is important in patterning the dorsal-ventral axis and ventral mesoderm in response to BMP4 signalling (Mead et al., 1996). However, *Mix.1* and *BMP4* do not have the same spatial expression pattern and Mix.1 overexpression represses both dorsal and ventral mesoderm markers. Moreover, inhibition of Mix.1 function with a more specific Mix.1 repressor (enRMix.1) (Lemaire et al., 1998) in which the Mix.1 protein is fused to the repressor domain of *Drosophila* Engrailed does not recapitulate the results in Mead et al., 1996. However, a severe reduction in endoderm formation was found. Therefore, experimental evidence from *Xenopus* suggests that vegetally expressed Mix.1 has a role in endoderm induction and suppresses mesoderm formation during gastrulation.

Subsequently a further 6 related transcription factors were identified in *Xenopus laevis* including Mix.2, Mixer/Mix.3, Bix/Mix.4, Bix2/Milk, Bix3 and Bix4, as well as the original Mix.1. In contrast, only one family member, *Mixl1*, has been identified in mammals (Henry and Melton, 1998; Rosa, 1989; Saka et al., 2000; Tada et al., 1998; Vize, 1996). Members of the Mix/Bix family of paired-like homeodomain transcription factors are transiently expressed in the blastula and gastrula vegetal cells with particularly high expression levels in the marginal region where the future mesoderm and endoderm will form (Ecochard et al., 1998; Henry and Melton, 1998; Lemaire et al., 1998; Mead

et al., 1998; Tada et al., 1998).

In *Xenopus* animal cap assays, overexpression of Xl.Bix1-4 and XlMixer, but not XlMix.1 can induce the endodermal markers such as *Xledd*, *XlSox17a* and *XIFABP* in naive ectoderm explants (Casey et al., 1999; Doherty et al., 2006; Ecochard et al., 1998; Henry and Melton, 1998; Tada et al., 1998; Trindade et al., 2003). In contrast, XlMix.1 must co-operate with other homeodomain proteins such as Siamois in order to induce endoderm (Lemaire et al., 1998; Mead et al., 1998). Although higher levels of Bix.1 and 4 can induce endoderm, low levels of Bix.1 and 4 are able to induce formation of ventral mesoderm in animal caps as judged by the marker genes such as *Vent1* (Casey et al., 1999; Tada et al., 1998). These observations are in accord with the finding that at the early gastrula stage *Bix.1* and *4* RNAs are more abundant in the prospective endoderm than in prospective mesoderm.

In addition, over-expression of *XlMix.1, XlBix1, XlBix2*, and *XlMixer* in the marginal region represses mesodermal gene expression, such as *XlBra* and *XlVent1* (Doherty et al., 2006; Ecochard et al., 1998; Lemaire et al., 1998; Tada et al., 1998). Even though XlMix.1 is able to repress mesodermal markers and induce the endoderm marker *Xledd* (Lemaire et al., 1998), experiments in which protein domains were swapped between XlMix.1 and XlMixer and the recombinant proteins were used to induce endoderm in animal caps indicated that XlMixer is the stronger endoderm inducer (Doherty et al., 2006). The homeodomain and last sixty-two amino acids within the carboxyl terminus (acid domain) of XlMixer are sufficient for induction of endoderm.

It should be note that *XlMixer* differs from other members in that it is expressed at later blastula stages and is activated by Activin/Nodal-like signalling but not maternal VegT (Xanthos et al., 2001).

The Mix family, especially XlMixer and XlMilk, but not XlMix.1, can mediate activin/TGF-β-induced signaling during prechordal plate formation. In this context, XlMixer interacts with activated Smad2/Smad4 via a conserved binding motif in XlMixer (SIM: Smad interacting motif) and forms a transcription complex (Germain et al., 2000; Randall et al., 2002) that induces gsc expression through the DE (distal element) of the *XlGsc* promoter region. Subsequently, Gsc downregulates *bra* expression thus promoting prechordal plate cell fate rather than mesodermal differentiation (Artinger et al., 1997). Loss-of-function experiments in which *XlMixer* was depeleted in a developing Xenopus embryo also suggested that XlMixer blocked mesoderm formation while promoting endoderm formation. qPCR (Kofron et al., 2004) analysis and microarrays (Sinner et al., 2006) performed on XlMixer-depleted embryos showed that they expressed higher levels of mesoderm-inducing signals and showed reduced expression of endoderm markers. Consistent with these findings, gain-of-function experiments performed in animal caps showed an increased formation of endoderm in the animal cap (Dickinson et al., 2006). Altogether these loss- and gain-of-function experiments suggested a role for Mixer in negatively regulating mesoderm genes while promoting endoderm gene expression.

Taken together; these results indicate that the *Mix-like* family play a role in mesendoderm induction and establishing the boundary between the future

endoderm and mesoderm in *Xenopus laevis*. Most of the key regulators of the Mix-like family are involved in mesoderm and endoderm specification and include members of TGFβ family, Activin, Nodal and BMP4 as well as the transcription factor VegT (Casey et al., 1999; Ecochard et al., 1998; Tada et al., 1998; Vize, 1996). However, multiple *Mix-like* genes with similar function and expression patterns suggest that there might be functional redundancy in this gene family (D'Souza et al., 2003; Poulain et al., 2006). Therefore, further loss of function analysis of other *Mix-like* genes and detailed studies of mesodermal and endodermal target genes will be important to resolve their particular function in the specification of endoderm and mesoderm.

Similar to *Xenopus laevis*, orthologs *Mix-like* genes have been found in *Xenopus tropicalis*; *XtMix*, *XtMixer*, *XtBix,* and in zebrafish; *Bon/Mixer*, *Mezzo*, *mtx1* and *mtx2* (D'Souza et al., 2003; Hirata et al., 2000; Kikuchi et al., 2000; Poulain and Lepage, 2002). The expression patterns of *Xenopus tropicalis Mix-like* genes are identical to their *laevis* counterparts (D'Souza et al., 2003). In zebrafish, the *Mix* orthologs are expressed in the prospective mesendoderm and the extraembryonic yolk-syncytial layer (YSL) (Hirata et al., 2000; Kikuchi et al., 2000; Poulain and Lepage, 2002). In zebrafish both *Bon/Mixer* and *Mezzo* expression require functional Nodal signals; however despite *mtx1* and *mtx2* being expressed in a domain that overlaps that of *mixer,* their expression is independent of Activin/Nodal signals. Moreover, the results indicate that the early expression of *mtx2* is dependent on Wnt signals and *mtx1* expression is not regulated by either Wnt or Nodal signals (Hirata et al., 2000). As immediate early targets of Nodal signalling, the function of *mezzo* is redundant with that of *Bon/Mixer,* indeed *mezzo* RNA

can partially rescue the *Bon/Mixer* mutant. However, morpholino knockdown results suggest that both act in parallel in the Nodal signalling pathway and are required for normal mesoderm and endoderm formation in zebrafish (Poulain and Lepage, 2002). Furthermore a novel molecular function of the *Mix* family was revealed by mtx2 morpholino knockdown which leads to disruption of epiboly movements (Bruce et al., 2005). This novel function of mtx2 is consistent with work in *Xenopus* demonstrating the importance of *Mix-like* genes in the control of gastrulation movements (Luu et al., 2008).

Previous studies suggest most members of the Mix family are transcriptional targets of Nodal signalling except for zebrafish *mtx1* and *mtx2* (Germain et al., 2000; Hart et al., 2005; Randall et al., 2002; Vize, 1996). Genetic studies reveal that the mouse *Mixl1* promoter is TGF-β responsive and this regulation requires the co-activator FoxH1 (Hart et al., 2005). Several Mix-like proteins physically interact with Smad proteins via SIM forming transcriptional complexes to regulate the transcription of other Nodal-dependent mesendoderm genes (Germain et al., 2000; Randall et al., 2002). However, this is not conserved across all Mix proteins, as aminote Mix orthologues do not have a SIM (Germain et al., 2000; Randall et al., 2002). The importance of the SIM in normal development has yet be determined; the SIM in *Xenopus laevis* Mixer has been shown to be necessary but not sufficient for endoderm formation (Doherty et al., 2006). Overall Nodal and Mix are closely linked with *Mix* genes acting as a transcriptional target and a downstream effector of Nodal signalling.

1.2.6 Brachyury

Brachyury, a T-box transcription factor, is one of the earliest candidate genes specifying the mesoderm (Herrmann et al., 1990). In the mouse embryo, *T/Brachyury* is expressed before gastrulation in the nascent posterior mesoderm of the primitive streak, and in the newly formed notochord (Herrmann, 1991). In *Xenopus*, *Brachyury* is expressed predominantly at the mid-blastula to neurula stages in the prospective mesoderm around the equator of the embryo. Expression is maintained in the developing notochord cells as they migrate anteriorly (Smith et al., 1991). During late gastrula and early neurula stages the expression of *Brachyury* continues in the prospective posterior and ventral mesoderm as a ring of cells around the closing blastopore (Smith et al., 1991).

In *Xenopus*, a dominant-negative construct (XlBra-EnR) inhibiting XlBra activity leads to incomplete gastrulation, a loss of posterior structures and impaired notochord differentiation (Conlon et al., 1996). Brachyury heterozygous mutant mice were first described by Dobrovolskaia-Zavadskaia in 1927 (Wilson et al., 1993). Heterozygous -/+ mutant mice are viable but have a truncated tail and notochord abnormality. Mice $T^{-/-}$ embryos die shortly after gastrulation and have severe mesoderm abnormalities, including a complete loss of the posterior mesoderm and defects in mesoderm migration (Showell et al., 2004; Wilson et al., 1993). Homozygous mutants of zebrafish *ntl,* the *Brachyury* ortholog, resemble mice $T^{-/-}$ embryos with defects in posterior somites and notochord development, but is not severe (Schulte-Merker et al., 1994). More recently, a second zebrafish *Brachyury* ortholog (*bra*) has been identified; however,

morpholino knockdown of the wild type bra does not cause any defect in somite and notochord formation in zebrafish embryos (Martin and Kimelman, 2008). Interestingly, a combined loss of the two paralogs, *ntl* and *bra*, fully recapitulates the phenotype of the mouse *T* mutant embryo (Martin and Kimelman, 2008).

Mis-expression of *XlBra* in *Xenopus* animal cap tissue diverts the prospective ectoderm into mesodermal lineages, in particular ventral mesoderm (Cunliffe and Smith, 1992). Therefore, both loss- and gain-of-function data suggest that in vertebrates Brachyury has a conserved dual role in the differentiation of posterior mesoderm and in the elongation of the posterior body axis. *Brachyury* orthologs have been found in all metazoans, including hydra and sea urchins (Bielen et al., 2007; Harada et al., 1995; Technau and Bode, 1999). In hydra, there are two *Brachyury* homologs; HyBra1 and 2, and HyBra1 is expressed predominantly in the endoderm and involved in head formation while HyBra2 is expressed in the ectoderm and involved in neural induction. However, HyBra is able to induce mesoderm in *Xenopus* animal caps as judged by animal cap elongation and the expression of mesodermal marker, muscle actin (Bielen et al., 2007; Technau and Bode, 1999). Microarray analysis identifies Brachyury's transcriptional targets; *kakapo*, *gesolin*, *APOBEC* and *OrCT* (Rast et al., 2002). Both Kakapo and gesolin play a role in modulating cell shape and motility; however, APOBEC and OrCT are expressed in the vegetal plate and then in the endodermal cells, involved in endoderm specification. The result illuminates the role that Brachyury plays in gastrulation and endoderm development in sea urchins. Comparative analysis of Brachyury suggests that the ancestral functions include

blastopore formation, mesoderm induction and axis elongation (Technau, 2001).

Molecular analysis in zebrafish and *Xenopus* reveals that *Brachyury* is an early immediate response gene to Activin, a member of the TGF-β superfamily (Smith et al., 1991) and maintained by a feedback loop with FGF (Casey et al., 1998; Latinkic et al., 1997; Schulte-Merker and Smith, 1995). Genetic studies in *Xenopus* have indicated that *VegT,* via TGF-β and Wnt signalling, induces the expression of *Brachyury* and consequently specifies the mesoderm (Vonica and Gumbiner, 2002). However, there is no *VegT* ortholog in mouse; rather Wnt3/β-catenin signalling is required for anterior-posterior axis and mesoderm formation, including the expression of *Brachyury* in normal developing mouse embryos (Lako et al., 2001; Morkel et al., 2003).

Brachyury has been shown to function at the molecular level as a classic transcriptional activator with the highly conserved T-domain at the N-terminal portion of the protein and a less conserved C-terminal activation domain (Showell et al., 2004; Technau, 2001). Brachyury binds through the T-box domain to a DNA consensus sequence regulating transcriptional levels of heterologous and downstream target genes in several different contexts (Conlon et al., 1996; Kispert et al., 1995; Kispert and Hermann, 1993). For example, in *Xenopus* Brachyury can repress *Goosecoid* expression by activating *XlVent2* through the N-terminal domain (Messenger et al., 2005). Other Brachyury target genes have been identified including the *Xenopus* *Bix1* and *Bix4* genes, *eFGF* and *Wnt11* (Casey et al., 1998; Isaacs et al., 1994;

Smith et al., 2000; Tada et al., 1998). Molecular analyses in *Xenopus* have suggested that some factors play important roles alongside Brachyury signalling in patterning mesoderm and endoderm. For example, XlMix.1 for endoderm specification and XlGsc for head mesoderm and anterior endoderm formation (Artinger et al., 1997; Latinkic and Smith, 1999; Lemaire et al., 1998). In addition to the negative regulation by XlMix.1 (Lemaire et al., 1998), evidence shows that XlMix.1 can act on XlBra indirectly, in part through activation of XlGsc*.* XlGsc acts as transcriptional repressor, directly repressing the transcription of *XlBra* in order to pattern head formation (Latinkic and Smith, 1999). Altogether, Brachyury acts as a mesodermally expressed transcription factor with putative roles in specifying the mesoderm from the mesendoderm during the early embryogenesis.

1.2.7 FGF signalling family

Fibroblast growth factors represent a large family of secreted molecules and induce their biological responses by binding to and activating FGFRs, a subfamily of cell surface receptor tyrosine kinases (RTKs). FGFs are involved in the regulation of many developmental processes including apoptosis, cell migration, chemotaxis, differentiation and proliferation. FGF receptors transduce signalling by three main pathways, the Ras/MAPK pathway, the PLCγ/$Ca2^{+}$ pathway and the PI3 kinase/Akt pathway (Bottcher and Niehrs, 2005). FGF signalling plays important roles in early developmental processes during the gastrulation of *Xenopus*, zebrafish, chicken, and mouse, including mesoderm formation and gastrulation movements themselves, neural induction and AP patterning, and endoderm formation (Bottcher and Niehrs, 2005; Fletcher et al., 2006).

In *Xenopus* and zebrafish, perturbation of FGF signalling by overexpression of a dominant negative FGFR strongly affects body axis formation (Amaya et al., 1993; Amaya et al., 1991; Griffin et al., 1995). In these embryos, phenotypic changes are observed mostly in posterior regions, and most trunk and tail mesoderm fail to form. In addition, FGFs control mesoderm specification and maintenance by regulation of the T box transcription factor, T/Brachyury (Amaya et al., 1993; Ciruna and Rossant, 2001; Griffin et al., 1995; Griffin et al., 1998; Smith et al., 1991). In *Xenopus*, multiple FGF ligands are involved in regulating mesoderm formation, including FGF8 and FGF4, which are necessary for mesoderm formation (Fisher et al., 2002; Fletcher et al., 2006; Isaacs et al., 1994; Isaacs et al., 2007), and bFGF (FGF2), the first identified mesoderm inducer (Kimelman and Kirschner, 1987; Slack et al., 1990).

In mouse, few *Fgfr1*$^{-}$*/*$^{-}$ cells contribute to mesoderm and endoderm cell lineages indicating Fgfr1 is not absolutely required for mesoderm and endoderm formation as judged by the expression of *Brachyury* and *GATA4* (Ciruna and Rossant, 2001; Deng et al., 1994; Esner et al., 2002). Similar to *Fgfr1* mutants, *Fgf8*$^{-}$*/*$^{-}$ embryos have severe gastrulation defects in mesoderm and endoderm migration and cell fate determination (Meyers et al., 1998; Sun et al., 1999). In addition, *Fgf4*$^{-}$*/*$^{-}$ embryos die shortly after implantation and fail to form detectable mesoderm and endoderm, a phenotype similar to the targeted disruption of *Fgfr2* (Feldman et al., 1995); therefore, FGF4 may be involved in mesoderm and endoderm formation. A further study supports the role of FGF4 in the specification of the primitive

endoderm in the mouse whereby recombinant FGF4 can induce the differentiation of endoderm in a concentration-dependent manner (Wells and Melton, 2000).

In *Xenopus*, studies in vegetal explants give conflicting results regarding the role of FGF signalling in endoderm (LaBonne and Whitman, 1997). Modulation of FGF signalling does not affect the expression of endodermal marker *Mixer* and *XlHbox8* in the vegetal mass (Kavka and Green, 2000), and FGF does not induce a number of endodermal genes in animal caps (Sasai et al., 1996). In summary, it is suggested that different FGFs may mediate mesoderm induction, but FGF signalling is not essential for endoderm formation; however, defined levels of FGF activity may be required for endodermal patterning.

1.2.8 GRNs for mesendoderm formation

Development is controlled by a variety of inter-cellular signalling pathways and intra-cellular gene regulation. The development of the specific body plan for each species is the outworking of regulatory gene interactions encoded in the genomic DNA (Davidson et al., 2003). Genes encoding transcription factors interpret DNA codes at specific times and places to determine cell fates throughout the whole animal. The DNA code consists of enhancers, silencers and insulators that serve as target sites for transcription factors (Levine and Davidson, 2005). Together, these interactions form genetic regulatory networks and these reveal a logic map governing cell specification and patterning in development. In addition to the *cis*-regulatory sequences, chromatin remodeling and modification further increase the complexity of a GRN. Therefore, the architecture of a GRN is not a simple linear or branching

pathway for explaining developmental process. Rather, gene regulatory states change over time to define the different fates of cells composing the various spatial elements of the system (Davidson et al., 2003; Levine and Tjian, 2003).

GRNs have been compiled for multicelluar organisms, such as the sea urchin, *Xenopus* and *Drosophila* embryos (Bonn and Furlong, 2008; Davidson et al., 2002; Loose and Patient, 2004; Sethi et al., 2009) GRNs representing early development can be generated through the use of appropriate genomic, genetic, and biochemical tools as demonstrated by networks for the specification of endomesoderm in sea urchin embryos (Davidson et al., 2002) and dorsal-ventral patterning in *Drosophila* embryos (Stathopoulos et al., 2002). More recently systematic informatic approaches have been utilized to develop integrated models of the GRNs underlying *Xenopus* (Koide et al., 2005; Loose and Patient, 2004) and zebrafish (Chan et al., 2009) development. The architecture of GRNs offer a systematic view of how development proceeds and the comparison of GRNs between different species may provide us with further insights into vertebrate development and evolution (Davidson and Erwin, 2006).

In recent years, gene regulatory networks controlling the induction of the mesendoderm and specification of endoderm and mesoderm in the sea urchin embryo have also been constructed (Davidson et al., 2002; Sethi et al., 2009). These GRNs provide a vast quantity of information about the genetic interactions that control biological processes. In addition they demonstrate how genomic components define functional connections between the various

regulatory genes that conduct the dynamic developmental program. Though developmental GRNs governing the formation of various tissues and organs are different among species, similar inductions and genetic regulatory links reveal conserved linkages used in many different networks. Such conserved components have been termed network kernels (Davidson and Erwin, 2006). It is suggested that these kernels, because of their developmental role and specific internal structure, are impervious to change during the types of change that lead ultimately to speciation (Davidson and Erwin, 2006). The underlying molecular events of mesoderm and endoderm specification can be explored by comparison of conserved GRN kernels, which underlie development of the major body plan.

The amphibian *Xenopus laevis* has long been used as a model to study vertebrate early development and has contributed greatly to the elucidation of gene regulation. In recent years, the gene regulatory network approach has also been applied to mesendoderm specification in *Xenopus laevis* embryos (Figure 1.2) (Koide et al., 2005; Loose and Patient, 2004). Although there are many differences between the *Xenopus* and the sea urchin GRNs, several key components of mesendoderm induction are conserved (Loose and Patient, 2004). For example, the essential role of maternal *β-catenin* and the observation that nuclearization of β-catenin is necessary to specify the future endomesoderm in both species. Indeed, recent study has indicated that in addition to its roles in axis specification and the establishment of left-right asymmetry (Duboc and Lepage, 2008), sea urchin Nodal is crucial for patterning of the endoderm and mesoderm (Duboc et al., 2010), like the *Xnrs* in *Xenopus.* Other genes such as the T-box gene *Brachyury*, the *GATA*

family and *Sox* factors also play important roles in both the sea urchin and *Xenopus* mesendoderm GRNs (Davidson et al., 2002; Levine and Davidson, 2005; Loose and Patient, 2004). In addition, repression of mesoderm fate by the winged helix Foxa family, a key factor for the endoderm specification, has been reported in sea urchin as well as *Xenopus* (Oliveri et al., 2006; Suri et al., 2004). However, many non-conserved components between sea urchin and *Xenopus,* such as the *Mix* family, are key for the specification of endoderm and mesoderm in *Xenopus*. For example, a *Mix* ortholog has not been identified to date in the sea urchin.

Defining the signalling pathways and transcriptional interactions that determine cell fate are primary goals of developmental biology. Information from experimental data and mathematical predictions have been compiled to build up much more intricate models of the GRN controlling vertebrate development (Chan et al., 2009; Koide et al., 2005; Vavouri and Lehner, 2009). Development in *Xenopus* has been studied extensively and large amounts of experimental data are available providing a useful starting point for reconstructing the GRNs underlying development. The *Xenopus* mesendoderm GRN has provided a good model to represent the interactions between transcription factors and embryonic signals specifying the mesendoderm and has been used as a model for understanding vertebrates in general (Koide et al., 2005; Loose and Patient, 2004).

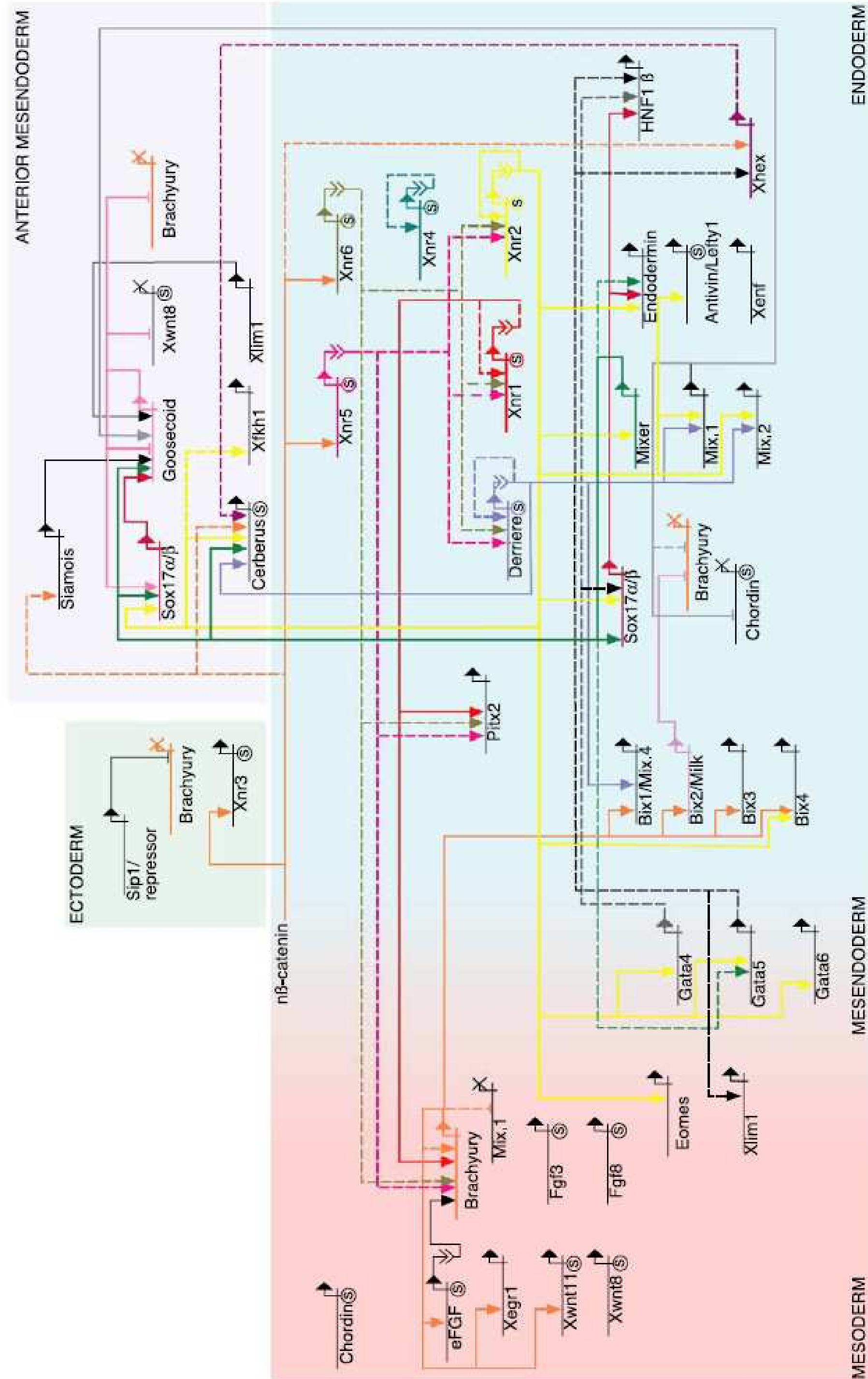


Figure 1.4 - A scheme of the *Xenopus laevis* mesendoderm GRN

The network for mesoderm and endoderm specification during the gastrula stage in *Xenopus laevis* (Loose and Patient, 2004). Over this period, the mesendoderm is subdividing into mesoderm and endoderm. The separation involves mutual repression between particular genes in the future mesoderm (Left) and endoderm (Right). In the centre, the interactions represent genes which are expressed in both mesoderm and endoderm, supporting the existence of a mesendodermal population in *Xenopus*.

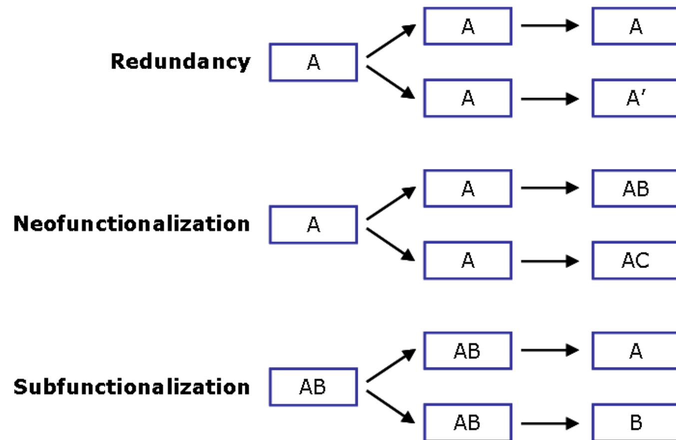
1.3 A simplified GRN

Vertebrate embryogenesis proceeds through a series of inductive events leading to changes in gene regulation. Gene regulatory networks (GRNs) provide a useful method to reveal the mechanisms of mesoderm and endoderm specification in vertebrates. *Xenopus* has been used as an excellent model to elucidate the gene regulations in different development events (Koide et al., 2005; Loose and Patient, 2004). However, the fact that *Xenopus* is allotetraploid and that a large number of gene duplications appear to have occurred make further genetic understanding of the network complex (Hirsch et al., 2002). Similarly, the ancestral genome duplication in teleosts makes zebrafish a less than ideal system for genetics (McClintock et al., 2001; Woods et al., 2000). As in *Xenopus laevis*, multiple copies of genes also can be detected in other organisms, such as *Xenopus tropicalis* and zebrafish (D'Souza et al., 2003; Kikuchi et al., 2000; Poulain and Lepage, 2002). Importantly, these multiple gene copies in *Xenopus laevis* are known to be only single copies in amniotes. During the evolution of vertebrates, two or three whole genome duplications have been proposed (Blomme et al., 2006). Either individual gene duplication or whole genome duplication has produced significant differences in gene retention which might coincide with the evolution of complex vertebrates (Blomme et al., 2006). Three main types of mechanism have been proposed involving gene duplication (Chain and Evans, 2006), innovation (neofunctionalisation), subfunction degeneration (subfunctionalisation), and genetic buffering (redundancy) (Figure 1.5A). However, natural selection must favor the retained expression of both paralogs, otherwise mutations will tend to silence one gene copy soon

after duplication (Chain and Evans, 2006). The presence of multiple copies of the *Mix* and *Nodal* genes in *Xenopus laevis* and *Xenopus tropicalis* with slight differences in expression patterns and functions suggests that the purifying selection in these genes is through a process of subfunctionalisation and/or neofunctionalisation and therefore consequently been relaxed to allow functional divergence and their presence in multiple copies that complicates the underlying GRN. Recent study in *Xenopus* has supported the idea of sub-functionalisation that results from the expansion of *Nodal* family (Luxardi et al., 2010).

A simplified gene regulatory network (sGRN) for mesoderm and endoderm formation has been constructed and is the working hypothesis of the laboratory (Figure 1.5B). The regulatory interactions in this network are based on the functional relationships between the transcription factors and embryonic signals involved in *Xenopus* mesendoderm formation. In the sGRN, the *Nodal* and *Mix* family are simplified into a single gene as in mouse and human since we propose these gene families represent sub-functionalisation from an ancestral gene. The *GATA* family, *GATA-4*, *5* and *6* are treated as a single copy gene due to the functional redundancy of these factors in endoderm formation (Zorn and Wells, 2007). The simplified network is proposed to resemble the ancestral mesendoderm network and can be used as a model system to test the underlying GRNs in other vertebrates.

A.



B.

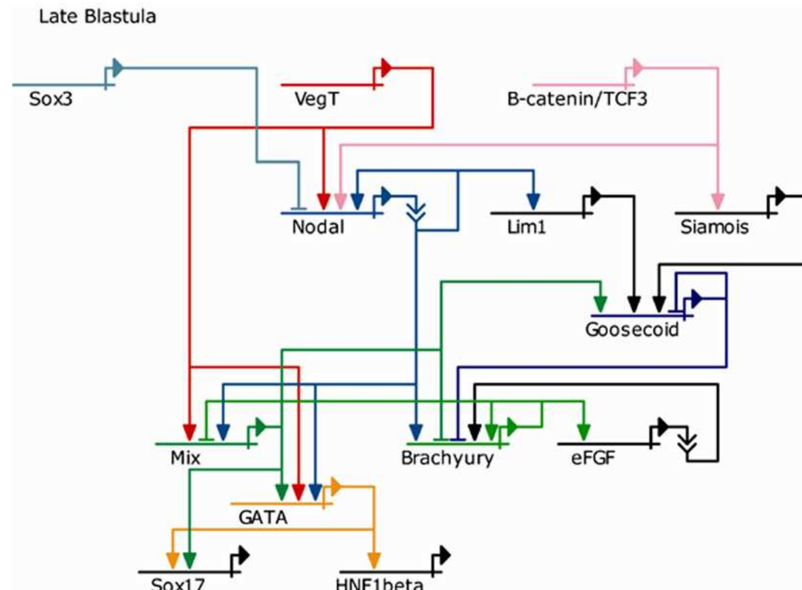


Figure 1.5 – Simplified mesendoderm GRN

(A) Duplicated genes can be maintained in the genome through redundancy, neofunctionalisation and subfunctionalisation. Gene redundancy is the process in the evolution of gene duplication and is produced by duplication of individual genes. With redundancy the duplicated genes have the same function with the one compensating for loss of the other. Gene duplication also allows for relaxed selection owing to redundancy, and this can result in two alternative evolutionary fates: one copy acquires a novel function (neofunctionalisation), or each copy adopts part of the tasks of their parental gene (subfunctionalisation). Figure modified from (Chain and Evans, 2006). **(B)** A simplified GRN for mesendoderm induction, however genes with multiple copies have been represented as single copies.

1.4 The axolotl as a model organism

Amphibians offer an excellent model system to study early embryonic development with several advantages, including easy adaption to laboratory, proper length of breeding season, easy manipulation and external larval stages which turn out it to be experimental animal. In addition, *Xenopus* has been widely used as a model for vertebrate early development; however, features of mesoderm patterning, gastrulation movement and primordial germ cell (PGCs) development suggest that the axolotl might represent as a primitive mode of vertebrate development.

At the beginning of the 20th century, under the influence of Spemann's studies in particular the discovery of the organizing center in 1924, the amphibian gastrula became a prevailing model for experimental embryologists. Neural induction during gastrulation and mesoderm induction at pregastrula stages was characterized later (Nieuwkoop, 1969). At first, urodele amphibians were used much less frequently than anurans. However, with the inspiration of Harrison's studies in the United States and Spemann's outstanding contributions in Germany, scientists began extensive studies on the newt egg and early embryo. Those studies finally led to the prominence of urodele embryos in general experimental embryology during the period 1920-1950. After 1960, it progressively changed, due to the increasing importance of *Xenopus laevis* in laboratories, initially because it is easier to obtain embryos all year long in *Xenopus*. In spite of the advantages of the *Xenopus* model system, the comparison of axolotl (urodele) and *Xenopus* (anurans) by Scott and Osborn (1879) had first described the differences in

gastrulation movements and the formation of germ layers between the urodele and anurans (Beetschen, 2001). The comparison of axolotl and *Xenopus* during gastrulation, mesoderm induction and primordial germ cell development will be further discussed in the discussion and highlight the axolol as a model system to test the hypothesis of a simplified GRN existing in the ancestral vertebrate.

1.5 Objectives

Previous studies have proposed that the conserved mechanisms shared between anamniote and amniote reveal that the ancestral amniote might be a urodele-like amphibian. However, the anuran amphibian, *Xenopus laevis*, has been used as a predominant model system to investigate embryo development in vertebrates. Many experimental results have been compiled to develop a GRN describing the transcriptional regulation of mesoderm and endoderm specification in the *Xenopus laevis* (Loose and Patient, 2004). In contrast to the amniotes, the multiple copies gene families, such as the *Mix-like* family and *nodal-related* gene family have highlighted the complexity of the network for mesoderm and endoderm formation. We reasoned that the increase in gene number in *Xenopus laevis* may be a consequence of sub-functionalisation. Following this logic, we predicted a simplified network which may more closely represent the underlying ancestral mechanism of mesoderm and endoderm development in amniotes.

Taken together we predicted that the urodele amphibian, the axolotl, would have a simplified GRN in comparison to that found in *Xenopus*. The aim of this thesis is to test whether the sGRN does exist in the urodele amphibian, the axolotl. First we needed to clone and characterize the *Mix* and *Nodal* genes in the axolotl and characterize their expression during gastrulation in relation to other mesoderm and endoderm genes. The roles of upstream regulation of *Nodal* and *Mix* genes, such as *Xenopus* maternal factors orthologs; AxVegT and AxVg1, and β-catenin in the axolotl mesoderm induction will also be investigated in comparison to their functions in *Xenopus laevis* and mouse mesoderm induction.

Chapter 2. Methods

2.1 Solutions and Buffers

1x Modified Barth's Solution (MBS)	88 mM NaCl; 1 mM KCl, 2.4mM $NaHCO_3$; 15 mM Hepes; 0.3 mM $CaNO_3$; 0.41 mM $CaCl_2$; 0.82 mM $MgSO_4$. pH 7.8 with NaOH and autoclave
10x MMR	1 M NaCl, 20 mM KCl, 20 mM $CaCl_2.6H_2O$, 10mM $MgCl_2$, 50 mM Hepes to pH 7.5
Axolotl antibiotics	10 mg/ml penicillin/streptomycin; 10 mg/ml fungizone; 10 mg/ml kanamycin
Xenopus antibiotics	10 mg/ml penicillin/streptomycin; 10 mg/ml kanamycin
Agarose plates	2% agarose in dH_2O + 0.1% Tris-HCl pH8
4x Collagenase/Dispase	80 mg collagenase type II (Sigma), 48 mg dispase II , dissolved in 10 ml of 1x MBS. Store at -20 in 5 ml aliquots and dilute to 1x solution with 1x MBS before use.
20x SSC:	3 M NaCl; 0.3 M sodium citrate. Adjust to pH with NaOH
Bouins fixative reagent	25% formaldehyde; 5% glacial acetic acid; 5% methanol; 1% picric acid. dH_2O to final volume
Hybridisation mix for ISH	50% (v/v) formamide (Sigma); 4x SSC (pH 4.5); 5mM EDTA; 0.05 mg/ml tRNA (Sigma); 0.1 mg/ml heparin (Sigma); 1% SDS
Blocking Reagent	MAB containing 2% (w/v) Boehringer Block (Roche)
10x PBS	27mM Potassium Chloride; 14.7 mM Potassium Phosphate monobasic (KH_2PO_4); 1.38 M NaCl; 80.6 mM Sodium Phosphate dibasic (Na_2HPO_4-7H2O) (Invitrogen)
PBS-Tween	1x PBS; 0.1% Tween
Bleaching solution	5% formamide; 1xSSC pH4.5; 3 - 5% H_2O_2

4% PFA	4% paraformaldehyde in 1xPBS
1x MAB	0.1 M Maleic Acid; 0.15 M NaCl adjusted to pH 7.5 with NaOH
1x NTMT	0.08 M NaCl; 0.1 M Tris-HCl (pH 9.5); 0.05 M $MgCl_2$; 1% (v/v) Tween
SOC	20 g Bacto Tryptone; 5 g Bacto Yeast; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM Glucose
50x TAE	2 M Tris-acetate; 0.05M EDTA
RNA-gel loading dye	95% formamide; 0.025% xylene cyanol; 0.025% bromophenol blue; 18 mM EDTA; 0.025% SDS
DNA-gel loading dye	0.1% Bromophenol blue, 0.1% Xylene Cyanol FF, 30% glycerol
Denaturing solution	1.5 M NaCl; 0.5 M NaOH
Neutralising solution	1 M Tris-HCl (pH 7.5) 1.5 M NaCl
1x Hybridisation buffer (HPB):	0.5 M NaCl; 0.1 M $Na_2HPO_4/7H_2O$; 5 mM EDTA
Mu Broth	10 g Bacto Tryptone, 5 g Bactoyeast, 10 g NaCl in 1 litre distilled water. To pH 7.0 with NaOH
Mu Agar	Mu Broth containing 15 g Bacto Agar/litre
Elution buffer	10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 50 mM NaCl
Homogenisation buffer	50 mM NaCl; 5ml dH_20; 300µl protease inhibitor cocktail (Sigma); 0.5 M PMSF (phenylmethylsulfonylflouride);
Resuspension buffer	50 mM NaCl; 50 mM PMSF; 1% TritonX
2x SDS gel-loading buffer	100 mM Tris-Cl (pH 6.8); 4% w/v SDS; 0.2% w/v bromophenol blue; 20% glycerol; 200 mM Dithiothreitol (DTT)
Resolving Gel (10%) pH 8.8	375 mM Tris-HCl pH 8.8; 10% acrylamide (from 30%:0.8% acrylamide:bisacrylamide stock (Protogel – BioRad); 0.1% SDS; 375 mM. Immediately before pouring add to 10 ml gel;

	100 µl 10% Ammonium persulphate; 10 µl TEMED
Stacking Gel (4%) pH 6.8	125 mM Tris-HCl pH 6.8; 4% acrylamide (from 30%: 0.8% acrylamide:bisacrylamide stock (Protogel – BioRad); 0.1% SDS. Immediately before pouring add to 5ml gel: 50 µl 10% Ammonium persulphate; 5 µl TEMED
SDS-PAGE running buffer	0.1% SDS; 0.25 M glycine; 25 mM Tris-HCl pH 8.3
Electrophoresis Buffer – aka Laemmli electrolyte buffer	25 mM Tris-HCl; 192 mM Glycine; 0.1% w/v SDS; H_20 to volume; pH to 8.3 using Glycine
Wet Transfer Buffer	25% Methanol; 24 mM Tris-HCl; 153 mM Glycine
TE buffer	10 mM Tris-HCl (pH 7.5); 1 mM EDTA
20x SET buffer	3 M NaCl; 1 M Tris-HCl pH8; 0.02 mM EDTA

2.2 Preparation and manipulation of DNA

2.2.1 Large and small scale preparation of plasmid DNA

Plasmid DNA was prepared using the QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer's protocol. For large scale production of high quality DNA, QIAprep® Spin Midiprep Kits (Qiagen) were used according to the manufacturer's protocol.

2.2.2 Determination of DNA and RNA concentration

DNA and RNA concentrations were measured using a Nanodrop 1000 Spectophotometer. 260/280 values were used to confirm purity of sample.

2.2.3 Amplification of DNA by Polymerase Chain Reaction (PCR)

PCR reactions [Saiki *et al.* 1985] were carried out in a final volume of 20 µl and consisted of 1x REDTaq® Ready Mix™ PCR reaction mix (Sigma), 7 µl dH_20, typically <100 ng DNA and 1µl each of forward and reverse primer at 1 mM final concentration. PCRs were run in Techne thermal cyclers according to the following program; after an initial denature of 95°C for 5 minutes the PCR typically consisted of 30 cycles with denature at 94°C for 45 seconds, annealing at the appropriate temperature for the primers (Ta: annealing temperature -4°C) for 45 seconds and the extension at 72°C for 90 seconds.

2.2.4 Restriction enzyme digestion

DNA was digested using restriction enzymes (NEB) according to the manufacturer's instructions and with the buffers provided. Digested DNA was analysed on 1.2 – 2% (w/v) agarose gels.

2.2.5 Agarose gel electroporesis

Digested DNA and transcribed RNA both for probes and embryo injections were analysed on 1.2 – 2.0% (w/v) agarose in 1x TAE gels and were run in 1x TAE. Ethidium bromide was added to gels, to intercalate with nucleic acids, at a final concentration of 1 µg/ml. For electrophoresis, DNA samples were mixed to give 1x DNA-loading buffer. For electrophoresis of RNA, gel tanks and combs were rinsed with fresh 1x TAE to prevent RNase contamination. RNA samples were mixed with 1x RNA loading buffer and heated to 72˚C to denature prior to electrophoresis. 100bp and 1Kb DNA ladders (NEB) were run alongside samples to identify sizes. Pictures were taken by placing gels

in a MultiMageTM light cabinet and photographed using an AlphaImagerTM 1220 Documentation & Analysis System (Alpha Innotech Corporation).

2.2.6 Phenol:Chloroform clean-up of DNA

An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) (Fluka) was added to the DNA and vortexed thoroughly and spun for 5 minutes at 13,000rpm at room temperature in a bench-top microfuge to facilitate phase formation. The aqueous phase containing DNA/RNA was removed and added to a fresh tube and re-extracted as above. An equal volume of chloroform:isoamylalcohol (24:1) was added, vortexed thoroughly and spun for 5 minutes, 13,000rpm, room temperature in bench-top microfuge. The aqueous phase containing DNA/RNA was aliquoted into a fresh tube.

2.2.7 Ethanol precipitation of DNA

DNA and RNA were precipitated from solution by adding 10 M ammonium acetate (pH5.2) to a final concentration of 0.3M and adding 2.5 volumes of ethanol and 1µl glycogen. The samples were mixed thoroughly by pippetting and incubated at -20°C for 20 minutes. Samples were spun for 15 minutes, 13,000rpm, room temperature in bench-top microfuge. The supernatant was discarded and the pellet washed with 70% ethanol and spun again for 2 minutes. The supernatant was removed and the pellet resuspended in dH_2O.

2.2.8 Purification of DNA from agarose gels

DNA run on agarose gels was visualised using a low intensity UV transilluminator and excised from the gel using a scalpel. DNA fragments were extracted from agarose gels using Spin columns from QIAquick gel extraction Kit (QIAGEN) according to manufacturer's guidelines.

2.2.9 Ligation of DNA fragments

Insert and vector were mixed together at a ratio of approximately 3:1. 1 μl T4-DNA ligase and buffer to 1x (NEB) were added to 10 μl reactions and incubated for 5 hours at room temperature or overnight at 14°C.

2.2.10 Transformations

70 μl of competent E. coli (strain DH5α) were incubated on ice with 50-100 ng of plasmid DNA for 30 minutes. The cells were heat shocked at 42°C for 45 seconds to allow plasmid uptake and then cooled on ice for 90 seconds. 250 μl of SOC media was added and the cells were incubated at 37°C for 1 hour. 50 μl to 100 μl of the transformation mixture were spread onto Mu agar plates containing the appropriate antibiotic which were incubated at 37°C overnight. For blue/white selection, 30 μl of 20 mg/ml X-GAL and 30 μl 100 mM IPTG were spread onto the agar prior to plating the transformation.

2.2.11 DNA Sequencing

DNA sequencing was carried out entirely by MWG or GeneService. PCR reactions contained 50 ng of DNA, 5 μM of primer (T3/T7/SP6), 1 μl sequencing buffer (Applied Biosystems) and 1 μl Big Dye Mix (Applied Biosystems) made up to final 10μl volume with dH_2O. PCR program was: 25 cycles at 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. DNA was precipitated in 50 μl ethanol and Sodium Acetate (pH5.6) to a final concentration of 0.1 M for 15 minutes at room temperature. Tubes were spun at 13,000 rpm for 5 minutes at room temperature in a benchtop microfuge and the supernatant removed. The pellet was washed with 150 μl of 80%

ethanol and spun at 13000 rpm for 5 minutes at room temperature in a benchtop microfuge. The supernatant was removed and pellet air dried at room temperature and sent to GeneService for sequencing using ABI 3730 DNA sequencing technology.

2.2.12 Linearising vectors for *in-situ* probe/mRNA synthesis

Up to 10µg vector was digested with the appropriate enzyme to linearise (see Table 2-1 for mRNA, Table 2-4 for in-situ hybridization (ISH). Linearised templates were verified by agarose gel electrophoresis and cleaned by phenol:chloroform extraction and ethanol precipitation. Precipitated linearised vectors were resuspended in 30 µl dH_2O and stored at -20°C until required.

2.2.13 Rapid Amplification of cDNA ends (RACE)

5′ RACE was carried out using BD SMART RACE Kit (Clontech). 5′ RACE-ready cDNA was made (by M. Loose) following manufacturer's guidelines from poly-A^+ RNA extracted from stage 10.5 embryos. Primers (see Table 2-1) were designed with a Tm >70°C and RACE was carried out following manufacturer's guidelines using the PCR program; 5 cycles at 94°C for 30 seconds; 72°C for 3 minutes, 5 cycles at 94°C for 30 seconds; 70°C for 30 seconds; 72°C 3 minutes and 25 cycles at 94°C for 30 seconds; 70°C for 30 seconds; 72°C for 3 minutes.

Table 2-1 – degenerate primer pairs

Degenerate primer pairs	Sequence (5'-3')
AxGsc deg.F	AACCAGCTGCAYTGYMGVMGG
AxGsc deg.R	CCARACCTCBACYTTYTCCTC
AxSox17 deg.F	ATGAAYGCBTTYATGGTBTGG
AxSox17 deg.R	GTARTTKGGRTGRTCYTGCAT
AxNodal deg.F	TGGATCRTYYACCCVMARMAGTWC
AxNodal deg.R	GGCAVCCRCAYTCBTSBACRAYCA
StVegT deg.F	GARATGATCATCACYAARTC
StVegT deg.R	CCYYTVGCAAADGGRTTGT
StVg1 deg.F	CCHYCTRTRYTHTGGARGAT
StVg1 deg.R	CCACRTTGTCAYTRTTRTCAT
LfVegT deg.F	AAGTGGAAYAARRAYMARTGGGA
LfVegT deg.R	TGGCTCCAGTCTGRCATNSYR
LfVg1 deg.F	RTGRARGARYTBAATGTYCCWGGK
LfVg1 deg.R	CCACRTTGTCAYTRTTRTCAT

2.14 RT-PCR

Total RNA was isolated by TRI reagent (sigma) and RT-PCR was according to RedTaq readyMix PCR reaction (sigma). *Axolotl* development series contains 13 different samples: (EC) early cleavage: 4-8 cells, (LC) late cleavage: 8-16 cells, stage 8, 9, $10_{1/2}$, 12, 16, 20, 25, 30, 35, 40 and –RT (negative control). PCR primers used for gene expression are listed below.

RT-PCR primer pairs	Sequence (5'-3')
AxGsc-Forward	AAACGCAGGCATCGCACAATC
AxGsc-Reverse	GATTGTGCGATGCCTGCGTTT
AxSox17-Forward	AGGAGCGCAAGAGGCTGG
AxSox17-Reverse	GTGCTGCACGCGGAGTCTCTCG
AxNodal-1-Forward	ATGCTTACAGATGCGAAGGGCTGTGCC
AxNodal-1-Reverse	GGTGGCGCATCACCACCTCCCCATTCT
AxNodal-2-Forward	TACCGCTGTGATGGAAAGTGTCCCAGC
AxNodal-2-Reverse	CTCCTCGTGGTGATGAACCACAACCTG

2.2.15 Sequence analysis and comparisons

NCBI BLAST (version 2.2.18) was used to determine sequence comparisons. All sequences were analysed in BioEdit [Hall 1999]. Alignments were carried out using the ClustalW Multiple alignment application built in to BioEdit [Thompson *et al.* 1994]. Pairwise similarity/identity comparisons were carried out using the BLOSUM62 simularity matrix in Bioedit.

2.3 Treatment and preparation of embryos and oocytes

2.3.1 In vitro transcription of mRNA for injection

Sense RNA for injection was transcribed from linearised DNA vectors using the relevant (see table 2-2) RNA polymerase mMachine kit (Ambion) according to manufacturer's guidelines. All reactions were carried out for 2 hours at 37°C to obtain maximum yield. RNA was recovered with two phenol:chloroform extractions and isopropanol precipitation according to guidelines. RNA was resuspended in 20 µl non-DEPC treated nuclease free water (Ambion) and the concentration was determined. mRNA was stored in 2 µl aliquots at -80°C until required. mRNA was diluted to the concentration required and stored on ice until required for injection.

Table 2-2 – Vectors for mRNA synthesis

Species	Gene	Vector	Linear	Sense Transcribe	Reference
Axolotl	AxNodal-1; AxNodal-2; AxMix; AxBra; AxVegT; AxVegT-EnR; AxVg1; AxBVg1; Ax109111V	pBUT4-HA	Sfi1	T3	
Xenopus	VegT; VegT-EnR; Vg1; Xnr-4	pBUT4-HA	Sfi1	T3	
Xenopus	Activin	pSPJC2L	EcoRI	SP6	(A.Johnson)
Xenopus	β-catenin	pCS2+	NotI	Sp6	Kindly given by Dr.Stefan Hoppler (Yost et al., 1996)
Xenopus	△N-Tcf-3	pCDNA	XbaI	T7	Kindly given by Dr.Martin Gering (Molenaar et al., 1996)
	GFP	pCSGFP2	NotI	SP6	(A.Johnson)

2.3.2 Morpholinos

Morpholinos were hydrated in non-DEPC treated nuclease-free water (Ambion) to 40 ng/nl and resuspended by heating to 65°C and cooling to 4°C twice. Hydrated morpholinos were stored at 4°C until required. Before injection, morpholinos were heated to 65°C, centrifuged and aliquots taken and stored at 37°C until they were mixed with the appropriate mRNA and injected.

AxNodal-1 Sp1 Morpholino: 5′-TAGACAGGCTGTGGGAAGAGAAGAC-3′
AxNodal-1 Sp2 Morpholino: 5′-TTGATGAAAGCATCTTACCTGCATG-3′
AxNodal-2 Sp1 Morpholino: 5′-AGATTCCATATTTCTTACCTGCATG-3′
AxNodal-2 Sp2 Morpholino: 5′-AGACTCTGAAGAAGAAAAGGAGAAG-3′
AxBra Sp1 Morpholino: 5′-TGATCTGTAGAGAGAGAAGGACAGT-3′
AxBra Sp2 Morpholino: 5′-TCCCCCACCACCACTCACCGCTCCT-3′
Control axolotl Morpholino: 5′-GGATTTCAAGGTTGTTTACCTGCCG-3′

The efficacy of the splice morpholinos was tested by PCR in each experiment.

The primers used were:

AxNodal-1: FP 5′-AAGCCCCACCTGCTCTTGCGTTCA-3′
RP 5′-GGTGGCGCATCACCACCTCCCCATTCT-3′
AxNodal-2: FP 5′-AGAGCACCCCGCCGCCAGAGAAGAT-3′
RP 5′-CTCCTCGTGGTGATGAACCACAACCTG-3′
AxBra: FP 5′-TGCACAAGTATGAACCCCG-3′
RP 5′-TCGCCATTATCCAGAACATC-3′.

2.3.3 Micro-injeciton

Injections were done using micromanipulation and needles pulled using a micropipette puller. Injections were carried out in injection plates; petridishes with a well for stability under 1x MBS + 4% $Ficoll_{400}$ (Sigma) with appropriate antibiotics (see section 2.1).

2.3.4 Oocytes

Large adult axolotl or *Xenopus laevis* females were immersed in 0.5% (w/v) aqueous solution of tricaine sulphonate (MS 222) and were sacrificed by rapid decapitation. Oocytes were liberated enzymatically by placing ovary tissue into a solution of type II collagenase (Sigma) and dispase (Gibco BRL). *Xenopus* oocytes were staged according to (Dumont, 1972), and axolotl oocytes were staged according to (Armstrong and Malacinski, 1989). In both systems, vitellogenesis begins at stage II. Lungfish and Sturgeon oocytes were kindly given by Dr.Jean Joss and Dr.Frank Chapman.

2.3.5 Axolotl embryos

Male and female axolotls were housed separately. Natural matings were set up by co-housing a male and female. Fertilised embryos were manually dejellied using forceps and maintained at 10°C in 1xMBS + antibiotics until required. One or two cell embryos were injected in the animal hemisphere with 2x 4 nl injections (one per blastomere) in 1x MBS + 4% Ficoll + antibiotics. Injected embryos were cultured at 18°C in 1x MBS + 4% Ficoll + antibiotics until stage 9 when they were washed down to 0.2x MBS + antibiotics and cultured until they had reached the required stage. Embryos were staged according to (Armstrong and Malacinski, 1989).

2.3.6 *Xenopus* embryos

Female *Xenopus laevis* were injected into the dorsal lymph sac with 500U human chorionic gonadotrophin and kept at 19°C overnight. Testes removed from sacrificed males were kept at 4°C in 70% L-15 medium (CAMBREX) supplemented with antibiotics. Testes stored this way are normally viable for

1 week. Eggs were squeezed from the female and fertilised immediately with macerated testes in 0.1x MBS + antibiotics (Smith and Slack, 1983). After 20 minutes, fertilised eggs were dejellied using 2% cysteine adjusted to pH 7.8 with NaOH. Dejellied embryos were rinsed with multiple washes of 1xMBS + antibiotics to remove cysteine and jelly before being stored in 1x MBS + antibiotics at 14°C until injected. Embryos were injected in 1x MBS + 4% Ficoll + antibiotics with 1x 4nl injection into the animal hemisphere. Embryos were cultured at 24°C until stage 8 when they were washed down to 0.1x MBS + antibiotics and cultured at 24°C. Stage 9 embryos were capped submerged in 0.5x MMR + antibiotics. Caps were cultured on agarose plates submerged in 0.5x MMR + antibiotics at 24°C until sibling embryos at appropriate stage for collection. Embryos were staged according to (Nieuwkoop and Faber, 1994)

2.3.7 Cycloheximide treatment

To inhibit protein synthesis, whole embryos were pre-incubated with 10 μg/ml cycloheximide (CHX) (Sigma) from stage 7 (before the mid-blastula transition) and the animal explants were cut when the sibling embryos reaching stage 9. The cap explants were incubated in 0.7XMMR with 10 μg/ml cycloheximide and collected at stage 10.5, and gene expression was analyzed by qPCR.

2.3.8 Microscopy and Photography

Embryos were visualised under Nikon SMZ 1500 microscopes. Photographs were taken using a Nikon DXM 1200F camera. Embryos/caps were photographed on agarose plates to allow orientation of embryos/caps.

2.4 Extraction of protein and RNA

2.4.1 RNA extraction

Axolotl and *Xenopus laevis* embryos or animal caps (caps) were collected and placed in autoclaved 1.5 ml eppendorf tubes with a minimal amount of liquid and were snap-frozen at -80°C and stored at -80°C until required.

Axolotl: Five frozen axolotl embryos (up to stage 20) were homogenised in 500 µl TRI-REAGENT™ (Sigma) using homogenising sticks. A further 750 µl of TRI-REAGENT™ was added to bring the final volume to 1.25 ml before spinning in a bench-top centrifuge at 13,000 rpm for 5 minutes at room temperature. The supernatant was decanted into a fresh 2.0 ml tube and made up to 1.5 ml with fresh TRI-REAGENT™ and left to stand for 5 minutes at room temperature. 0.2x volume of chloroform was added to the supernatant which was vortexed briefly and left to stand for 5 minutes at room temperature before spinning at 13,000 rpm for 10 minutes at room temperature. The aqueous colourless phase containing RNA was decanted and phenol:chloroform extracted. 0.5x volume of isopropanol was added to the aqueous phase collected after phenol:chloroform extraction, vortexed and precipitated at 4°C for 15 minutes and spun for 10 minutes at 13,000 rpm at room temperature. The pellet, containing RNA, was resuspended fully in 250 µl non-DEPC treated nuclease free water (Ambion) and an equal volume of 8M lithium chloride was added to precipitate the RNA. Eppendorfs were vortexed to mix and precipitation took place at 4°C for 24 hours.

After precipitation samples were spun at 13,000rpm for 15 minutes at room temperature. The supernatant was carefully removed (the pellet is

transparent) and the pellet washed with 70% ethanol at room temperature, vortexed, and spun for 5 minutes at 13,000rpm at room temperature. Ethanol was removed and the pellet allowed to briefly air dry before being resuspended in 10 µl non-DEPC treated nuclease free water (Ambion) per embryo extracted.

Xenopus RNA extraction: Up to 10 frozen *Xenopus laevis* caps were homogenised in 300 µl TRI-REAGENT™ using either homogenising sticks or by pippetting up and down. Samples were left to stand for 5 minutes at room temperature. 0.2x volume chloroform was added and the samples mixed by inversion before being left to stand for a further 5 minutes at room temperature. Samples were then spun for 15 minutes at 13,000rpm at room temperature and the top aqueous phase was decanted into a fresh tube. 1 µl of glycogen was added before adding 0.5x volume of isopropanol and the tubes mixed by inversion. Samples were left to precipitate for 25 minutes at room temperature before being vortexed briefly and spun for 15 minutes at 13,000rpm at room temperature. After precipitation the supernatant was discarded and the pellet washed with 70% ethanol at room temperature, vortexed, and spun for 5 minutes at 13,000rpm at room temperature. Ethanol was removed and the pellet allowed to briefly airdry before being resuspended in 2.5 µl non-DEPC treated nuclease free water (Ambion) per cap.

Whole embryos were extracted as above except they were homogenised in 300µl TRI-REAGENT™ per embryo and resuspended in 10 µl non-DEPC treated nuclease free water (Ambion) per embryo.

2.4.2 DNase1 treatment of RNA

The extracted RNA was treated with recombinant (r) DNase1 (Ambion), to remove genomic contamination. rDNase1 was removed with DNase inactivation reagent (Ambion) according to manufacturer's guidelines. Concentrations were determined and quality checked on 1.2% agarose gels. Samples were stored at -80°C until required.

2.5 Analysis of gene expression by Reverse Transcriptase (RT-PCR)

2.5.1 RT-PCR

PCR reactions were carried out in a final volume of 20 µl and consisted of 10 µl REDTaq® Ready Mix™ PCR reaction mix (Sigma), 7 µl dH_20, 1 µl cDNA and forward and reverse primers at a final concentration of 1 mM. RT-PCRs were run in Techne thermal cyclers according to the following program; after an initial denature of 95°C for 5 minutes the PCR consisted of 20 cycles with denaturing at 94°C for 45 seconds, annealing at Tm -5°C for 45 seconds and the extension at 72°C for 90 seconds. Samples were then run on a 1.2% agarose gel for virtual Northern analysis. 30 cycle PCRs were carried out to visualise DNA by ethidium bromide staining.

Table 2-3 - Southern Blot Probe PCR primers

Probe	Forward (5'-3')	Reverse (5'-3')
AxNodal-1 Eoxn2-Eoxn3	CCTGTCTACAACAGAAGGACCGAT	GCACCCACACTCCTCCACAACCA
AxNodal-2 Eoxn2-Eoxn3	ATGGGAGATAGATGGATCCTCACG	GCACCCACACTCCTCCACAACCA
AxMix Intron2	GTGAGACCCGTGGATAGGC	AGAGTGAGCAGAGTAAAATACC
AxMix Eoxn3-3'UTR	AATCAAATCCACCCAGAAGCC	TGGTCATTGAGCAGATGGAAGA
AxMix Homeobox-Exon3	CGCAGGAAGAGGACGAGCT	TGGTCATTGAGCAGATGGAAGA
XlMix.1 Homeobox-Exon3	GCATCTCAGCGCAGGAAAA	TCAAAGGTGGAGGAGCACA
XlBix.1 Homeobox-Exon3	ACGTCCAATCGCAGGAAGA	TCAGAAGAGAGTTCTAATA

2.5.2 cDNA synthesis

cDNA synthesis was carried out using SuperScript™ III (invitrogen) as described by the manufacturer. In a 20 µl reaction, 500ng total RNA was used to make cDNA from animal caps and 1µg RNA was used to make cDNA from whole embryos in the presence of 200 ng of random hexamer primers. Reverse transcriptase reactions took place at 50°C for 60 minutes with the synthesised cDNA stored at -20°C until required. For qRT-PCR, cDNA synthesis reactions were set up as described and mixed together and diluted with 30 µl non-DEPC treated nuclease free water (Ambion) per reaction. cDNA samples were then stored at -20°C.

2.5.3 Real-Time qPCR

For relative quantification of gene expression in morpholino assays and on developmental series' qRT-PCR was performed using the ABI 7500 Sequence Detection System (Applied Biosystems) with TaqMan fluorescence resonance energy transfer (FRET) technology. qRT-PCRs were carried out in triplicate on 96 well Fast plates (Applied Biosystems) in 25 µl reactions. All tubes were vortexed briefly and spun down prior to use. Reactions contained 1 µl cDNA; 1x qPCR mix with ROX (ABgene), 200 nM final concentration of both forward and reverse primers, 5 pmol of probe. Reactions were made up to 25 µl with non-DEPC treated nuclease free water (Ambion). Plates were sealed with optical adhesive film (Applied Biosystems) briefly spun to remove air bubbles and run on an AB 7500 sequence detection system. The program followed is: 50°C for 2 minutes, 94°C for 15 minutes followed by 40 cycles of 94°C for 15 seconds and 60°C for 1 minute.

Mouse sequences were assayed using the following standardized PCR assays from Applied Biosytems (UK). Mixl1 (Mm00489085_m1), Brachyury/T (Mm00436877_m1), Sox17 (Mm00488363_m1), FGF4 (Mm00438917_m1), Actin (Mm02619580_g1).

2.5.4 Primer and probes

Primers and probes were designed using Primer Express version 3.0 software (Applied Biosystems) according to manufacturer's instructions. Primers (Invitrogen) were resuspended to a final concentration of 10 μM, aliquoted and stored at -80°C. Probes (Sigma) are dual-labelled fluorogenic probes (5' FAM; 3' TAMRA) and HPLC purified. Probes were aliquoted and stored at -80°C until required. Working stocks were stored at -20°C. Primer and probe sequences are shown in (Table 2-4).

Table 2-4 – Primers and probes for qPCR

Axolotl		Real-Time qPCR	
Gene	Forward (5'-3')	Reverse (5'-3')	Probe (5'-3')
AxNodal-1	CCCAGTGGATGAAACGTTCAG	GGGTCGGGTGGTACAGCTT	CGACGAATCATGCCTACATGCAGAGC
AxNodal-2	CATACCGCTGTGATGGAAAGTG	CCCGCTCTGGAATGTACAATTT	AGCATTTCCAGCCCACCAACCATG
AxBrachyury	CATTGACCACATGTACCAATTGC	GATCAAGGGTCAATCGTGAGTTC	TACCCATAGTTCTTTTGTGCAGCATCCACG
AxMix	GTCCAGGATCCAGGTCTGGTT	GCTTCTGGGTGGATTTGATTTATAA	AATAGGCGTGCCAAGTCCCGCC
AxFGF8	TGCAGGTCCTTGGCAACAA	AAGGTGTCCGTTTCCACAATTAA	ACGGCGACTCGCACGCCA
AxSox17	TGGATACGACGCTCCACAGA	CTCCCTGTAGTGGCCGATGT	CATGAGCAGCAGTTCCAGCAGGACAAC
AxGsc	GCCTCTTCCAGGAGACCAAGT	TGGCTCTGCGGTTCTTGAAC	CACCCGAGAGCAGCTGGCCC
AxODC	ATGCCCGTCATGAGTAGTACCA	CCCGGACCCAGGTTACG	TGACAGTTCCAAGGTTTCATTCAATTGCTG
AxVegT	TCCATCTCCTTCCATAAGCTCAA	GCATGGAGTGCAGGATGATGT	CTTACCAACAACACCTTGGACCAGCAGG

Xenopus		Real-Time qPCR	
Gene	Forward (5'-3')	Reverse (5'-3')	Probe (5'-3')
Xnr-2	GGGAGCCTGTCCTATTCCTTTAA	TTCCACTTTCTCTTGATCGTACAACT	AAACCTTCAAGCCAACGAACCATGCC
Xnr-4	CCCGAGTCCAGTTAATGAAAGC	TCAGGAGCCTTTCCTTTAACATAGTAG	TGAAGCCAAATAACCACGCATACATGCA
Xlbra	GACCTGTGGACGAGGTTCAAG	TCACCTTCAGAACTGGAAACATTC	AGCTCACCAACGAGATGATCGTCACCA
XlMyoD	TCTACCCGGTTCTGGAACATTAC	TCATGCCATCGGAGCAGTT	ACTCAGATGCCTCAAGCCCCAGGTC
XlMix.1	GCTAGCGAGGCACATCTATATCC	CGTCTGACCTTTGCTCTTCTGTT	TCCCGCATTCAGGTCTGGTTCCA
XlMixer	TCTTCCAGACCAACATGTATCCA	CGCTCCTTTGCTCTTCTGTTCT	CACCACCGGGAAGAACTGGCTAAACG
XlSox17	CAAGAGACTGGCACAGCAGAAC	CCACGACTTGCCAAGCATCT	CGACCTGCACAACGCCGAGCT
XlODC	GTAAAATGCAACGATGGCAAAG	GATTTCAGTCTTACTGGCACAGTCA	AGACTCTCTCCATTCTTGGTGCCGGC

2.5.5 Data analysis

qRT-PCR data was analysed by the comparative C_T method (Livak and Schmittgen, 2001). Validation experiments were carried out on a 4-fold dilution series of cDNAs from 1 to 1/256 to ensure the PCR efficiencies of the

target and endogenous reference, (ODC), were approximately equal. The data was analysed in excel (Microsoft) and graphs were plotted of the ratio of gene expression relative to uninjected for morpholino-injected embryos, and relative to stage 12 for the developmental series. Error bars are one standard deviation of the sample.

2.6 *In situ* hybridization (ISH)

2.6.1 Preparation of axolotl embryos for *in situ* hybridization

Embryos were collected and placed into 2 ml round bottom eppendorfs with a maximum of 5 embryos per tube. Embryos were fixed in 4% PFA at 4°C for a week; the PFA replaced with fresh PFA after 2-3 days. Embryos were then washed twice with 100% methanol and stored at -20°C. Storage for up to several months before in situ hybridisation is carried out does not seem to affect WISH. There is no need to remove vitelline membranes of embryos until late neurula stages as in situ hybridisation efficiency does not seem to be affected.

2.6.2 Preparation of DIG-labelled RNA probes

DIG labelled antisense or sense probes were transcribed from 1μg linearised plasmid using 1-2 units of appropriate polymerase (Promega) (Table 2-5) and 2x DIG-UTP NTP RNA labelling mix (Roche). The supplied buffer was used with a final concentration of 10 mM DTT. 20 units of RNase OUT (Invitrogen) was added to reactions to protect RNA degradation. Reactions were made up to 20μl final volume with dH_2O and incubated at 37˚C for 2 hours. 1 unit of rDNase1 (Ambion) was added to reactions to remove DNA

template and reactions stopped with a final concentration of 20mM EDTA (pH8). Reaction volumes were made to 50µl with dH_2O and free nucleotides were removed using G_{50} spin columns (GE Healthcare) according to protocol. Probes were analysed on a fresh 1.2% gel and stored at -80˚C until use.

Table 2-5 - Vectors for *in-situ* hybridisation (ISH) probes

		Antisense		Sense		
Gene	Vector	Linearise	Transcribe	Linearise	Transcribe	Reference
AxNodal-1	pGEMT-Easy	SacII	Sp6	SpeI	T7	
AxNodal-2	pBS-SK+	XhoI	T3	XbaI	T7	
AxMix	pBS-SK+	HindIII	T3	XbaI	T7	
AxBra	pBS-SK+	XhoI	T3	XbaI	T7	
AxVegT	pBS-SK+	XhoI	T3	NcoI	Sp6	
AxVg1	pBS-SK+	XhoI	T3	NotI	T7	
LfVegT	pBS-SK+	XhoI	T3	NotI	T7	
LfVg1	pGEMT-Easy	NcoI	Sp6	SpeI	T7	
StVegT	pBS-SK+	XhoI	T3	PstI	T7	
StVg1	pGEMT-Easy	NcoI	Sp6	SpeI	T7	
XlVegT	pBS-SK-	NotI	T7	NheI	T3	Kindly given by Professor Jim Smith (White et al., 2002)
XlVg1	Psp70	NcoI	T7	EcoRI	Sp6	Kindly given by Professor Janet Heasman (Tannahill and Melton, 1989)

2.6.3 Hemisectioning

Axolotl embryos were hemisectioned following a modification of the protocol described for *Xenopus laevis* (Lee et al., 2001). Embryos stored in 100% methanol were rehydrated to PBS-Tween through a methanol series consisting of 5 minute washes in 75:25, 50:50 and 25:75 methanol:PBS-Tween before being washed in PBS plus 0.3 M Sucrose three times each wash lasting 1 hour. Embryos were embedded and orientated in 2% low melting point agarose (Promega) in 1x PBS plus 0.3 M sucrose before being bisected using a disposable scalpel under 1x PBS plus 0.3 M sucrose. Bisected embryos were stored in 100% methanol at -20°C until used (no longer than 1 week). *In situ* hybridisations were performed on hemisectioned embryos according to the whole mount protocol.

2.6.4 Whole-Mount in situ hybridization (WISH)

Whole mount *in situ* hybridisations were performed following a modified method from the Harland website (http://tropicalis.berkeley.edu/home/gene_expression/in-situ/insitu.html accessed 31st March 2008).

WISH was carried out in 2 ml round bottom eppendorfs for hemi-sections and glass vials for whole embryos. Embryos stored in 100% methanol were rehydrated to PBS-Tween through a methanol series, consisting of 5 minute washes in 75:25, 50:50 and 25:75 methanol:PBS-Tween, before being washed in PBS-Tween three times for 5 minutes. Embryos were re-fixed for twenty minutes in 4% PFA and then washed five times for 5 minutes to remove all traces of PFA. Embryos were equilibrated for five minutes in 50:50 hybridisation mix:PBS-Tween at 60°C which was then replaced with fresh hybridisation mix and pre-hybridised for 6 hours at 60°C. After pre-hybridisation the hybridisation mix was saved and stored at 60°C overnight for use the next morning. The embryos were hybridised overnight at 60˚C in fresh hybridisation mix containing DIG-UTP labelled RNA probe (0.5 µg/ml).

After hybridisation the buffer containing labelled probes was saved and stored at -20°C for re-use (maximum five times). Embryos were rinsed briefly in the pre-hybridisation mix saved from the previous day, followed by two brief rinses with 2x SSC (pH 4.5) at 60°C. Embryos were washed three times in 2x SSC (pH 4.5) for twenty minutes at 60°C, and then twice in 0.2x SSC (pH 4.5) for 30 minutes at 60°C before being washed twice for 15

minutes in MAB at room temperature. Embryos were then blocked for at least 5 hours in MAB plus 2% Block reagent (Boehringer Mannheim). To detect the DIG labelled probes 2% MAB blocking reagent was replaced with fresh 2% Blocking reagent in MAB with 1:3,000 dilution of anti-DIG antibodies conjugated to alkaline phosphatase (Roche) and embryos were left overnight at 4°C.

After incubation with the anti-DIG antibody, embryos were washed for 5 hours with MAB at room temperature with ten changes of MAB required to completely remove all traces of antibody. Embryos were washed twice for 5 minutes in NTMT at room temperature before being incubated in the dark at room temperature in BM Purple (Boehringer Mannheim) to allow colour development; typically overnight to three days depending on the probe.
After staining embryos were briefly washed in MAB at room temperature before being fixed overnight in Bouins reagent at room temperature. Once fixed the embryos were washed with 70% buffered ethanol at room temperature until the yellow staining of the Bouins reagent was removed, and rehydrated to 1x SSC (pH 4.5) through an ethanol series consisting of 5 minute washes in 75:25, 50:50 and 25:75 ethanol:PBS-Tween. Embryos were then bleached in bleaching solution on a light box at room temperature until most of the pigment had been removed. The bleaching solution was removed and the embryos washed twice with 1x SSC (pH4.5) before being stored at 4°C in 80% glycerol solution. Photographs were taken either before storage or after being stored in glycerol for a couple of days.

2.7 Handing and manipulating genomic DNA

2.7.1 Genomic DNA extraction

Adult axolotls were desanguinated according to home office guidelines and the blood was resuspended in 1x SET buffer and spun for 3 minutes at 5,000rpm at room temperature. The pellet was resuspended in 1x SET buffer. Proteinase K (Sigma) was added to 200 µg/ml and SDS to 0.5% and incubated overnight at room temperature. Genomic DNA was phenol:chloroform purified twice and precipitated with 0.1x volume 3 M sodium acetate and 2x volume 95% ethanol. The DNA was washed several times with 70% ethanol and once with 95% ethanol and air dried. The pellet was resuspended in TE buffer over 2 days at room temperature with gentle rocking.

2.7.2 Genomic Intron PCR

PCRs used Thermo SCIENTIFIC Extensor Hi-Fidelity PCR Master Mix Buffer 1 with reactions containing 150 – 200 ng genomic DNA. PCRs were run in Techne thermal cyclers according to the following program; after an initial denature of 94°C for 2 minutes the PCR consisted of 28 cycles with denaturing at 94°C for 10 seconds, annealing at Tm -5°C for 30 seconds and the extension at 68°C for 5 minutes. Primer pairs (see Table 2-6) used for the axolotl genomic PCR are listed below.

Table 2-6 – Genomic PCR primers

Genomic PCR Primers	Sequence (5'-3')
AxMix Intron1-PF4	GGATGAGCAGGATGCCCGCAGACA
AxMix Intron1-R2	AGCTCCTCCCGCAGGTGGATGT
AxMix Intron2-FP3	CGGACATCCACCTGCGGGAG
AxMix Intron2-R	GCGGGACTTGGCACGCCTATTCT
AxMix Eoxn3-3'UTR-FP2	GGTTCCAGAATAGGCGTGC
AxMix Eoxn3-3'UTR-RT1	TGGTCATTGAGC AGATGGAAGA
AxNodal-1 Intron1-Exon1-FP1	AAGCCCCACCTGCTCTTGCGTTCA
AxNodal-1 Intron1-Exon2-RP	CCATCCGACCTGCTCAAAATCCACAAA
AxNodal-1 Intron2-RT&RACE FP	ATGCTTACAGATGCGAAGGGCTGTGCC
AxNodal-1 Intron2- RT&RACE RP	GGTGGCGCATCACCACCTCCCCATTCT
AxNodal-2 Intron1-Exon1-FP1	AAGGCAGAAAGGGGGAGCAAAGCACA
AxNodal-2 Intron1-Exon2-RP	TGTAGGCATGGTTGGTGGGCTGGAAAT
AxNodal-2 Intron2-RT&RACE FP	TACCGCTGTGATGGAAAGTGTCCCAGC
AxNodal-2 Intron2- RT&RACE RP	CTCCTCGTGGTGATGAACCACAACCTG
AxBra Intron3 and 4- Exon3 FP	TGCACAAGTATGAACCCCG
AxBra Intron3 and 4- Exon5 RP	TCGCCATTATCCAGAACATC

2.8 Blotting

2.8.1 Southern blotting

Southern blots were carried out to visualise genomic DNA. For Southern blots 30 μg genomic DNA was digested for each lane with all possible combinations of *PstI*, *BsrGI* and *MscI* (NEB). Once run, the top right-hand corner of the gel was removed for orientation and the gel was washed in denaturing buffer once for 45 minutes at room temperature with shaking. The gel was then washed in neutralising buffer once for 30 minutes at room temperature with shaking and once for 15 minutes at room temperature with shaking. The gel was then placed wells down onto a sponge overlaid by 3 mm Whatmann

paper and soaked in 20x SSC (pH7.0). Hybond-N membrane (GE Healthcare) cut to the size of the gel was placed on top of the gel, with the appropriate corner removed for orientation. Air bubbles were removed by rolling and 3mm Whatmann paper covered the membrane and paper towels and a weight were placed on top. Capillary transfer of DNA from agarose gels occurred overnight at room temperature for transfer onto membranes. Membranes were dried on 3 mm Whatmann paper for 1 hour at room temperature and cross-linked using a GCLM-8 Crosslinker at 120 mJ for 30 seconds. Crosslinked membranes were wrapped in Saran wrap and stored at 4°C prior to use or at -20°C after use.

2.8.2 Preparation of salmon sperm DNA

Salmon sperm DNA (Sigma) was dissolved in water to a concentration of 10 mg/ml and adjusted to a pH of 0.1M NaOH. DNA was phenol:chloroform extracted and sheared by passing rapidly 12 times through a 17-gauge hypodermic needle before being ethanol precipitated in 2x volume of ice-cold ethanol. DNA was recovered by centrifugation and re-dissolved to a final concentration of 10 mg/ml in dH_2O.

2.8.3 Pre-Hybridizaiton

1x HPB + 1% Sarkosyl was heated to the pre-hybridisation temperature of 65°C in Techne Hybridiser ovens. Salmon sperm DNA (50 µg/ml) was boiled and quenched on ice before being added to the pre-heated 1x HPB + 1% sarkosyl. The pre-hybridisation mix was then added to the hybridisation tubes containing membranes with DNA/plaques facing into tube. Pre-hybridise for a minimum of 3 hours at 65°C.

2.8.4 Radiolabelled probe synthesis and hybridization

RT-PCR was carried out to amplify DNA sequence for probe synthesis (see Table 2-2 for primer information). The DNA was run on agarose gels and gel extracted. The DNA template was diluted to a concentration of 0.5 ng/µl in dH_2O to a final volume of 45 µl. DNA was boiled to denature and quenched on ice for 5 minutes before being added to a Rediprime labelling mix (GE healthcare). The Rediprime labelling mix and template DNA was pippetted to mix and 2-5 µl $\alpha^{32}P$ dCTP (250 µCi) (GE healthcare or Perkin Elmer) was added to reactions. The reactions were mixed by pipetting and incubated at 37°C for 30-60 minutes to synthesise probe. The reaction was stopped by adding EDTA to a final concentration of 1mM EDTA (pH8) and the free unincorporated nucleotides were removed by passing through a G50 spin column according to manufacturer's instructions. The probe was boiled to denature, quenched on ice and added to fresh, pre-warmed 1x hybridisation buffer + Sarkosyl + salmon sperm DNA. Membranes were hybridised overnight at 55°C.

2.8.5 Washes

Unbound excess probe was removed by washing membranes in hybridisation tubes for 20 minutes per wash at increasing temperatures. Wash buffers consisted of various concentrations of SSC with 0.1% SDS and low stringency wash procedure was followed: two washes in 2x SSC, 0.1% SDS at room temp, once in 1x SSC, 0.1% SDS at room temp and once in 1x SSC, 0.1% SDS at 50°C. Membranes were washed until counts using a Geiger counter were between 10-50 cps.

2.8.6 Autoradiography

The washed radioactive membranes were wrapped in Saran wrap and taped securely, DNA side up, into a cassette containing calcium tungsten intensifying screens. In a dark room under red light, x-ray film (SuperRX Fujifilm) was placed on top of the membranes and exposed for up to 72 hours at -80°C. The x-ray film was taken out of the cassette in a dark room and developed automatically using an SRX-201 Xograph.

2.9 Cell culture and manipulation

2.9.1 Cells

CGR8 mouse ES cell lines were maintained on gelatin-coated dishes (0.1%) in ESCs medium as described in Table 2-7 (Tada et al., 2005; Turksen, 2006). Embryoid bodies were generated via the hanging drop method, cultivating 600 cells in a 20 µl drop. ES cells were expanded and differentiated as previously described (Tada et al., 2005). Mixl1 specific shRNA sequences were designed as previously described (Izumi et al., 2007).

Table 2-7 - CGR8 mouse ES cell medium and differentiation medium

(⁎ Products from Invitrogen)	ESCs medium (200ml)	Differentiation medium (200ml)
⁎ Knock-out DMEM	160 ml	152 ml
⁎ KSR (knock out serum replacement)	30ml (15% final)	40ml (20% final)
⁎ FCS (fetal calf serum)	2ml (1% final)	
BSA (bovine serum albumin) (Sigma)		2 ml form 10% stock (0.1% final)
⁎ NEAAS (nonessential amino acids)	2ml from stock (0.1 mM final)	2ml from stock (0.1 mM final)
⁎ L-Glutamin	2ml from stock (2 mM final)	2ml from stock (2 mM final)
⁎ P/S (penicillin/streptophan)	2ml from stock (0.1 mM final)	2ml from stock (0.1 mM final)
⁎ Sodium pysuvate	2ml from stock (1 mM final)	
2-Mercaptoethanol (Sigma)	200 µl (0.1 mM final)	100 µl (50 µM final)
LIF (leukemia inhibitory factor) (Produced by hybridoma cells)	1 ml (1000 units/ml in final)	
Activin A (R&D Systems)		200 µl (10 ng/ml final)

Mouse Mixl1 siRNA target sequence:

GTATTCGTCTCTCTCTGAAGA (637-657)

Mouse Mixl1 Scramble siRNA sequence:

GTCGATCCTTCCGGTAATTAT

2.9.2 Transfection and stable cell line selection

The tet-regulated vector; PLVCT-tTR-KRAB (Addgene), was used to express the appropriate shRNA. A Pol III promoter-small hairpin RNA cassette allows for drug-controllable RNA interference (Tet-on shRNA). Because the vector does not include a drug selection marker, a zoecin selection cassette (Invitrogen) was incorporated into the NotI site. The Mixl1 shRNA expression vector was linearized with Sfi1 and cultured CGR8 mouse ES cells were transiently transfected by use of lipofectamine 2000 (Invitrogen). Empty and scramble expression vectors were also generated as controls. Stable transfection was carried out according to the manufacturer's guidelines. 24 hours prior to transfection, cells were split into 6 well plates (Fisher) at $2x10^5$ cells/well. After 2 days, the ES cells were expanded and selected in ESC medium with 25 μg/ml zeocin and 1 μg/ml doxycycline. The zeocin resistant and GFP positive colonies were isolated. Subsequently, the stable ES cell lines that maintained the highest level of GFP expression were expanded and maintained in ESC medium with 25μg/ml zeocin for further analysis.

2.9.3 Luciferase assays

Promoter reporter plasmid DNAs and mRNAs were injected into the animal pole at the one or two cell stage. 40 pg of firefly luciferase reporter construct in combination with 4 pg of renilla luciferase (renilla-TK: Promega) per embryo were injected into animal pole with 500pg mRNA. Whole embryos were collected at stage 10.5. Luciferase levels were measured from lysates of three or more whole embryos in duplicate or triplicate using the Dual Luciferase Assay Kit (Promega). Dual-Luciferase Reporter assays (Promega) were carried out according to manufacturer's guidelines on a GloMax 96

microplate luminometer (Promega). Briefly, embryos were collected and lysed using 100 µl 1x Passive lysis buffer (Promega) in 1x PBS. The embryo lysate was subjected to 1 or 2 freeze–thaw cycles to accomplish lysis of cells. Luciferase activity was assayed in 96 well plates according to the manufacturer's instructions. Data was processed in Excel (Microsoft). Data was normalised within experiments and results shown are a single experiment representative of two or more repeats.

Chapter 3. Characterizing a single Mix gene and cloning Nodal genes in axolotls

3.1 Introduction

The Nodal signalling pathway is integral to the processes of pattern formation and differentiation as gastrulation proceeds during chordate development. Genetic studies in *Xenopus*, zebrafish, chick and mouse have established its importance and functional conservation in various species (Shen, 2007; Tian and Meng, 2006). As already discussed (see introduction), whilst there is a single *Nodal* gene in mouse and human, there are at least six *Xenopus nodal-related* genes (*Xnr1, Xnr2, Xnr4, Xnr5, Xnr6*, and *Derriere*), and two zebrafish nodals, *cyclops (cyc)* and *squint (sqt)*, implicated in mesendoderm development (Feng and Derynck, 2005; Schier, 2003). Similarly, the Mix family of homeobox transcription factors have undergone expansion in anurans and teleosts. *Xenopus laevis* has seven *Mix-like* genes (*Mix1, Mix2, Bix1/Mix4, Bix2/Milk, Bix3, Bix4,* and *Mixer/Mix3*) (Casey et al., 1999; Ecochard et al., 1998; Henry and Melton, 1998; Latinkic and Smith, 1999; Mead et al., 1998; Rosa, 1989; Tada et al., 1998; Vize, 1996), and zebrafish has four (*bonnie* and *clyde, mezzo, mxt1*, and *mxt2*) (Hirata et al., 2000; Kikuchi et al., 2000; Poulain and Lepage, 2002), whereas only one is found in mammals, such as mice (*Mixl1*) and humans (*MIXL*) (Guo et al., 2002; Peale, Jr. et al., 1998; Pearce and Evans, 1999; Robb et al., 2000). Analysis of *Nodal* genes in teleosts and tetrapods indicate that the teleost and tetrapod *Nodal* genes are derived from a single ancestral gene (Fan and Dougan, 2007). At least in teleosts, the nodal-related genes provide an example of

evolution by gene duplication followed by subpartitioning of gene function (Fan and Dougan, 2007), and similarly in *Xenopus*, the expansion of the *nodal-related* genes represents the sub-functionalizaiton in evolution (Luxardi et al., 2010).

Work in urodele amphibians as already discussed, suggests that early patterning events including germ cell induction and mesoderm movement are conserved with mammalian systems, unlike *Xenopus* (Johnson et al., 2003a; Shook et al., 2002; Shook and Keller, 2008b; Wakahara, 1996). Indeed, recent studies suggest that ancestral amniotes arose from an urodele-like anamniote amphibian (Shook and Keller, 2008b). Taken together, this suggests that the underlying transcriptional regulatory network for mesoderm formation in the axolotl may be more similar to that of amniotes. If this is the case, the increased numbers of *Mix* and *Nodal* genes in *Xenopus* arose after the last common ancestor with axolotls and may represent the subfunctionalisation of an ancestral gene from an urodele-like common ancestor (Chain and Evans, 2006; Fan and Dougan, 2007; Luxardi et al., 2010). As such genes are found as single copies in mammals, will they be present in single copies in the axolotl?

To test this hypothesis, we identified the axolotl orthologs of the *Mix-like* and *nodal-related* genes supported by sequence analyses. Sothern blotting results provide evidence that *AxMix* (accession number:GU256640) is present as a single copy in the axolotl. In contrast, there are two *nodal-related* genes, *AxNodal-1* (accession number:GU256638) and *AxNodal-2* (accession number:GU256639), found in the axolotl genome.

3.2 Cloning of AxMix

The *AxMix* gene was previously cloned by G.Swiers in the lab (G.Swiers 2008, PhD thesis). Three positive colonies were identified from a stage 10.5 axolotl cDNA library screened using a full length mouse (*Mm Mixl1*) probe. Based on sequence analysis, all clones were derived from one *Mix* gene. The same library was re-screened using the axolotl Mix sequence as a probe and revealed 200 positive colonies. Ten *Mix-like* candidate genes were randomly picked and sequenced. All of the sequences matched with the original *AxMix* cDNA providing the first preliminary evidence that *AxMix* may be present as a single copy gene in the axolotl genome. Furthermore, provisional axolotl 454 transcriptome data from Dr.Elly Tanaka, indicates no other *Mix* genes are expressed during gastula stages in axolotls.

3.3 Genomic analysis for *AxMix*

Given that only one *Mix* ortholog has been identified in the axolotl, we asked if the genomic structure of *AxMix* is most like human and mouse, or *Xenopus*? Initially, we considered two possible models for the genomic structure of *AxMix*, either the human/mouse-like structure with two exons and one intron, or the *Xenopus*-like structure with three exons and two introns (Figure 3.1A). Firstly, we compared the *AxMix* coding sequence with the exon regions of human *MIXL1*, mouse *Mixl1* and *Xenopus tropicalis Mix.1*. Based on sequence alignments, we only detect a single conserved exon-intron boundary (Figure 3.1A– blue circle), suggesting there might be only one intron in *AxMix* as with the human and mouse. To distinguish between these two possibilities, we designed three primer pairs (see Methods) covering the genomic region of

AxMix to confirm the distribution of exons and introns in *AxMix* (Figure 3.1B). The genomic fragments of *AxMix* were cloned by PCR from genomic DNA extracted from axolotl erythrocytes. These PCRs revealed the presence of a second intron; intron 1 is 2.1 kb and intron 2, dividing the homeodomain, is 1.4 kb. This result reveals that the genomic structure of *AxMix* is more similar to *Xenopus* than the human and mouse (Figure 3.1B). The proteins encoded by the various *Mix-like* genes vary in size, but share a highly conserved paired-type homeodomain and a conserved carboxy-terminal acidic domain (Sahr et al., 2002; Tada et al., 1998). These sequence results alongside our conserved domain predictions, allowed us to characterize the genomic organization of *AxMix* (Figure 3.2).

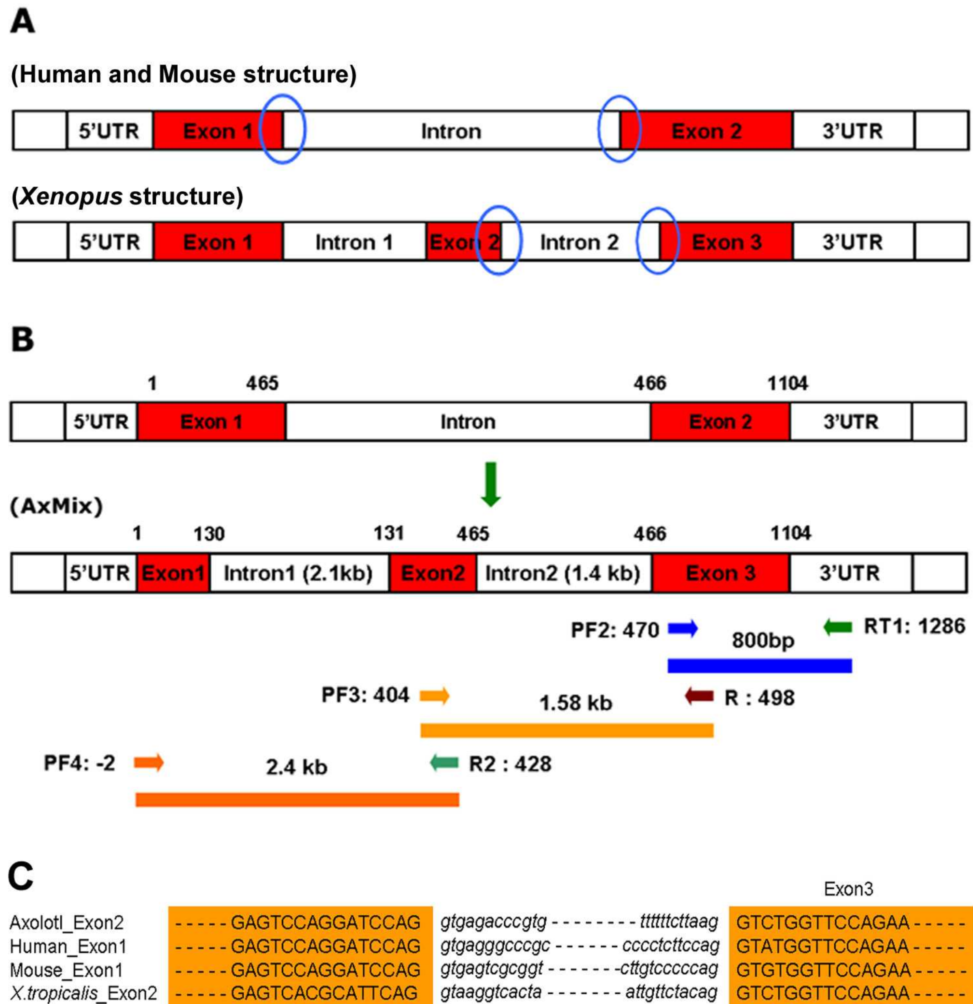


Figure 3.1 – The genomic structure of AxMix is more like amphibians than mammals

(A) Two possible arrangements for the genomic structure of AxMix. In the human and mouse, here is only one intron dividing two exons. In *Xenopus tropicalis* mode, there are two introns dividing three exons. The blue circle indicates the only conserved exon-intron boundary.

(B) Scheme of the primer pairs designed to amplify the *AxMix* genomic fragment. There are three pairs of forward and reverse primers, PF2-RT1, PF3-R and PF4-R2. The size of the amplified fragments is indicated. The genomic structure of *AxMix* is similar to *Xenopus*, having three exons and two introns. The exon-intron junctions have a perfect splice donor and acceptor consensus identified between nucleotides 130 and 131, and nucleotides 465 and 466. Exon 1 is predicted to be 130 bp, exon 2 to be 335 bp and exon3 to be 639 bp.

(C) Sequences within the exon-intron boundary in the axolotl, human, mouse and *X.tropicalis Mix* gene. Exon: upper case with the orange highlight. Intron: Italic font in lower case.

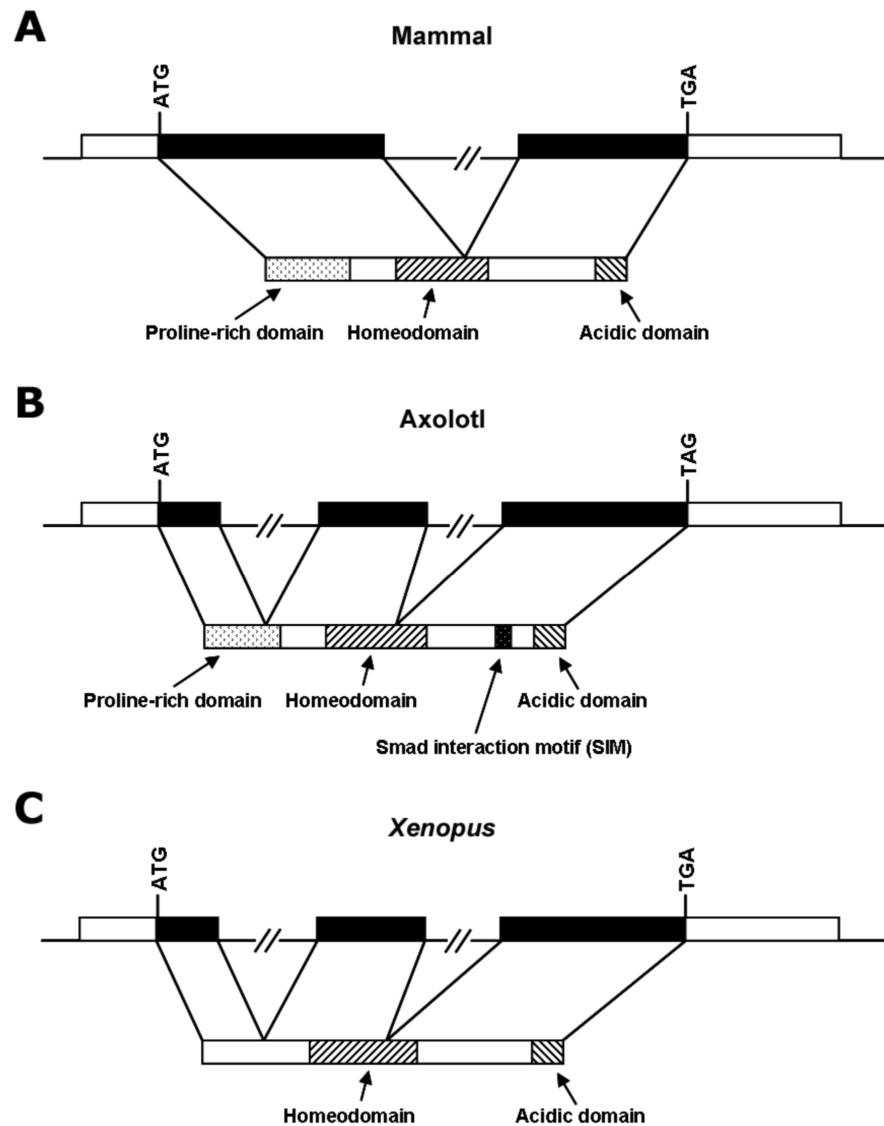


Figure 3.2 – The genomic organization of the Mix-like genes

(A) The genomic organization of mammalian *Mixl1*. Mammalian *Mixl1* contains only one intron, whereas genomic DNA analysis reveals the presence of two introns in *AxMix* **(B)** as well as *Xenopus tropicalis Mix.1* **(C)**. Intron 2 of *AxMix* and *XtMix.1* resides in the same place as the mammal intron, bisecting the homeodomain. Both the Mix-like homeodomain and carboxy-terminal domain are evolutionarily conserved. The amino-terminal proline-rich domain, not found in *Xenopus Mix.1*, is unique to *MIXL, Mm Mixl1* and *AxMix*. A conserved SIM (smad interacting motif) domain is also identified in AxMix indicating AxMix is able to mediate TGF-β transcription (Germain et al., 2000; Randall et al., 2002).

3.4 AxMix is present as a single copy

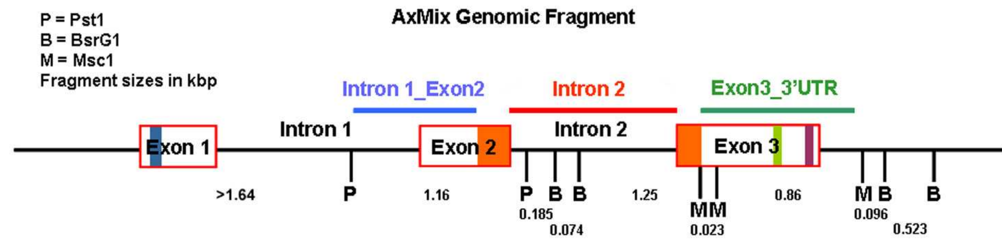
The results from cDNA library screening indicated that *AxMix* is a single copy gene in the axolotl genome. Definitive proof that *AxMix* is single copy in the axolotl relies on full genome sequence which is currently not available. In the absence of this, we carried out genomic southern blotting to investigate *AxMix* copy number in the axolotl.

Prior to carrying out genomic southern blot analysis with *AxMix* probes, we amplified the entire *AxMix* genomic region (4.767 kb) and created a restriction map using all possible combinations of the enzymes used for the southern blots (Figure 3.1B). This allowed us to predict the restriction fragments we would expect in genomic southern blots (Figure 3.3A). Various different regions of the *AxMix* sequence, both coding and non-coding, were used to probe the digested genome. All southern blots were performed using low stringency procedures (see Methods).

Southern blotting with the full-length coding sequence (CDS) and several short PCR-generated *AxMix* probes (see methods) was used to analyze the genomic structure of *AxMix*. Using the *AxMix* full-length CDS as a probe identified not only the expected bands (see Figure 3.3A), but also additional unexpected fragments (data not shown). To resolve these bands, we used a subset of probes to simplify the result. Probes containing part of intron 1 and some of exon2 (excluding the homeodomain), and a probe designed to anneal in intron 2 were used to detect *AxMix* fragments containing the intron regions (Figure 3.3B). We excluded the homeodomain in order to avoid potential cross-hybridization with other homeobox proteins. The intron2

probe detects fragments matching our prediction, but for the intron1_exon 2 probe, as well as the predicted fragments, we could detect several small fragments of 600-700bp (data not shown). These bands cannot be explained based on the *AxMix* sequence we have cloned and we discuss their identity later. For further clarification, we used a probe designed to a partial exon3 region and some of the 3'UTR containing the SIM domain and the acidic c-terminal region, which are conserved amongst all Mix-like family members in all species. The exon3-3'UTR probe should identify fragments in common with the intron2 result and indeed we find this to be the case (indicated in yellow on Figure 3.3B and C). Taking the results of the intron2 and exon3_3'UTR probes together, all fragments corresponded to the size we predicted and must be *AxMix* specific. Therefore, we conclude that the southern blotting results for the intron2 and exon3-3'UTR probes support our hypothesis that *AxMix* is a single copy gene in the axolotl.

A.



B. **C.**

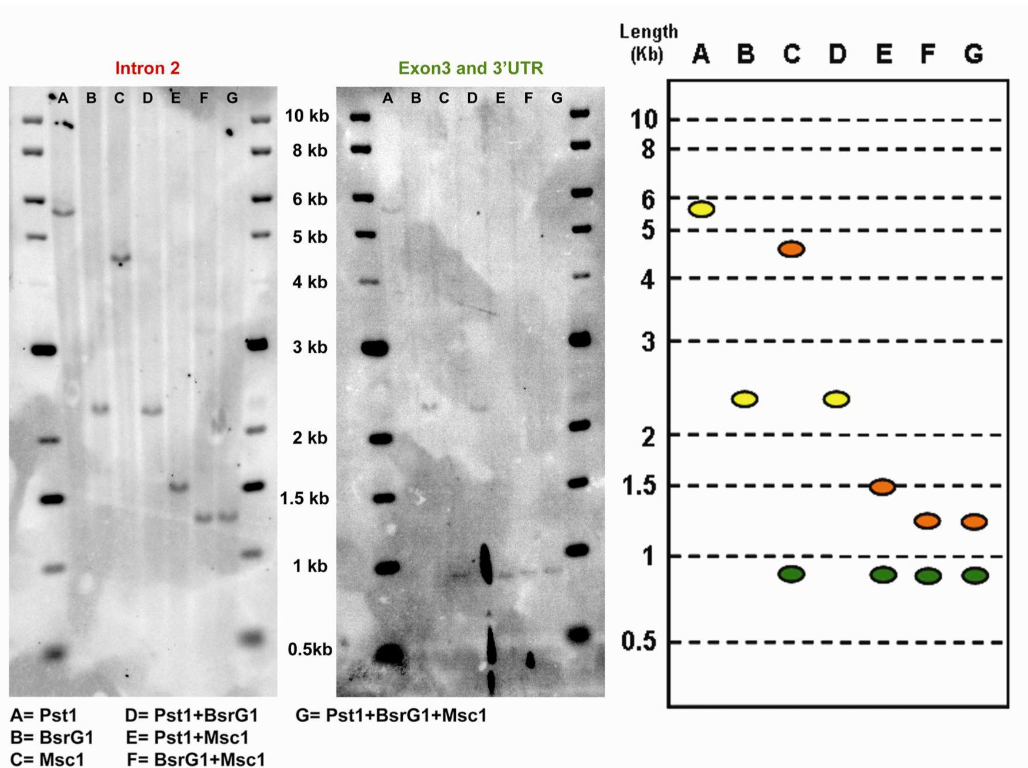


Figure 3.3 – Southern blotting result for AxMix

(A) Axolotl genomic DNA was digested using all combinations of three enzymes; Pst1 (P), BsrG1 (B), Msc1 (M). The probe regions are marked on the cartoon in blue, red and green as well as the identified band sizes are indicated in the restriction map of the *AxMix* genomic fragment. **(B)** Digested axolotl genomic DNA probed with two different regions of *AxMix* sequence – intron 2 probe (left hand blot – red) and exon 3_3'UTR probe (right hand blot – green). **(C)** The combined results of intron 2 and exon3_3'UTR. The intron2 result is indicated with orange; exon3-3'UTR is indicated with green. The yellow indicates bands that be identified by both probes. There were no unexpected bands on either blot.

To further support our hypotheses, we designed another probe including the homeodomain and exon3 region. The homeodomain-exon 3 probe identified the same fragments as the exon3-3'UTR probe, but failed to detect two expected bands (the 1.16 kb fragment in the Pst1 digest and the 1.25 kb fragment in the BsrG1+Msc1 digest). These missing bands will be explored in the discussion. Under the same conditions, we repeated the southern blotting analysis with the equivalent region (homeodomain-exon3) of *Xenopus laevis Bix.1* and *Mix.1* as a probe on *Xenopus tropicalis* genomic DNA. Working with the known *XtMix.1* and *XtBix.1* genomic sequences allowed us to predict the size of the restriction fragments with every combination of enzymes. Both results reveal the multiple copies of *Mix-like* gene members in the *Xenopus tropicalis* genome (Figure 3.4). This suggests that the southern blotting conditions would detect other copies of *Mix-like* genes in axolotls and further supports our hypothesis that *AxMix* is present as a single copy in the axolotl genome.

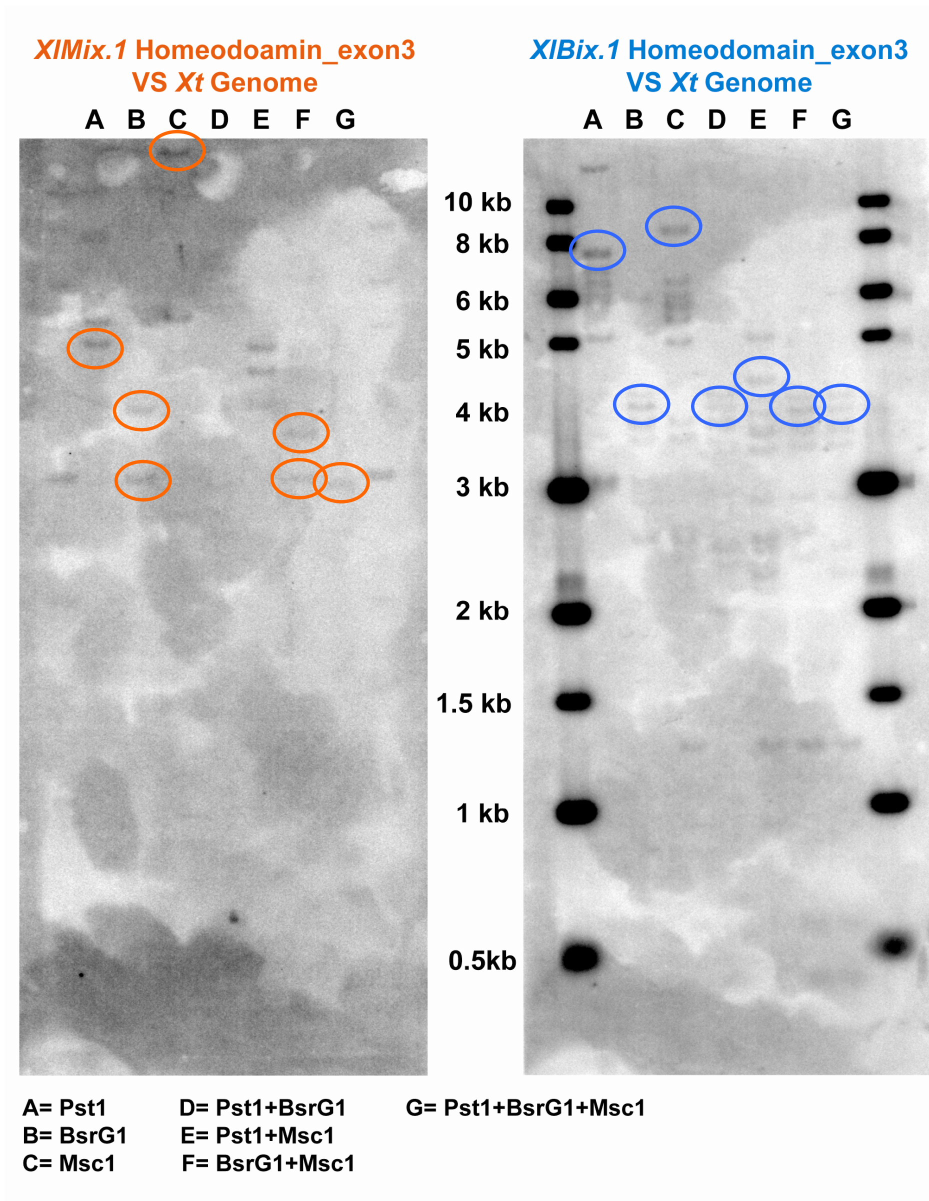


Figure 3.4 – Southern blot results for XlMix.1 and XlBix.1 on *Xenopus tropicalis* genomic DNA

Xenopus tropicalis genomic DNA was digested with the same combination of enzymes as that of the axolotl. Digested *Xenopus tropicalis* genomic DNA was hybridized with the probe covering the highly conserved regions; homeodomain and exon 3, from *Xenopus laevis Mix.1* and *Bix.1*. The left hand blot indicates the result for *XlMix.1* hybridization and the right hand blot indicates the result for *XlBix.1*. Orange circles represent the bands from *XtMix.1*, and blue circles represent the bands from *XtBix.1*. All unexpected bands in both blots illustrate that there are multiple copies of *Mix-like* genes in the *Xenopus tropicalis* genome.

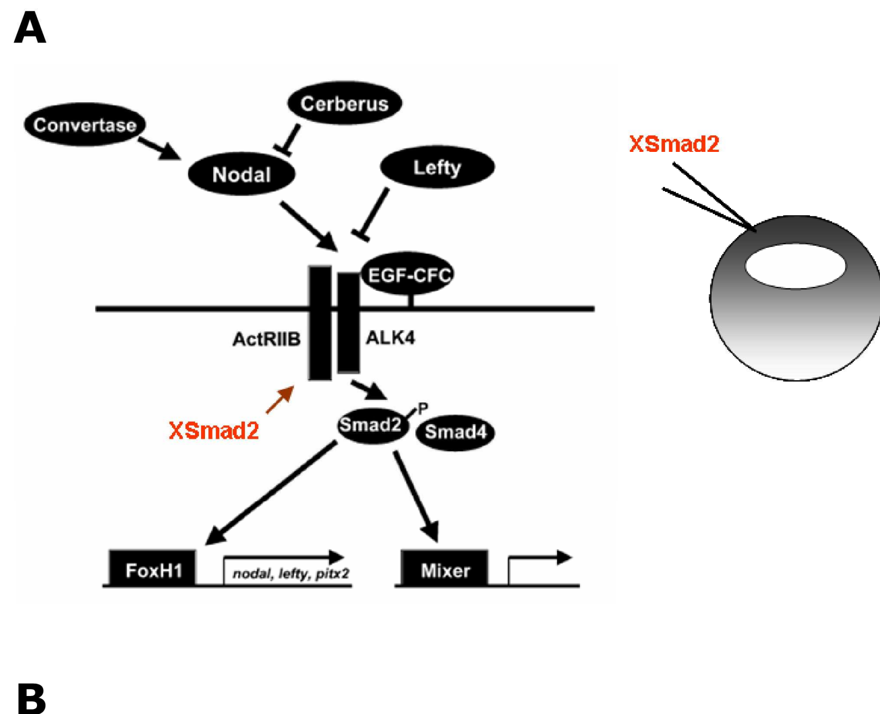
3.5 Cloning and characterization of *AxNodal*

After identifying a single *Mix* gene in the axolotl, we turned our attention to the Nodal family. We set out to identify and clone the axolotl *Nodal* gene or genes and identify if only one *Nodal* gene exists in the axolotl as with *AxMix*.

Prior attempts to clone axolotl *Nodal* sequences by library screening had not been successful (G.Swiers pers. Comm.). In the absence of axolotl EST or genomic sequences homologous to *Nodal*, we used a PCR-based approach with degenerate primers designed against the conserved regions of *Nodal* from different species including human, mouse, chick, *Xenopus* and zebrafish (see methods). Initially this was also unsuccessful; therefore we used an approach designed to enrich Nodal transcripts in axolotl animal caps. Previous studies have demonstrated that a constitutively active form of Smad2 can be made by the replacement of phosphorylated serines with acid amino acids (Ser465,467Glu) (Funaba and Mathews, 2000). We over-expressed this constitutively active *Xenopus laevis* Smad2 mRNA (100pg) to activate endogenous *Nodal* gene expression in axolotl embryos (Figure 3.5A). Using cDNA derived from these embryos we obtained a degenerate fragment. After cloning this fragment into T-vector, we randomly picked 16 clones and sequenced them. Seven sequences had no homology to any known sequence. The remaining nine sequences were all orthologs of *Nodal*. Eight of these clones were identical and are referred to as *AxNodal-2.* The remaining one unique sequence was called *AxNodal-1* due to its close homology to other amphibian *Nodal* genes (see below). This suggests that at least two *Nodal* genes exist in the axolotl. To obtain the complete 5′ and 3′

CDS sequence of these two *AxNodal* genes, rapid amplification of cDNA ends (RACE) was carried out using 5' and 3' RACE ready cDNA from stage 10.5 axolotl embryos. With the combination of 5' and 3' RACE reactions, complete sequences for these two genes were assembled. *AxNodal-1* contains an open reading frame (ORF) of 1,248 bp, giving a predicted polypeptide of 416 amino acids. *AxNodal-2* has a 1,200 bp ORF giving a predicted polypeptide of 400 amino acids. Phylogenetic analysis shows that *AxNodal-1* is most closely related to a *Nodal* gene identified in *Cynops* (Ito et al., 2006), another urodele, and then to the *Xenopus nodal* genes *Xnr1,2,3,5* and *6*. *AxNodal-2* clusters with *Xnr4* from *Xenopus* (Figure 3.6). To confirm these sequences are *Nodal* family sequences, databases were searched using NCBI BLAST which provided the nucleotide alignments (Figure 3.7A) and conserved domain prediction identity (Figure 3.7A), confirming these two clones are Nodal candidates. Both AxNodal-1 and AxNodal-2 contain the TGF-β family propeptide, the RXXR cleavage site and 7 cysteine residues which form a cysteine knot in the C-terminal region. This cleavage site and cysteine knot is conserved among all TGF-β superfamily proteins (Kingsley, 1994) (Figure 3.7B).

A



B

Gene	Blast program	Hit	Species	Gene	Accession Number	Identities	E-value
AxNodal-1	BLASTN 2.2.22+	1	Cynops	CyNodal	AB212661.1	80%	0
		2	Cynops	Cynr-1	AB114684.1	86%	2E-80
		3	Polypterus	squint	AB500934.1	77%	6E-62
		4	Xenopus laevis	Xnr-1	NM_001085796.1	76%	3E-53
		5	Chicken	NODAL	XM_424385.2	69%	1E-52
	BLASTP 2.2.22+	1	Cynops	CyNodal	BAD99300.1		0
		2	Polypterus	squint	BAH58785.1		2E-111
		3	Xenopus laevis	Xnr-2	NP_001081436.1		1E-109
AxNodal-2	BLASTN 2.2.22+	1	Horse	Nodal	XM_001503737.1	73%	5E-38
		2	Paralichthys	southpaw	AB232903.1	72%	2E-36
		3	Mouse	Nodal	NM_013611.4	73%	8E-35
		4	Pig	Nodal	XM_001928024.1	71%	8E-35
		5	Rat	Nodal	NM_001106394.1	72%	8E-35
	BLASTP 2.2.22+	1	Polypterus	squint	BAH58785.1		9E-71
		2	Polypterus	southpaw	BAF35855.1		7E-69
		3	Xenopus laevis	Xnr-4	NP_001081816.1		3E-68

Figure 3.5 – AxNodal gene cloning and phylogenetic anaylsis

(A) Scheme of the basic nodal signalling pathway. With the overexpression of *Xenopus laevis Smad2* RNA, we expected to induce the expression of Nodal genes via FoxH1 downstream signalling in the axolotl.

(B) NCBI BLAST results for the *AxNoda-1* and *AxNodal-2* coding sequence and translation amino acid sequence. BLASTN is a nucleotide blast, and BLASTP is an amino acid blast.

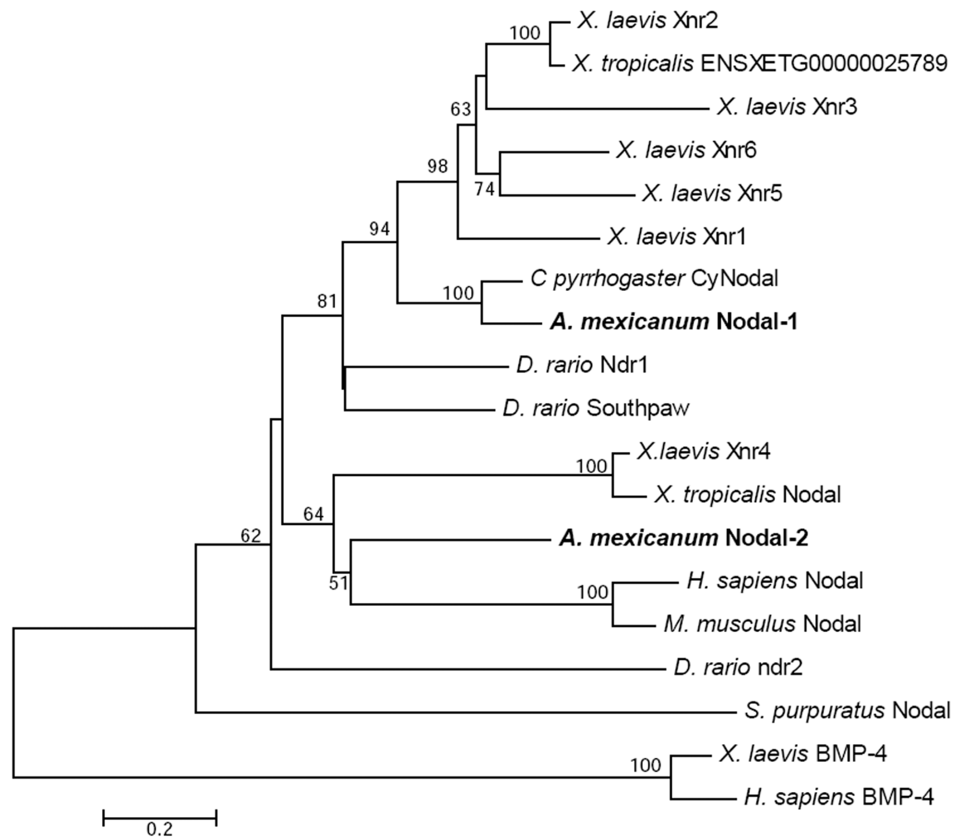
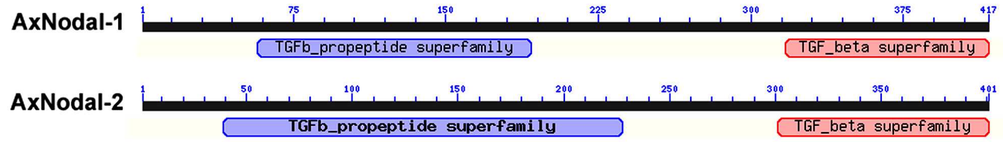


Figure 3.6 - Protein alignments of Nodal sequences from *X. laevis, C. pyrrhogaster, A. mexicanum, H.sapiens* and *M. musculus*.

Phylogenetic tree constructed using Neighbour-joining (JTT Matrix), bootstraps shown if the support is greater than 50. *Nodal* sequences from *X. laevis*, zebrafish, *Cynops*, mouse and human were used to construct the tree. *AxNodal-1* groups with the previously identified *CyNodal* and roots at the base of the *X. laevis* cluster of *Xnr1, 2, 3, 5* and *6* (and putative *X. tropicalis* sequence). *AxNodal-2* groups with *X. laevis Xnr4* and *X. tropicalis Nodal* and the mammalian *Nodal* sequences. Human and *X. laevis BMP-4* were used as an out-group.

A



B

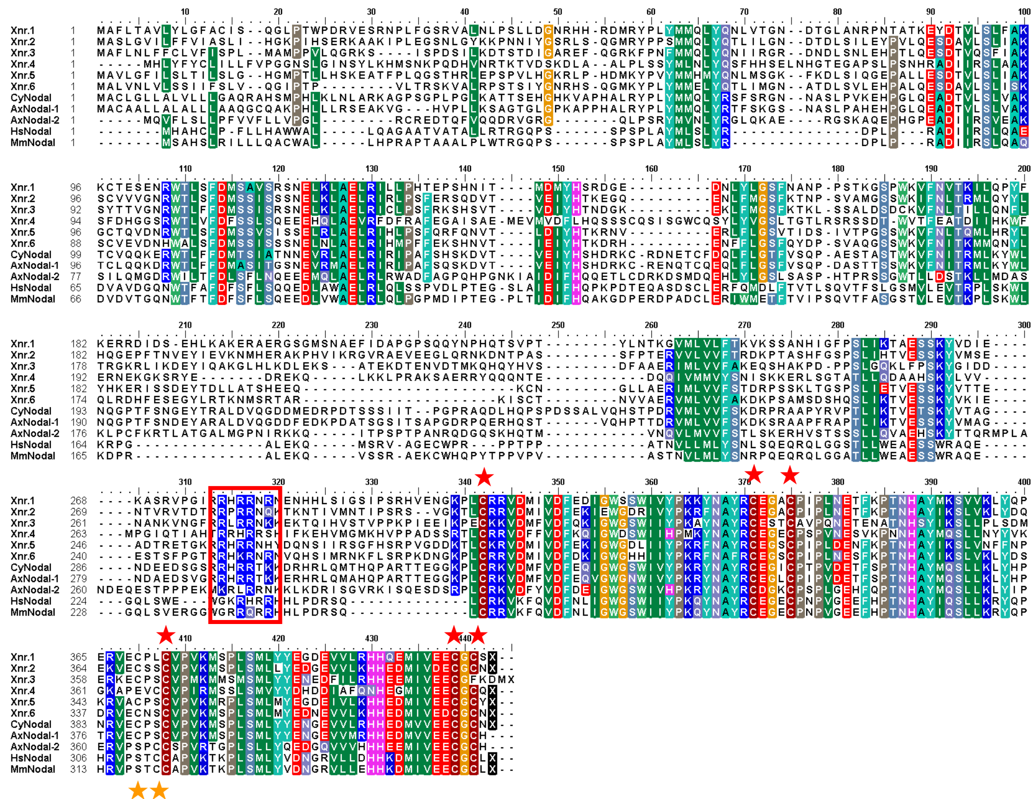


Figure 3.7 - Protein alignments of Nodal sequences from *X.laevis, C. pyrrhogaster, A. mexicanum, H.sapiens* and *M. musculus*

(A) The conserved domain prediction indicates both sequences are TGF-β family proteins. This is the amino acid sequence comparisons of conserved TGF-β pro-peptide domain (light purple) and conserved carboxy-terminus (pink) of TGF-β proteins between these two genes.

(B) The color regions indicate conserved amino acids and the red square is the conserved cleavage site of TGF-β family. In addition, the stars indicate the c-terminal conserved cysteines; red color is the conserved cysteine residue and yellow color is the alternative cysteine distribution.

As with *AxMix*, southern blotting was used to determine whether any other Nodal orthologs could be found in the axolotl genome. To understand the genomic structure of these two *Nodal-like* genes, we used the human, mouse and *Xenopus tropicalis nodal-related* gene structure to predict the possible exon-intron boundaries in the axolotl *Nodal* genes (Figure 3.8). The sequence alignments indicate both *Nodal* genes have two introns as do all other *Nodal* homologs. Primer pairs were designed to amplify the genomic fragments of these two genes. Genomic PCR indicated that *AxNodal-1* has two introns as found in *Xenopus nodal-related* genes, human and mouse *Nodal* genes. For *AxNodal-2*, we could only clone the intron2 fragment, which is 8.8kb in length. Based on the conservation of the exon-intron junctions, we suggest *AxNodal-2* has two introns like *AxNodal-1*. To date we have been unable to clone intron1 of *AxNodal-2* and I discuss this further below.

A probe including exon2 and exon3, which contains the conserved TGF-β pro-peptide domain and conserved carboxy-terminus of all TGF-β family members, was used to analyze the genomic structure of the *AxNodal* genes. As with *AxMix*, we constructed a restriction map for genomic *AxNodal-1* and *AxNodal-2*, allowing us to predict the restriction fragments we would expect in the southern blots (Figure 3.9A).

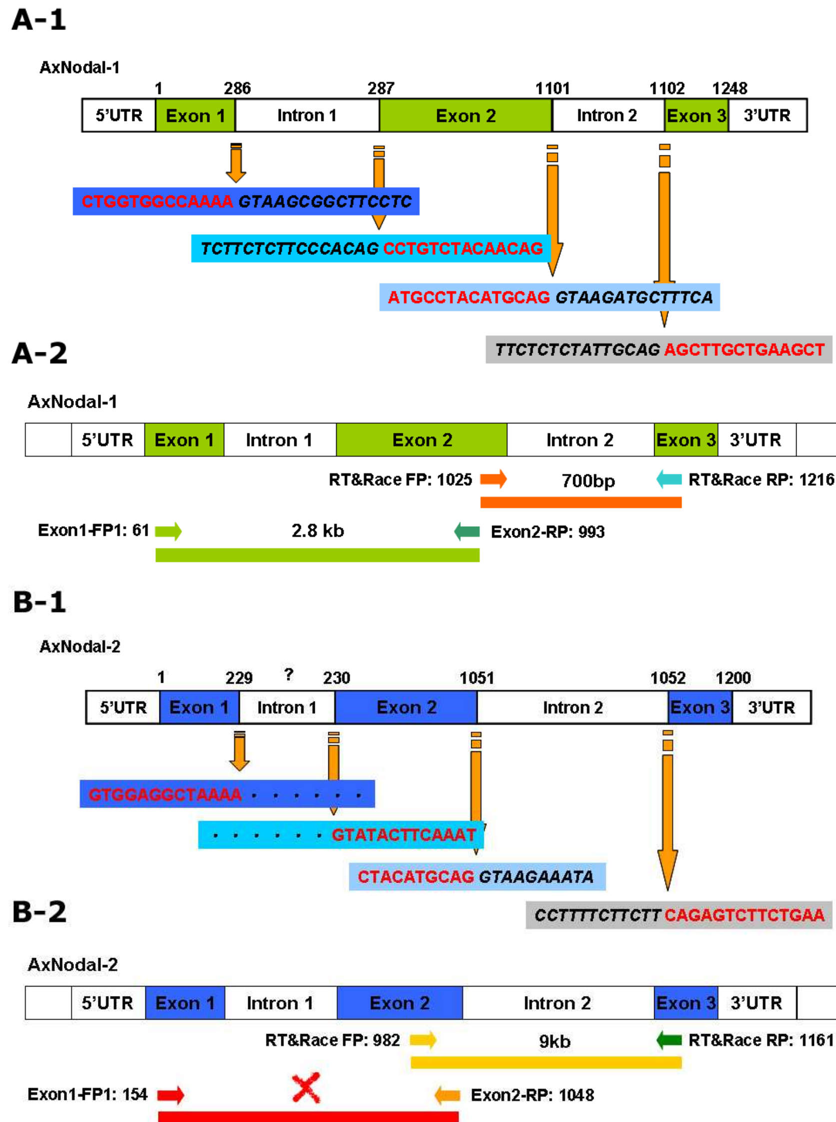


Figure 3.8 - Genomic organization of AxNodal-1 and AxNodal-2 genes

The sequence alignment study revealed the conserved splicing junctions of most *nodal-related* genes among different species. Two *AxNodal* genes; *AxNodal-1* **(A)** and *AxNodal-2* **(B)**, show that there are two introns among three exons. **(A-2)** and **(B-2)** show two primer pairs designed to amplify the intron region in each *Nodal* gene and the size of the amplified genomic fragments has been shown in this diagram. Scheme of **(A-1)** and **(B-1)** show the exon-intron junctions with perfect splice donor and acceptor consensus; the red sequences indicate the exon sequence near the exon-intron junctions and black italic sequence indicate the intron sequence.

Firstly, a probe containing the *AxNodal-1* exon2 and exon3 was used to detect if *AxNodal-1* is present as a single copy gene. As well as the *AxNodal-1* specific bands, we identified a number of additional bands of varying intensity, indicating the presence of other *nodal-related* members in the axolotl. The obvious source of these unexpected fragments is the second *Nodal* gene, *AxNodal-2*. The southern blot with *AxNodal-2* exon2_exon3 was therefore carried out (Figure 3.9B). Alongside the fragments matching our prediction, the *AxNodal-2* exon2_exon3 probe also detected several other fragments. However, these match to specific fragments from AxNodal-1 and vice-versa (Figure 3.9C). All the fragments in both blots can be explained and are derived from the two *AxNodal* genes. Therefore, the southern blotting results of the exon2-exon3 probes for *AxNodal-1* and *AxNodal-2* confirm the observation that there are two, not one, *AxNodal* genes in the axolotl genome.

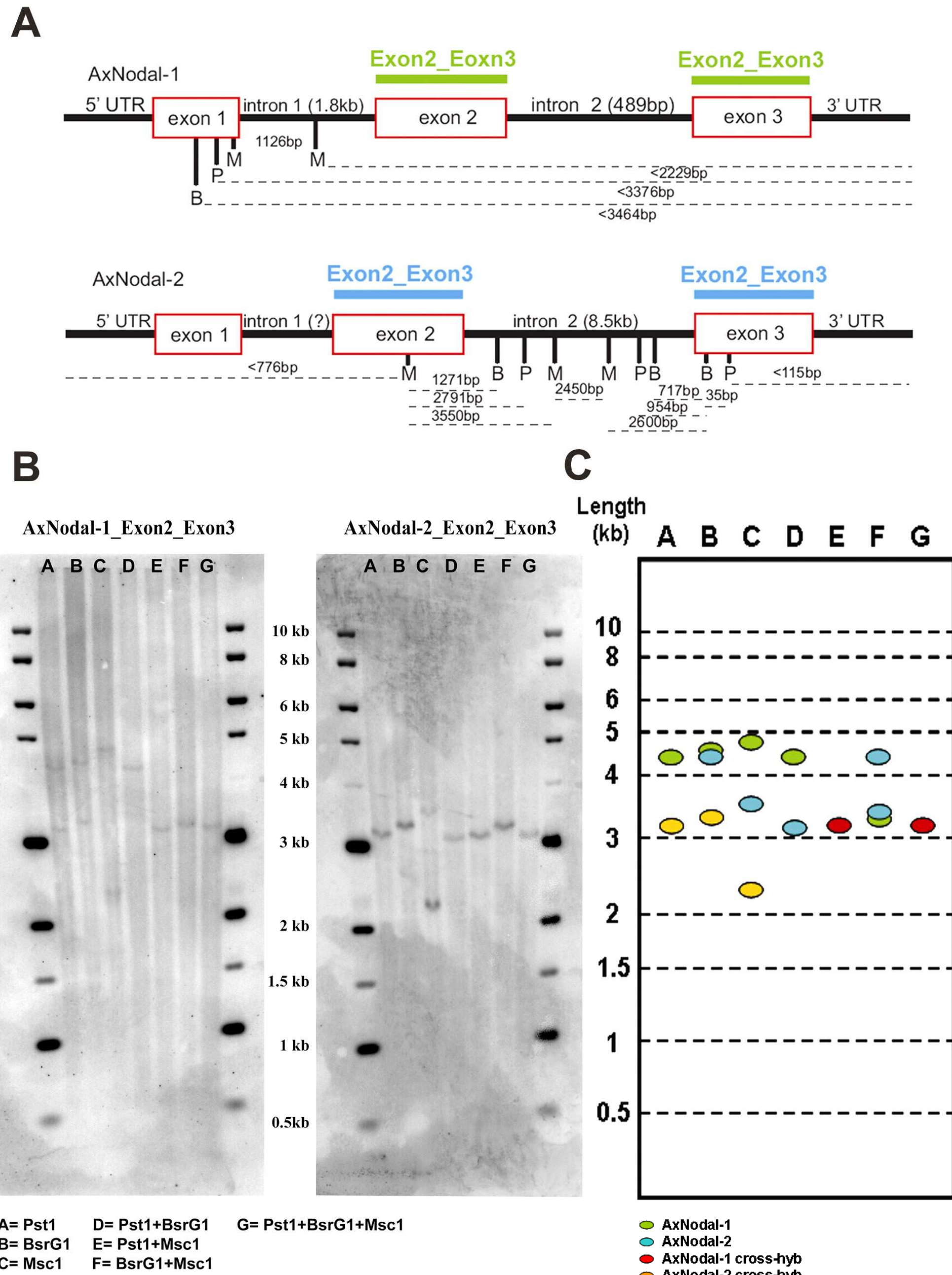


Figure 3.9 - Southern blotting results for AxNodal-1 and AxNodal-2 **(A)** Axolotl genomic DNA was digested using all combinations of three enzymes; Pst1 (P), BsrG1 (B), Msc1 (M). The probe region is marked on the cartoon in green (*AxNodal-1*) and blue (*AxNodal-2*) and the band sizes are indicated in the restriction map of the *AxNodal-1/2* genomic fragment. **(B)** Digested axolotl genomic DNA was probed with Exon2_Exon3 region – *AxNodal-1* probe (left hand blot) and *AxNodal-2* probe (right hand blot). **(C)** shows the combination results of *AxNodal-1* and *AxNodal-2*. Green represents *AxNodal-1*, blue, *AxNodal-2*, red, *AxNodal-1* sequence that cross-hybridises with *AxNodal-2* and yellow, *AxNodal-2* sequence that weakly cross-hybridises with *AxNodal-1*.

3.6 The syntenic relationships of the *Nodal* and *Mix* homologues in human, mouse and *Xenopus tropicalis*

Comparison of the amino acid sequences of *AxMix* and the *AxNodals* with other vertebrate homologs have illustrated their phylogenetic relationship (G.Swiers 2008, PhD thesis and this work). Investigating the genomic structure of *AxMix* and the *AxNodals* is another method to compare the gene homologues in different vertebrates. Sequencing projects have provided substantial genome information enabling comparative sequence analysis. Synteny analysis, meaning the identification of a set of genes that share the same relative arrangement on the chromosomes of two or more species, has been used to distinguish between homologs, orthologs and paralogs (Frazer et al., 2003).

Recently the draft genome sequence assembly of *Xenopus tropicalis* has been published, revealing substantial shared synteny with human and chicken (Hellsten et al., 2010). Here we have investigated the syntenic relationships for the *Nodal* and *Mix* genes among human, mouse and *Xenopus tropicalis* (Figure 3.10A and 3.11). As also reported by (Hellsten et al., 2010), the ENSEMBL synteny view displays *Xtnr4* on scaffold 204 is the ortholog of the single human and mouse *Nodal. Xtnr4* is located between the *EIF4EBP2* / Q6P382_XENTR and *PALD* (KIAA1264 / X99364 / TGas002h03.1) genes. Interestingly, this *Nodal* syntenic location appears to have been deleted from the chicken genome. Instead, chick nodal has synteny to a separate nodal gene cluster including *Xtnr1, 2, 3, 5* and *6* on scaffold 34,

lying between *EIF4EBP1* and *ASH2L* genes (Figure 3.10B).

Similarly, human and mouse *Mixl1* share synteny between the *LIN9* and *ACBD3* genes (Figure 3.11). Unfortunately, due to the gene annotation for *Xenopus tropicalis* genome is incomplete, *XtMix* syntenhy on scaffold 2320 is not available. Notably, unlike the two conding exons seen in the human and mouse *Mix* homologs, both *XtMix* and *AxMix* have three coding exons. Although genome sequence is not yet available from the axolotl, synteny analysis among several species provides us with new insights into evolutionary relationships and can help identify homologous genes and regulatory elements (Frazer et al., 2003).

A

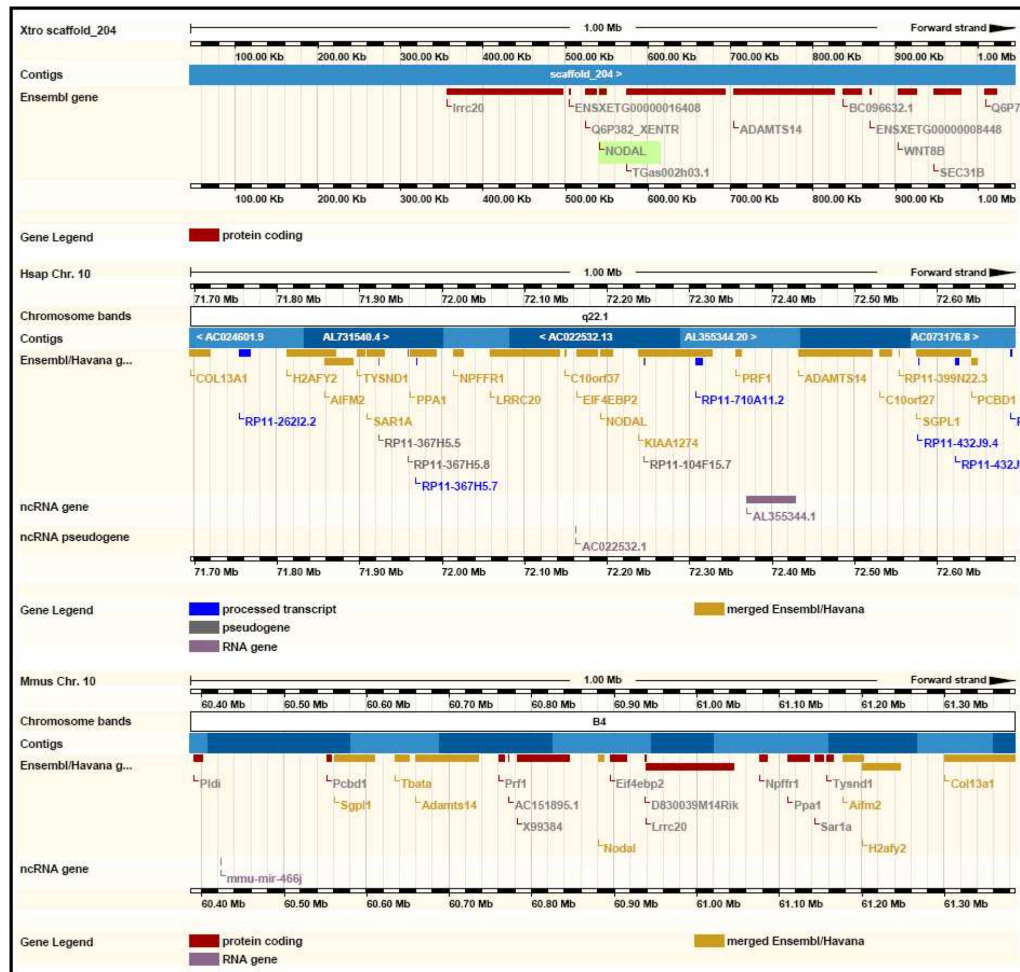


Figure 3.10A - Syntenic relationships of the *Nodal* homologs in *Xenopus tropicalis*, human and mouse

The figure shows that the *nodal* locus *Xtnr4* in *Xenopus tropicalis* shares sytheny with the nodal locis in the human and mouse genome, i.e. that they have the same gene neighbours *Nodal* homologs, namely EIF4EBP2 / Q6P382_XENTR and PALD (KIAA1264 / X99364 / TGas002h03.1).

B

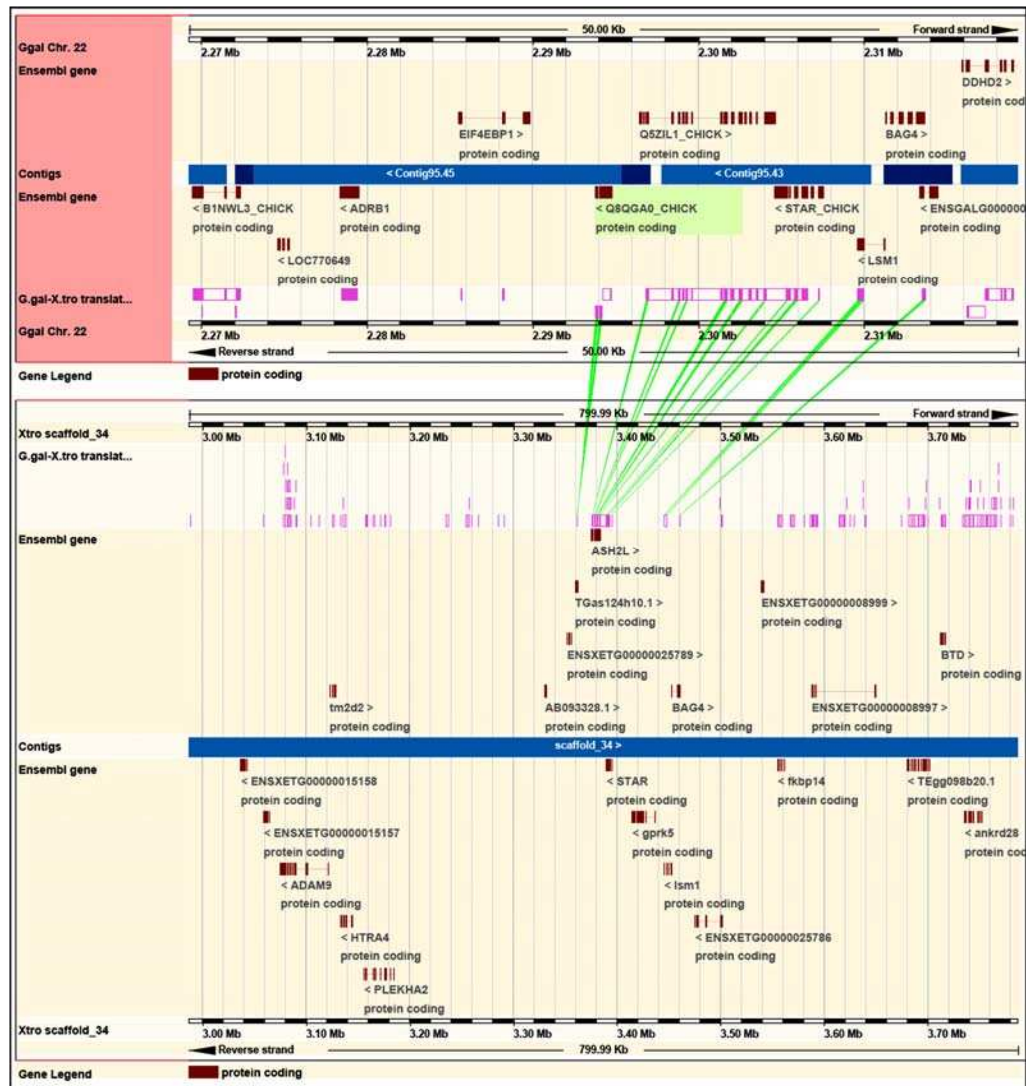


Figure 3.10B - Syntenic relationships of the *Nodal* homologs in *Xenopus tropicalis* and chicken

The figure summarizes the other *nodal* loci in *Xenopus tropicalis*, in which *Xtnr3* (AB093328.1) shares the same neighboring gene, *ASH2L* (Q5ZIL1_CHICK), as that of the chicken Nodal homolog. Due to repeated *Xtnr5* and *Xtnr3* as well as incomplete gene annotation, we cannot assemble and define the other neighboring gene, *EIF4EBP1* in this *nodal* locus. As the consequence, it can only demonstrate one *Xtnr3*, adjacent to *ASH2L*.

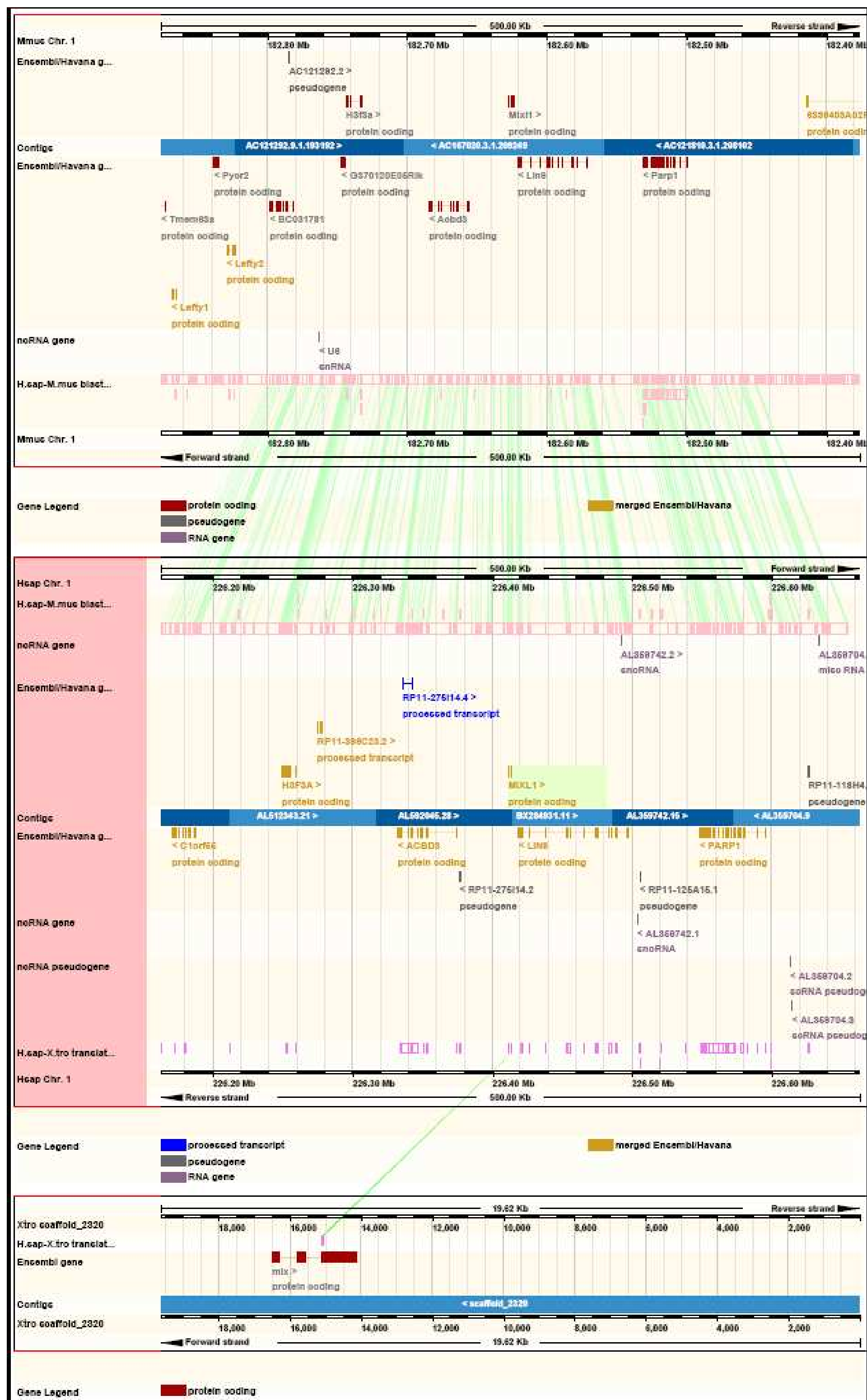


Figure 3.11 - Syntenic relationships of the *Mix* homologs in *Xenopus tropicalis*, human and mouse

The figure summarizes the *Mix* locus in mouse and humans, in which different *Mix* homologs share the same neighboring genes, *LIN9* and *ACBD3*. However, due to incomplete gene annotation in *Xenopus tropicalis*, the *XtMix* synteny cannot be determined.

3.7 Discussion

The *Mix-like* axolotl ortholog, *AxMix*, had previously been identified by G.Swiers (G.Swiers PhD thesis 2008). Here, we also describe the cloning and characterization of two *nodal-related* genes, *AxNodal-1* and *AxNodal-2.* Southern blotting analyses provide evidence to show that *AxMix* is present as a single copy gene. *AxNodal* southern blotting supports that there are only two *Nodal* orthologs in the axolotl genome. Note that this is unlike the one *Nodal* gene in mammals. These observations suggest a more detailed understanding of the role of the *Mix-like* and *nodal-related* family during early axolotl embryo development is required.

Interestingly, the screening of a stage 10.5 cDNA library with *Xenopus laevis Mix.1* failed (G.Swiers PhD thesis). The full length sequence of the axolotl *Mix* ortholog was only identified using the mouse *Mixl1* sequence as a probe. This is perhaps surprising given that we show here the length and genomic organization of *AxMix* is most like the amphibian *Mix* orthologs. However, the major functional domain, the homeobox, is more closely related to the amniote *Mix-like* genes (G.Swiers PhD thesis). To identify if *AxMix* is present as a single copy in the axolotl genome, southern blotting was carried out on axolotl genomic DNA using various regions of the *AxMix* genomic region as probes. The probe including the *AxMix* full-length CDS region should hybridize with the fragments containing exons. However, alongside the *AxMix* specific bands, there were also some nonspecific bands of approximately 600-700 base pairs that could not be explained. Probes containing intron2 generated fragments that perfectly matched our prediction. However, an

intron1-exon2 probe excluding the homeodomain also detects small fragments around 600-700bp. Given that the intron2, exon3-3'UTR and hoemobox-exon3 analyses all suggest that there is only one *Mix* gene in the axolotl, we conclude that pseudogenes or genes of distantly related subtypes with high copy number must exist in the axolotl genome. An additional consideration is the high GC content of exon 2 (71-75%) which may cause technical difficulties in a southern blot.

Nodal-related genes have been found in all vertebrates examined, including zebrafish, *Xenopus*, chicken, mouse and human. Although there are at least 6 *nodal-related* genes in *Xenopus laevis*, only single copy genes have been detected in the mouse and human genomes. How many *nodal-related* genes are there in the axolotl? Two *AxNodal* genes, *AxNodal-1* and *AxNodal-2* were identified from stage 10.5 cDNA pools, confirmed as *Nodal* orthologs (NCBI BLAST-Figure 3.5B), and the phylogenetic relationship between the *AxNodal* genes and other vertebrate *Nodal* genes identified (Figure 3.6A). Conserved domain predictions indicate both sequences are TGF-β family proteins, characterized with a conserved TGF-β pro-peptide domain and conserved carboxyl-terminus between these two genes.

The genomic organization of *AxNodal-1* and *AxNodal-2* was investigated by PCR. The intron1 and intron2 regions have successfully been amplified and sequenced in *AxNodal-1*. For *AxNodal-2*, two perfect exon-intron junctions exist. However, only intron2 has been successfully amplified and we are still unable to amplify intron1 from *AxNodal-2*. This could be caused either be extreme length of intron1, high GC content, or secondary structure of the

genomic DNA causing the PCR to fail.

In the axolotl, we now know there are at least two *Nodal* genes, *AxNodal-1* and *AxNodal-2*. To confirm these to be the only *Nodal* orthologs, genomic southern blots were performed. Low stringency hybridization was carried out and alongside the expected *AxNodal-1* and *AxNodal-2* specific bands, unexpected bands were found in each blot. However, the non-specific bands appearing in the *AxNodal-1* blot map to those expected for *AxNodal-2*, and vice versa. The cartoon illustrates the expected band sizes and indicates the specific bands from the *AxNodal-1* and *AxNodal-2* probes, and those that cross-hybridize between the *AxNodal-1* and *AxNodal-2* sequences (Figure 3.8C).

As complete genome sequences have become available it has become clear that two rounds (2R) of whole genome duplication have occurred during early vertebrate evolution (Dehal and Boore, 2005). As a consequence, a gene present in the vertebrate ancestor might be expected to be found in four copies in extant vertebrates. However, it is clear that the retention of all four copies is not generally true as copy number analysis and large scale evaluation of genome sequence does not reveal a peak at four copies of a gene per family (Dehal and Boore, 2005). The exact copy number of the nodal gene family has been subject to much discussion. For example, studies in the zebrafish identify that the three zebrafish nodal genes arose during two genome duplication events from a single ancestral nodal (Fan and Dougan, 2007). Synteny analysis reveals that *Xenopus tropicalis* and the lizard *Anolis carolinensis* possess two equivalent nodal loci that arose during one of the

whole-genome duplications during vertebrate evolution (Hellsten et al., 2010). Mammals and birds possess only one Nodal gene, with a different locus having been lost in mammalian and avian evolution (Hellsten et al., 2010).

Genome duplication generate a complete set of paralogs, but translocation, inversion and deletion are likely to modify the number of paralogues subsequently (Dehal and Boore, 2005). The vast majority of duplicated genes were deleted, suggesting that only a few genes may have been responsible for the increased complexity seen in vertebrates. Presumably each copy of a duplicate gene may be subject to distinct evolutionary constraints; however, study in *Xenopus laevis* suggests multiple genetic mechanisms such as neofunctionalisation, subfunctionalisation and redundancy could promote the retained expression of gene duplicates within the same genome (Chain and Evans, 2006). Notably, it is possible that genome duplications and the associated accelerated rate of sequence change chould have played an important role in increasing vertebrate complexity before returning to a single copy during the evolution of vertebrates. As there are two single copy *Nodal* genes and one *Mix* gene in the axolotl genome, it will be interesting to compare their functions and learn more about their syntenic relationship to their homologues in other vertebrates.

In summary, previously we had cloned an axolotl ortholog of the *Mix-like* family of paired-like homeodomain transcription factors and now we provide evidence to suggest that *AxMix* is present as a single copy in the axolotl. Furthermore, we identified two orthologs of Nodal TGF-β signalling molecules,

AxNodal-1 and AxNodal-2. Southern blotting result confirms these genes are single copies in the axolotl genome. Amino acid sequence comparisons have shown that *AxNodal-1* is most closely related to the amphibian *nodal-related* genes, while *AxNodal-2* clusters with more mammals-like *Nodal* orthologs.

Chapter 4. Investigating the role of Nodal in mesoderm and endoderm specification

4.1 Introduction

Data from *C.elegans* and the sea urchin suggests the existence of a bipotential germ layer, the mesendoderm, from which the endoderm and some mesoderm are derived (Angerer and Angerer, 2000; Maduro et al., 2001; Maduro, 2006). The existence of mesendoderm in vertebrates is suggested by the co-expression of endoderm and mesoderm marker genes during the late blastula, predominantly observed in *C.elegans*, zebrafish and *Xenopus* (Rodaway and Patient, 2001). Evidence from the zebrafish supports that a single wild type cell transplanted from the margin at sphere stage into the margin of a MZ*oep* mutant embryo can internalise and express mesendodermal markers as judged by the expression of *axial/foxa2* and *sox17* (Carmany-Rampey and Schier, 2001). Similarly, in *Xenopus* embryos the cells of the marginal zone contain precursors for endoderm and mesoderm as judged by their ability to express both the presumptive mesodermal marker *Bra* as well as endodermal markers such as the *Mix-like* genes (Kimelman and Griffin, 2000; Zorn and Wells, 2007).

In axolotls, the mechanism specifying endoderm and mesoderm from mesendoderm is unclear, although presumed to be similar to that of other amphibians. Given the results presented in chapter 3, the working hypothesis is that a simplified mesendoderm GRN may be operating in the axolotl, with fewer copies of *Mix* and *Nodal* and the network as a whole more

closely similar to a predicted amniote GRN (Figure 4.1). The temporal and spatial expression of *AxMix* and perturbation experiments to investigate its role in mesoderm and endoderm specification have already been carried out by G.Swiers in the laboratory, and I have shown that the *AxMix* gene is present as a single copy in the axolotl genome. Nodal signalling in mouse and *Xenopus* is important during gastrulation for the induction of endoderm and mesoderm (Artinger et al., 1997; Brennan et al., 2001; Jones et al., 1995; Kimelman and Kirschner, 1987; Kumano and Smith, 2000; Latinkic and Smith, 1999; Lemaire et al., 1998; Tian and Meng, 2006; Yamaguchi et al., 1999). Here I explore the role of the two *AxNodal* genes in mesoderm and endoderm specification and further investigate the role of the *AxMix* gene in mesoderm induction.

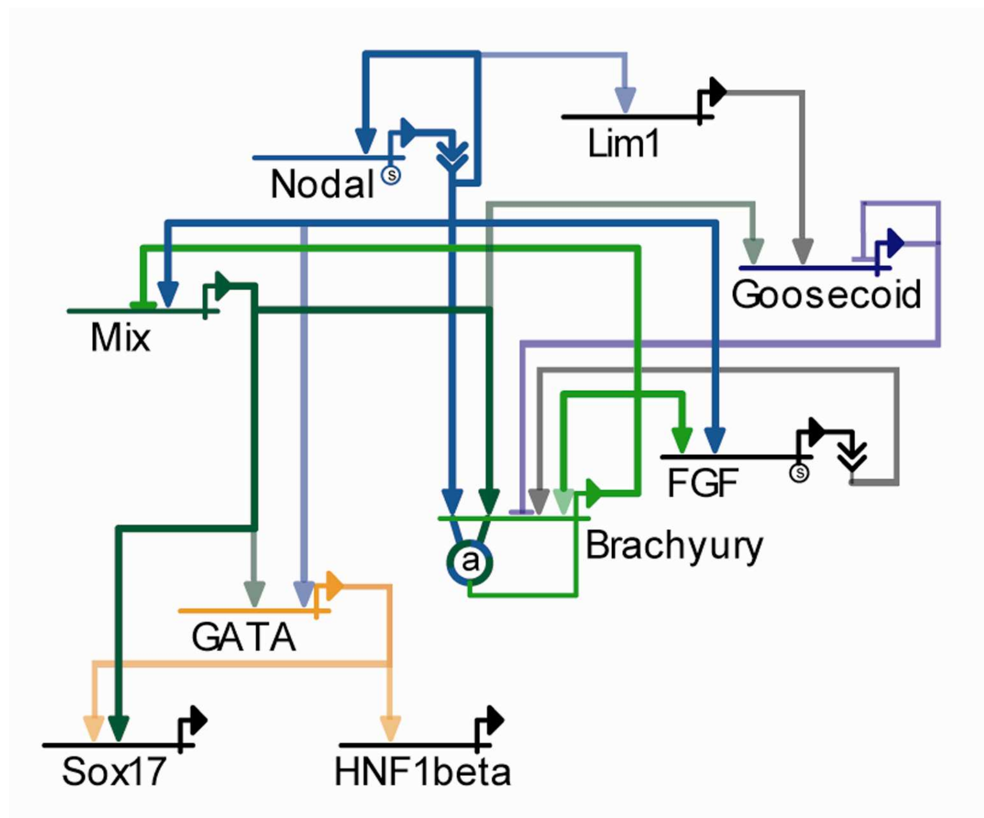


Figure 4.1 – A simplified mesendoderm GRN, downstream of Nodal

The *Xenopus* mesendoderm GRN can be simplified by presenting only single copies of genes. Here the network is illustrated downstream of *Nodal*. Nodal regulates the expression of a set of transcription factors and signals that establish boundaries of gene expression and consequently the regulation of the markers of mesoderm or endoderm genes.

4.2 AxNodal expression

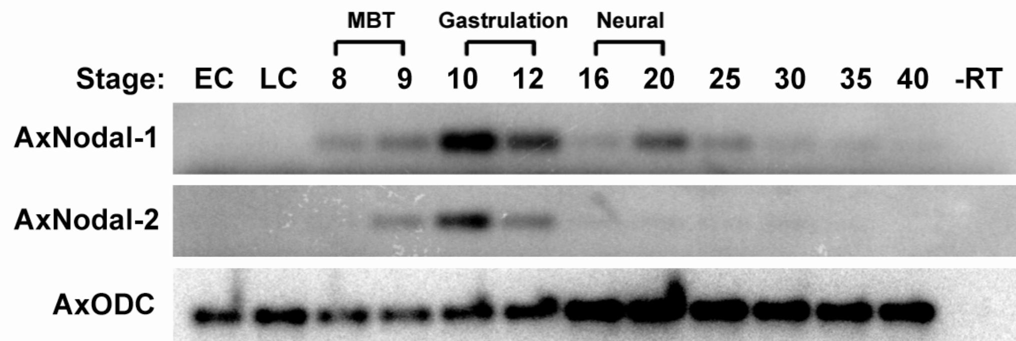
4.2.1 Temporal expression of AxNodal-1 and AxNodal-2

To investigate the temporal expression of the *AxNodal* genes during axolotl development, we designed primers and carried out RT-PCR followed by southern blotting (Figure 4.2A). Neither *AxNodal-1* nor *AxNodal-2* expression is detectable at early-cleavage (4-8 cell) or late-cleavage (8-16 cell) stages, showing that these two genes are not maternally expressed. *AxNodal-1* and *AxNodal-2* transcripts are first detected at a low level during the early blastula stage (stage 8) with *AxNodal-1* higher than *AxNodal-2*. The abundance of both *Nodal* transcripts increases abruptly at the late blastula stage (stage 9) and remains elevated until the end of gastrulation (stage 12). When embryo development reaches neural stages, the level of *Nodal* transcripts decreases significantly. A second phase of *AxNodal* expression occurs during late neural stages (stage 20), after which the levels of *AxNodal-1* transcripts return to a very low level.

To obtain better resolution over gastrula stages, and provide quantitation, we reanalyzed expression of these genes at more stages using quantitative real-time PCR (qPCR) (Figure 4.2B). The qPCR results confirm our observations of the expression pattern for the two Nodal genes showing that both commence expression at the mid-blastula stage (MBT) (stage 8), with transcript levels peaking in early gastrulae (stage 10). For a direct comparison of expression levels between *AxNodal-1* and *AxNodal-2*, both time courses are normalised to *AxNodal-1* at stage 12. This suggests that *AxNodal-1* is expressed at least two-fold higher than *AxNodal-2* at all stages. Therefore, we conclude that both *Nodal* genes are expressed zygotically and

predominantly during the late blastula and gastrula stages.

A.



B.

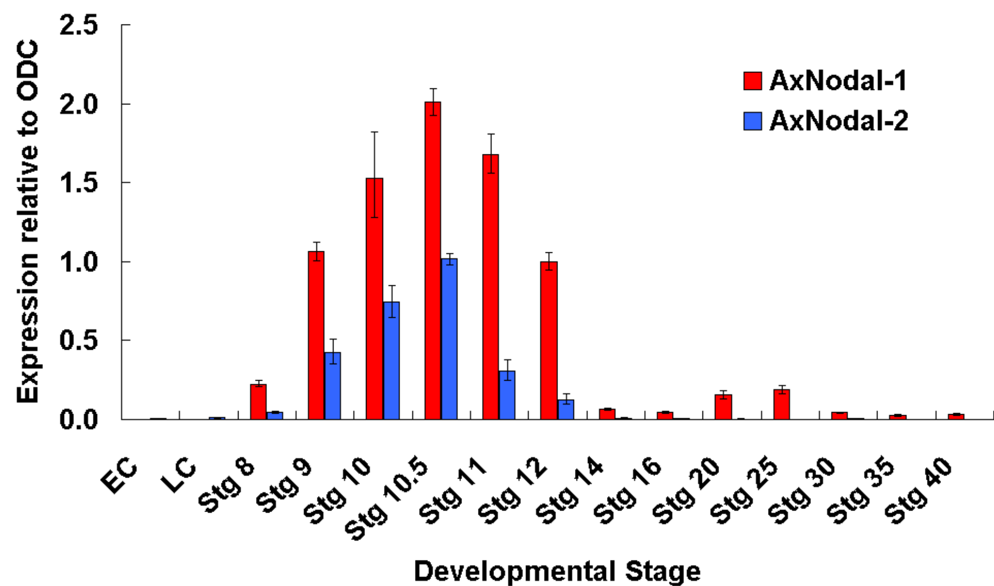


Figure 4.2 – Temporal expression pattern of Nodal-related genes through development

(A) The gene expression patterns of *AxNodal-1* and *AxNodal-2* were analyzed by RT-PCR (20 cycles) at various developmental stages. Both genes start zygotic expression at the MBT stage. The expression of these genes is very similar during gastrulation but it is not the same later on. *ODC* is used as a loading control. Reverse transcriptase-negative samples (-RT) show the absence of genomic DNA contamination. **(B)** qPCR was performed at the stages indicated and confirms the expression of *AxNodal-1* (red) and *AxNodal-2* (blue). Gene expression was normalised to *ODC* and then *AxNodal-1* at stage 12 to allow comparison of levels between *AxNodal-1* and *AxNodal-2*.

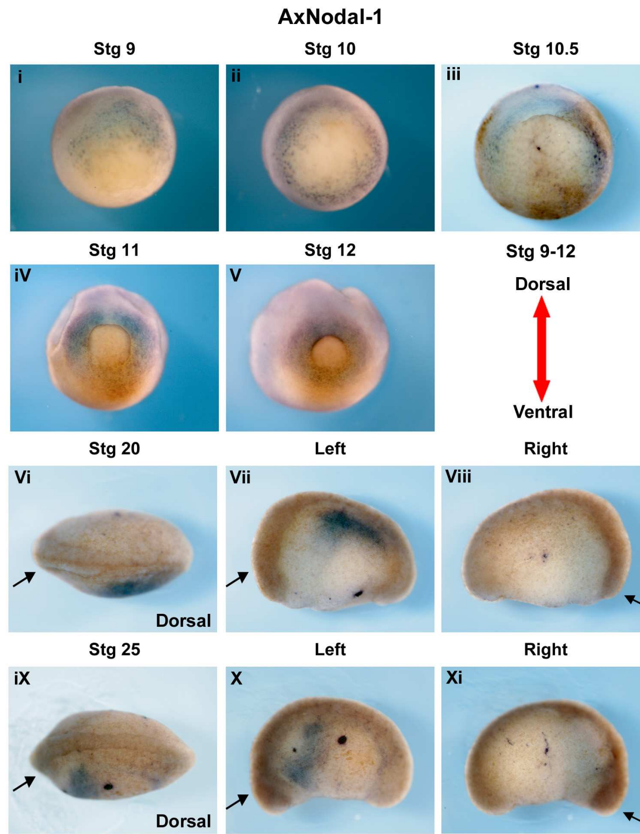
4.2.2 Spatial expression of AxNodal-1 and AxNodal-2 in early axolotl embryos

Whole-mount *in-situ* hybridization was carried out to investigate the spatial expression patterns of *AxNodal-1* and *AxNodal-2* in axolotl embryos at early developmental stages (Figure 4.3). At the late blastula stage (stage 9), both genes are expressed dorsally (Figure 4.3Ai and Bi). By stage 10, the dorsal expression of *AxNodal-1* and *AxNodal-2* spreads laterally around the marginal zone into the ventral region (Figure 4.3Aii and Bii). At stage 10.5 to 11, this expression is maintained in the dorsal-marginal zone with weak expression ventrally (Figure 4.3A and B iii-iv). This is reminiscent of the combined *Xenopus laevis Xnr1* and *Xnr2* expression patterns (Agius et al., 2000; Takahashi et al., 2006). By stage 12, *AxNodal-1* is strongly expressed around the blastopore, however, *AxNodal-2* is expressed dorsally and more towards to anterior than *AxNodal-1* (Figure 4.3Av and Bv) with little expression in the marginal and ventral zone. According to RT-PCR and qPCR results, the expression of *AxNodal-1* and *AxNodal-2* decrease by early neurala stages and only *AxNodal-1,* not *AxNodal-2,* shows the second later phase of expression. This observation is confirmed by whole-mount *in situ* hybridization (WISH). By stage 20 and stage 25, only *AxNodal-1* is detectable in the left lateral plate mesoderm, consistent with the well-characterized role for Nodal in left-right asymmetry (Figure 4.3A vi, vii and xi,x). *AxNodal-2*, which cannot be detected and lacks the later asymmetrical expression, has an expression pattern similar to *Xnr-4* (Joseph and Melton, 1997).

Figure 4.3 – WISH reveals expression patterns of AxNodal-1 and AxNodal-2

The localisation of *AxNodal-1* **(A)** and *AxNodal-2* **(B)** transcripts during embryogenesis. Whole-mount *in situ* hybridizations are shown sequentially for axolotl embryos at stages 9 (i), 10 (ii), 10.5 (iii), 11 (iv), 12 (v), 20 (vi - viii) and 25 (ix - xi). The mRNA expression is shown by the blue-purple staining. Gastrula embryos (i - v) are vegetal views. (vi – viii) Neurula and (ix – xi) tailbud embryos are dorsal and left-right side views with the black arrow indicating anterior. In gastrula stage embryos, *AxNodal-1* is first expressed in the future dorsal lip (Ai) and gradually extends to marginal and ventral areas (Aii – Av). *AxNodal-2* is first expressed in the future dorsal region with a little expression in the ventral site (Bi). During gastrulation, the expression pattern of *AxNodal-2* is more focused on the dorsal side and only weakly detected in lateral and ventral regions (Bii – Bv). In neurula and tailbud stage embryos only *AxNodal-1* shows the left-right asymmetrical expression pattern (A and Bvi – xi).

A.



B.

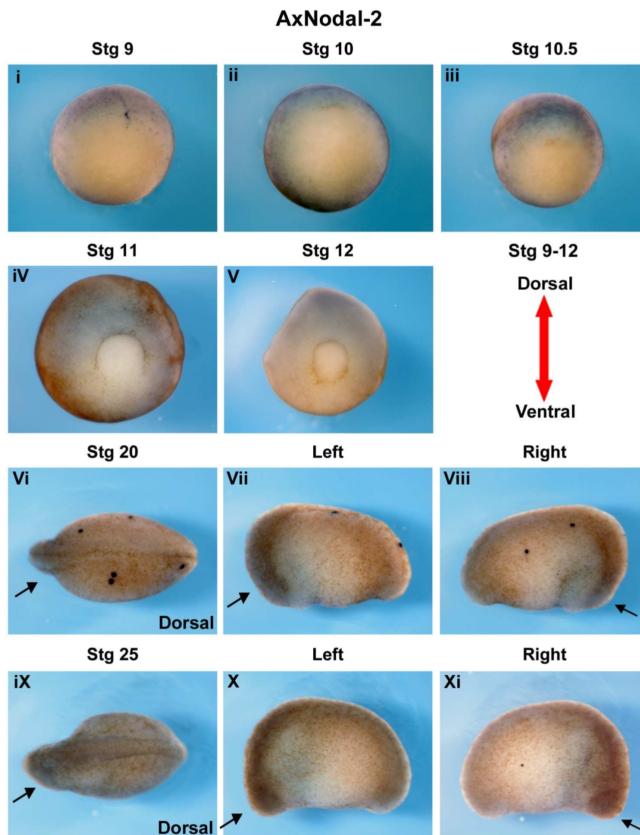
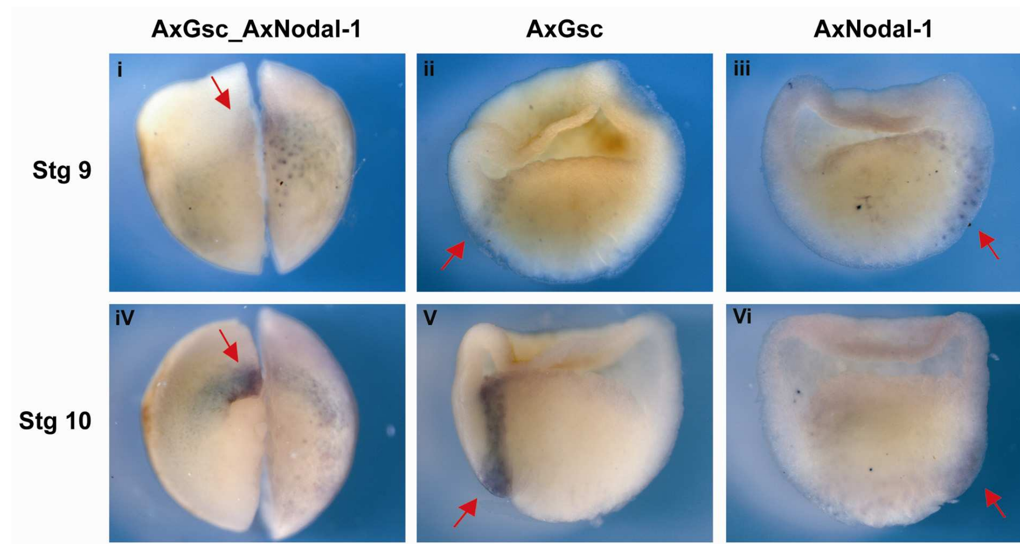


Figure 4.3 – WISH reveals expression patterns of AxNodal-1 and AxNodal-2

At stage 9, weak expression of *AxNodal-1* and *AxNodal-2* can be detected in the vegetal hemisphere, in particular around the marginal zone. To make sure the existence of graded AxNodal signals at this stage is on the future dorsal side of the embryo, *in situ* hybridizations for *AxNodal-1* and *AxNodal-2* on hemi-sectioned embryos were compared with *Goosecoid*, a dorsal mesoderm marker gene (Figure 4.4A). The expression of *AxNodal-1* and *AxNodal-2* were re-examined using a more sensitive *in situ* hybridization procedure, in which the embryos are fixed and hemisectioned facilitating the penetration of the probe into embryos (Figure 4.4B). At stage 9, both Nodal transcripts are detected in the future dorsal lip and dorsal vegetal region (Figure 4.4B i and iv). During early gastrulation *AxNodal-1* and *AxNodal-2* expression is detected in both the dorsal and ventral side of the embryo. As gastrulation proceeds the expression of both Nodal genes are stronger dorsally (Figure 4.4B iii and vi). At the end of gastrulation (stage 11 and stage 12), *AxNodal-1* can be detected in the posterior dorsal mesoderm, ventral mesoderm and weakly in the endoderm, whereas *AxNodal-2* cannot be detected in the dorsal mesoderm. *AxNodal-2* expression is restricted to the dorsal ectoderm and ventral mesoderm. Taken together, we conclude that *AxNodal-1* transcripts are detected in the dorsal marginal zone in a potential mesendoderm cell population. During gastrulation *AxNodal-1* expression spreads laterally and is mainly detected in both dorsal and ventral regions of the embryo. By stage 12 *AxNodal-1* expression circles the blastopore (Figure 4.3A v) and is detected in the posterior dorsal and ventral mesoderm (Figure 4.4B viii). Compared with *AxNodal-1*, *AxNodal-2* transcripts are detectable at the late blastula stage (stage 9) as with *AxNodal-1*. However, unlike *AxNodal-1, AxNodal-2* is only significantly

expressed in the future dorsal lip. By stage 11 and 12, the expression pattern of *AxNodal-2* is dorsally and ventrally much more restricted than *AxNodal-1* with slightly superficial expression in the axolotl embryo. By this time *AxNodal-2* is expressed in a narrow strip in the dorsal and ventral ectoderm whereas *AxNodal-1* is strongly expressed in the posterior dorsal and ventral mesoderm (Figure 4.4B vii-x). Whole mount and hemi-section *in-situ* hybridizations were also carried out with sense probes for both Nodal genes and no expression is detected in sense control embryos (Figure 4.5).

A.



B.

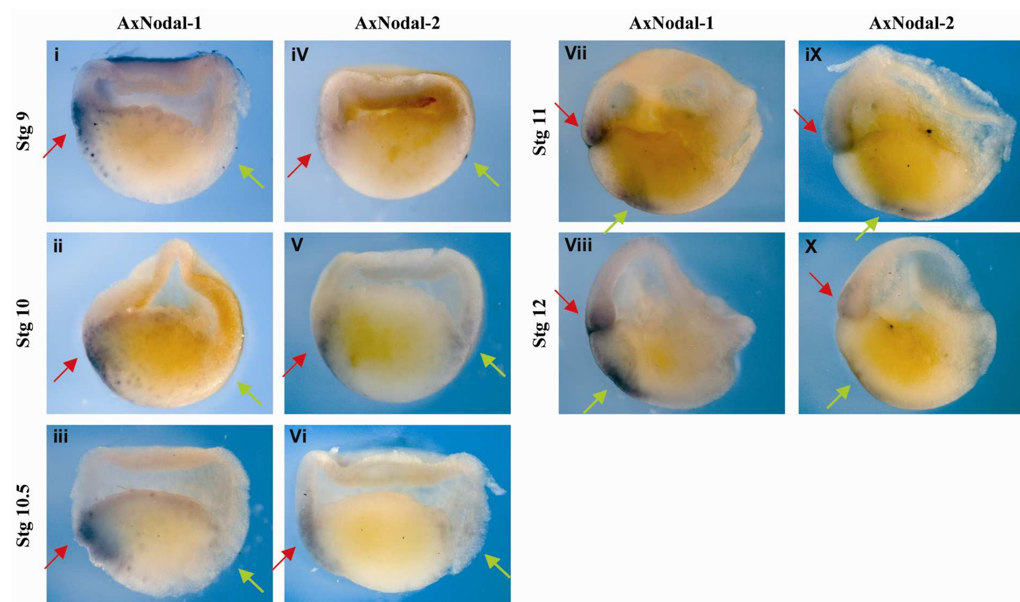


Figure 4.4 – Hemi-sections and *in situ* for AxGsc, AxNodal-1 and AxNodal-2 expression

(A) *AxNodal-1* is first detected on the future dorsal side (red arrow) of stage 9 embryos as marked by the expression of *AxGsc*. **(B)** *In situ* hybridization for *AxNodal-1* and *AxNodal-2* on hemi-sectioned embryos; one half stained for *AxNodal-1*, the other half stained for *AxNodal-2*. (red arrows = dorsal, and green arrows = ventral).

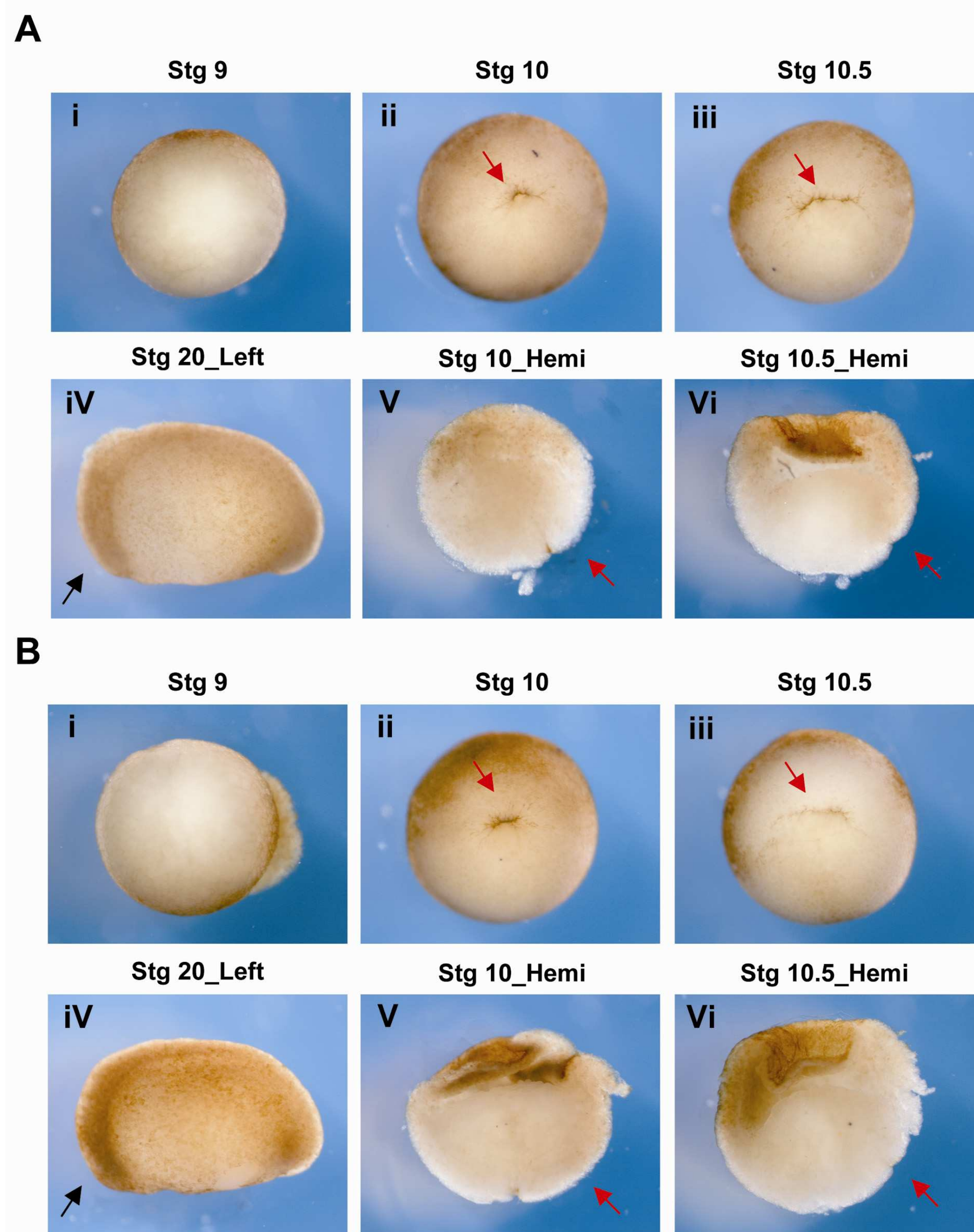


Figure 4.5 –WISH sense controls for AxNodal-1 and AxNodal-2

Whole-mount *in situ* hybridizations of gastrula and neurula stage axolotl embryos with sense probes recognizing *AxNodal-1* **(A)** and *AxNodal-2* **(B)** both on whole (i – iv) and hemi-sectioned embryos (v – vi). Stage 9,10 and 10.5 embryos are vegetal views with the dorsal blastopore lip to the top. Stage 20 embryos are shown as lateral views with anterior to the left. (Red arrows = dorsal, black arrows = anterior).

4.3 Functional analysis of AxNodal-1 and AxNodal-2

The spatial expression patterns of the two *Nodal* genes indicate they may have different roles during embryogenesis. To investigate the role of *AxNodal-1* and *AxNodal-2* in mesendoderm formation, the normal expression of *AxNodal-1* and *AxNodal-2* were perturbed through both overexpression studies and morpholino knock-down assays.

4.3.1 Overexpression of Nodal orthologues in *Xenopus laevis* caps

In *Xenopus*, *activin-like* signalling molecules of the TGF-β superfamily, such as *derriere* and the *nodal-related* genes (*Xnrs*), are essential for mesoderm and endoderm formation (Chang and Hemmati-Brivanlou, 2000; Faure et al., 2000; Kofron et al., 1999). The six known *Xnrs* (Jones et al., 1995; Joseph and Melton, 1997; Smith et al., 1995; Takahashi et al., 2000) are all strong inducers of mesendoderm with the exception of *Xnr3* (Hansen et al., 1997). We first used *Xenopus laevis* animal caps to investigate the possible function of *AxNodal-1* and *AxNodal-2* in mesoderm and endoderm induction as compared with *Xnr4* (Joseph and Melton, 1997).

Three different levels (2pg, 20pg and 200pg) of each AxNodal mRNA were injected into the animal pole of 1 or 2 cell stage *Xenopus laevis* embryos alongside 100pg GFP. As a positive control, *Xnr4* was injected at the same concentrations. Animal cap explants were cut at stage 9, cultured to stage 25 (Figure 4.6) and analyzed by qPCR (Figure 4.7). Control cap explants remain rounded, differentiating into atypical epidermis (Figure 4.6). In each group,

injection of low amounts of mRNA (2pg) fails to induce elongation in cap explants. However at 20pg and 200pg, *Xnr4* injected explants extend slightly compared to control caps (Figure 4.6), as reported elsewhere (Osada and Wright, 1999). For *AxNodal-1*, at 20pg the injected caps slightly elongate as with *Xnr4*. Higher dose (200pg) injected cap explants elongate extensively compared to controls and *Xnr4*, behavior mimicking the convergent extension of axial mesodermal cells in normal development (Keller and Tibbetts, 1989). In contrast, *AxNodal-2* mRNA injected cap explants do not elongate at any level (Figure 4.6).

Animal caps from injected embryos were then assayed for expression of various mesoderm and endoderm markers; *Brachyury*, a general mesoderm marker at early gastrula stages, *MyoD*, marker of dorsal mesoderm and presumptive muscle cells, and *Sox17*, a general endoderm marker. *Xnr4* and *AxNodal-1* (20pg and 200pg) injected animal caps express *XlBra*, showing the induction of mesoderm. The muscle-specific marker *XlMyoD* is induced at higher doses (20pg and 200pg), while low doses (2pg) fail to induce any mesoderm markers. Similarly, *AxNodal-2* mRNA injected caps fail to induce mesoderm at low doses (2pg). In contrast, higher doses (20pg and 200pg) only weakly induce *XlBra* expression, but cannot induce *XlMyoD* at all. The endoderm marker, *XlSox17*, is weakly induced in 20pg *AxNodal-1* mRNA injected caps and strongly expressed in 200pg injected cap explants. However, *Xnr4* and *AxNodal-2* injected caps never induce *XlSox17* (Figure 4.7).

AxNodal-1 induces a variety of dorsal mesodermal cell types in a

dose-dependent manner. Induced tissues range from pan-mesoderm, marked by the presence of *XlBra*, to more lateral paraxial mesoderm, marked by *XlMyoD*. Overexpression of *AxNodal-1* is also able to induce the expression of the endoderm marker *XlSox17* as previously described for *Xnr1* (Engleka et al., 2001). Taken together, *AxNodal-1* but not *AxNodal-2* represents a good candidate for a *Nodal* ortholog involved in mesoderm and endoderm induction in the axolotl.

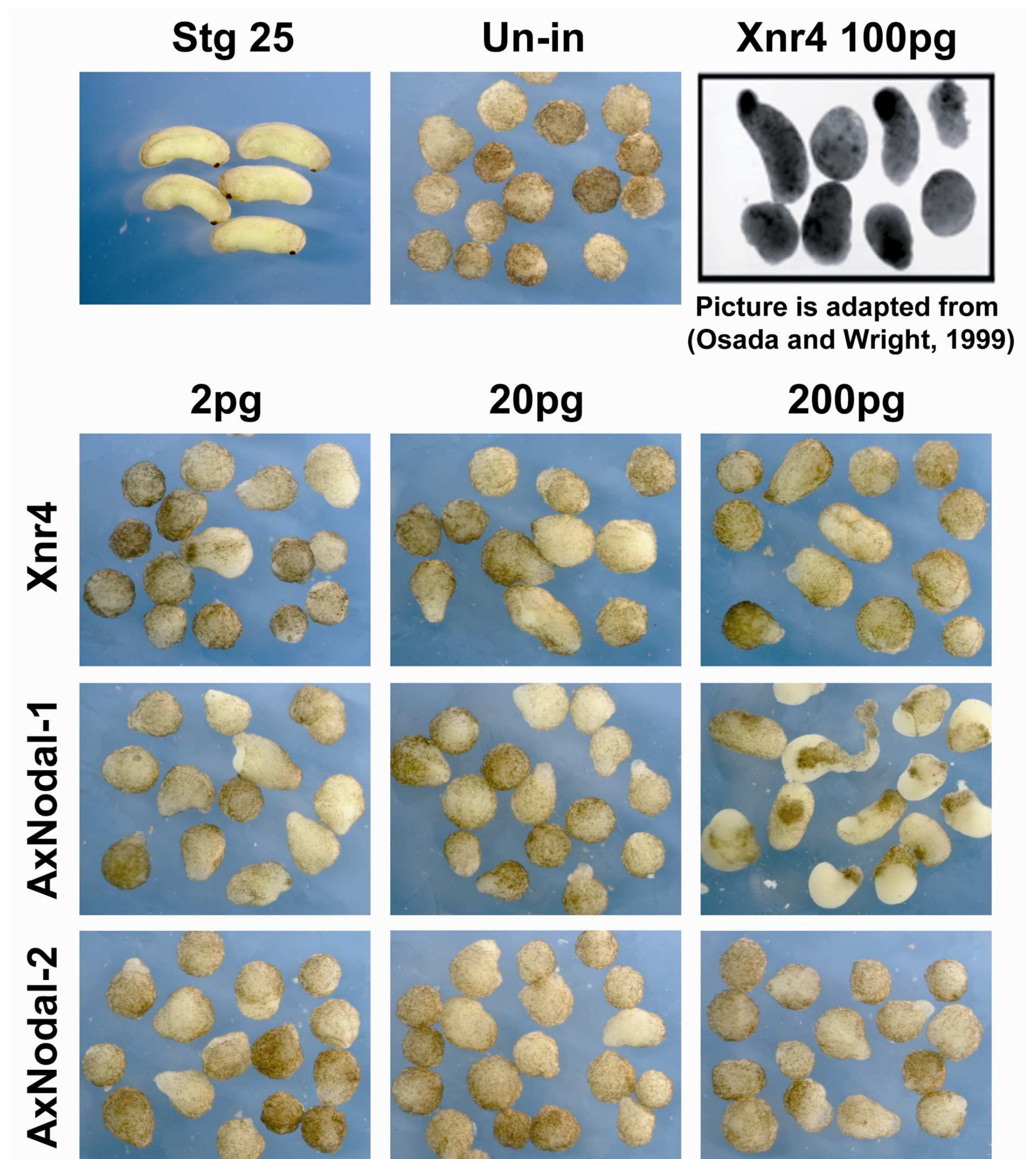


Figure 4.6 – Effects of Xnr4, AxNodal-1 and AxNodal-2 on *Xenopus laevis* animal caps

Animal cap explants are collected at stage 25 as control embryos. Animal caps were cut from stage 9 embryos injected with synthesized RNA; *Xnr-4*, *AxNodal-1* or *AxNodal-2*, into the animal pole at the one or two-cell stage with coinjection of 100pg GFP RNA (100%, *n*=11-18). Amounts of RNA injected (per embryo) are indicated on the top side of the panels. Animal caps slightly elongated by injection of 20 pg and 200pg of *Xnr4* or 20 pg of *AxNodal-1*RNA compared to control caps. 200pg *AxNodal-1* injection, cap explants show massively mesoderm elongation and endoderm formation. However, in *AxNodal-2* injected samples, explants showed no elongation under all conditions.

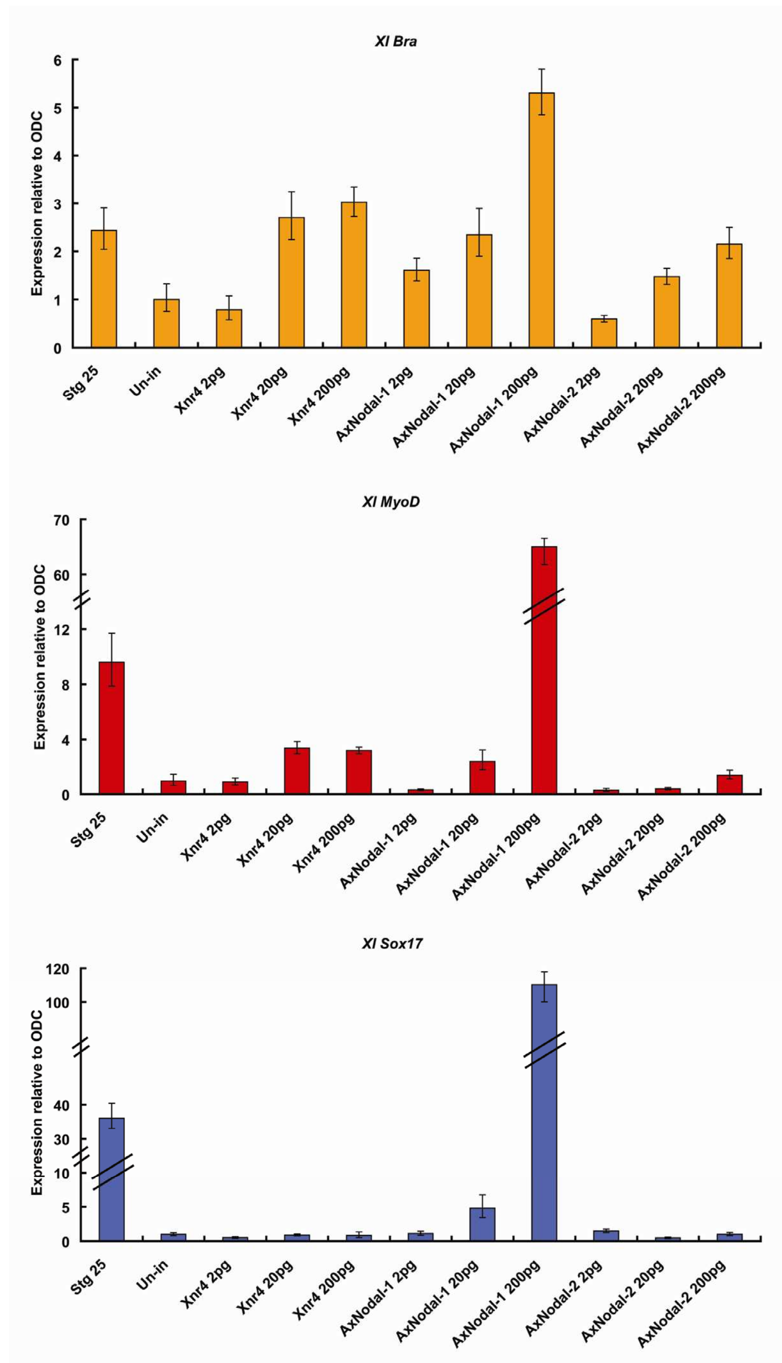


Figure 4.7 - qPCR analysis of mesodermal and endodermal markers in AxNodal-1 and AxNodal-2 injected caps

Cap explants were collected at stage 25 and gene expression level are relative to *ODC*, and then normalised to the uninjected samples. The cDNA prepared from these samples was tested sequentially using specific *XlBra*, *XlMyoD* and *XlSox17* qPCR primers and probes. The X-axis indicates the sequential cDNA samples; Stg 25 (whole embryos), Un-in (uninjected caps), *Xnr4* (2pg, 20pg and 200pg), *AxNodal-1* (2pg, 20pg, 200pg) and *AxNodal-2* (2pg, 20pg and 200pg).

4.4 AxNodal Knock-down

To further investigate the roles of AxNodal-1 and AxNodal-2 during early development, we used morpholino antisense oligonucleotides to inhibit these two genes *in vivo*. Morpholinos are synthetic oligonucleotides of about 25 bp that have been modified so that they are resistant to nuclease and are therefore stable in cells (Corey and Abrams, 2001; Heasman, 2002). Morpholinos can either block translation or splicing. Translation blocking morpholinos are designed to be complementary to the 5' end of mRNA sequences and block the initiation of translation. Knock-down by translation blocking Mos should be verified by western blot. Splice inhibiting morpholinos are designed to prevent pre-mRNA splicing and result in intron retention, exon skipping or cryptic splicing. The mis-spliced mRNA is designed to result in non-functional protein. RT-PCR can be used to confirm the knock-down efficiency for splice morpholinos. The morpholino approach has been widely applied to many organisms such as *Xenopus*, zebrafish, chick and mouse (Coonrod et al., 2001; Heasman et al., 2000; Kos et al., 2001; Nasevicius and Ekker, 2000).

In addition to the morpholino approach, SB431542, a chemical inhibitor of activin/nodal signalling, acts by specifically interfering with the type I receptors; ALK4, ALK5 and ALK7 (Callahan et al., 2002; Inman et al., 2002). SB431542 treatment can generate phenotypes resembling those of known perturbations in the nodal signalling pathway in *Xenopus* and zebrafish embryos (Ho et al., 2006).

4.4.1 Nodal inhibitor – SB431542

The formation of the mesoderm requires Nodal signalling, and this is conserved in all deuterostomes (Swalla, 2006). We have found two *Nodal* genes in the axolotl and believe these to be the only *Nodal* genes present. However, we cannot formally exclude the possibility of other *Nodal* genes existing in the axolotl genome. To determine the consequences of the loss of all Nodal signalling, axolotl embryos were treated with the soluble nodal signalling inhibitor, SB431542 (75 or 150 μM). In axolotl embryos, early SB431542 treatment results in a failure to form dorsal lips and the embryos do not gastrulate (Figure 4.8A) (100%, *n*=3x15), phenocopying *Xenopus* embryos (Ho et al., 2006). This phenotype was further characterized by investigating the expression of mesodermal (*AxBra*, *AxFGF8*) and endodermal (*AxMix*, *AxSox17*) marker genes at various timepoints with qPCR analysis. qPCR results (Figure 4.8B) showed that all four markers are significantly downregulated compared to the DMSO-treated control, indicating a block to the formation of both the mesoderm and endoderm in these embryos.

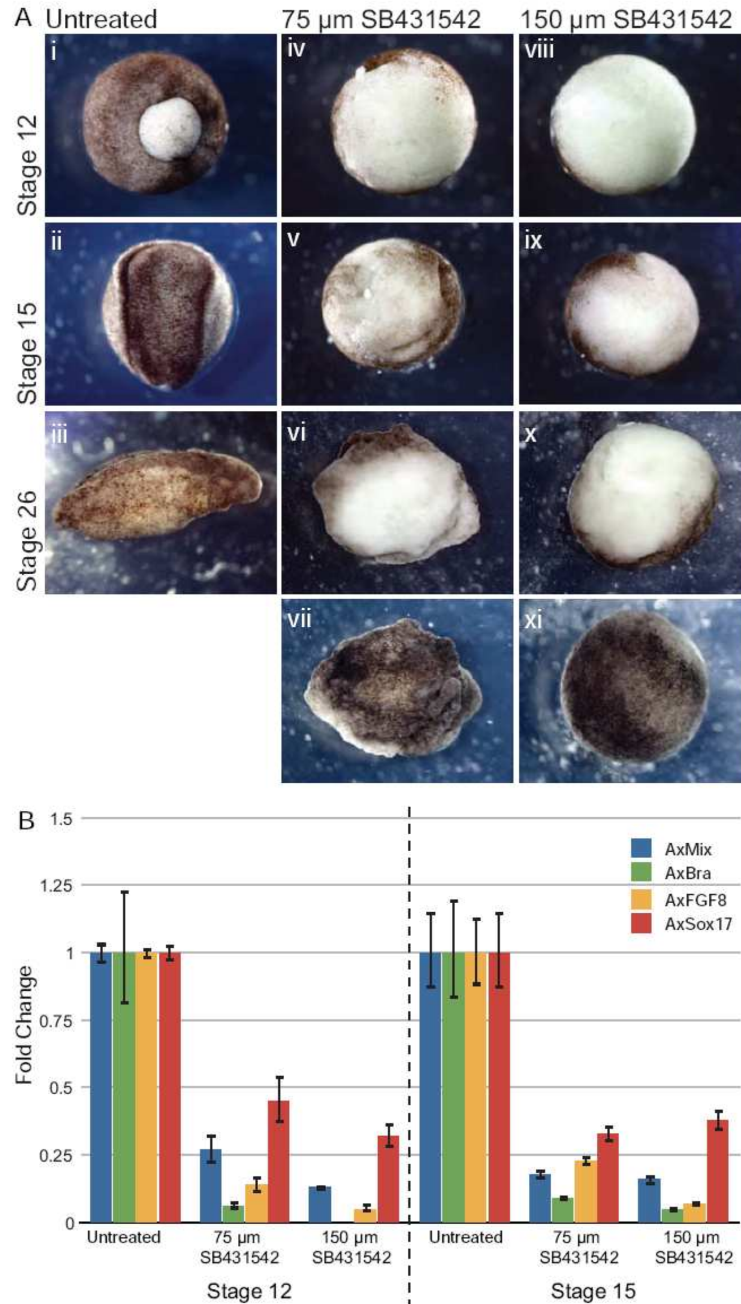


Figure 4.8 - Effect of SB431542 Nodal antagonist on axolotl development

(A) The axolotl embryos are treated with 75µM and 150µM SB431542 or DMSO only at the 2 cell stage. Representative embryos show the complete block to gastrulation caused by SB431542 treatment, as seen in *Xenopus* embryos. Panels i, iv, v, vi, vii, ix and x vegetal views. vii and xi show the animal view of the embryos in vi and x respectively. **(B)** qRT-PCR analysis of inhibitor treated embryos. Embryos were collected at stage 12 and stage 15, and expression of *AxMix*, *AxBra*, *AxFGF8* and *AxSox17* was analyzed. Expression levels are relative to *ODC*, normalised to the untreated embryos.

4.4.2 AxNodal splice blocking morpholinos

To disrupt *AxNodal-1* and *AxNodal-2,* ATG-morpholinos were designed targeted to the 5'UTR and start codon of *AxNodal-1* and *AxNodal-2.* To test if the ATG-morpholinos specifically knock-down *AxNodal* gene translation, morpholino and *in-vitro* transcribed HA tagged mRNAs were injected into *Xenopus tropicalis* oocytes. However, translation of *AxNodal-1* or *2* HA mRNA is not significantly affected by the ATG-AxNodal-1 or 2 morpholino as AxNodal-1 and AxNodal-2 are still translated in the presence of ATG-morpholinos (data not shown). As an alternative approach, two splicing morpholinos were designed for each *Nodal* gene. For *AxNodal-1*, one morpholino, Sp1, was designed across the intron1/exoxn2 boundary, targeting the splicing acceptor sequence. The second, Sp2, was designed to the exon2/intron2 boundary targeting the splicing donor (Figure 4.9A). For *AxNodal-2*, we are not able to identify the first intron (as described in the chapter 3), thus two splicing morpholinos, Sp1 and Sp2, were designed to target the splicing donor and acceptor sites between exon2/intron2 and the intron/exon3 boundary (Figure 4.9A).

The efficacy of splicing morpholinos targeted to *AxNodal-1* and *AxNodal-2* splice junctions can readily be determined by RT-PCR (Figure 4.9B). To validate the function of the splice morpholinos, a total of 80ng or 160ng of each morpholino set (40ng and 80ng of each morpholino) was injected into the animal pole of 1-cell stage embryos. Embryos were collected at control equivalent stage 12 and analyzed by RT-PCR. Each splice morpholino set for *AxNodal-1* and *AxNodal-2* functionally altered the splicing pattern at both 80ng and 160ng (Figure 4.9B). In subsequent MOs knock-down experiments,

80ng of each Sp1 and Sp2 were injected into 1 cell stage embryos, a total of 160ng morpholino, alongside 200pg GFP. In addition to knocking down each Nodal gene individually, both were knocked down at once (80ng of each morpholino set). For control embryos, 160ng of a mis-targeted morpholino was injected. This provided a timing control as morpholino injection causes a delay in gastrulation relative to uninjected sibling embryos. Embryos were collected at two different time points, first when uninjected and control embryos are at stage 12.5 and second when the controls are at stage 15. To investigate the consequences of the knockdown of each *Nodal* gene, or both in combination, qPCR was performed to examine the mesodermal and endodermal marker genes on those embryos.

Control morpholinos had no obvious effect on development, other than a slight developmental delay. AxNodal-1 Sp1/Sp2 morpholino injected embryos exhibit severely disrupted gastrulation resulting in a complete arrest of development at the onset of gastrulation with no blastopore formation (Figure 4.9). This phenocopies the effects of SB431542 treatment. By stage 20, sibling embryos have gastrulated normally, whilst the *AxNodal-1* knockdown embryos are halted at a pre-gastrula stage, resembling embryos at stage 9 (Figure 4.9). The similarity between the AxNodal-1 morphants and SB431542 treated embryos suggest a complete loss of mesoderm induction in AxNodal-1 morphants embryos. In the same experiment, AxNodal-2 morphants can form the dorsal lip and gastrulate normally, even though they are delayed with respect to uninjected siblings (Figure 4.9). By tail-bud stages, AxNodal-2 morphants are disrupted with abnormal axial patterning, having a shorter body axis with no head or tail

structures and a failure in closing the neural plate. Nevertheless, the ability of these embryos to complete gastrulation indicates that *AxNodal-2* is dispensable for mesoderm induction. Co-injection of both sets of morpholinos has no additional effects over injecting MOs targeted only to *AxNodal-1* (Figure 4.9).

To examine the consequences of *AxNodal-1* and *AxNodal-2* knockdown on gastrulation, mesoderm (*AxBra* and *AxFGF8*) and endoderm (*AxMix* and *AxSox17*) marker genes were analyzed by qPCR. In all cases gene expression was normalised to control morphants. At stage 12, AxNodal-2 morphants show a mild decrease in expression of *AxMix, AxBra, AxFGF8* and *AxSox17*, however, the expression levels of these genes are back to normal by stage 15. In contrast, AxNodal-1 morphants show an almost complete loss of expression from all four genes when assayed at stage 12, and expression is never recovered compared to controls at stage 15. These results are similar to those obtained with Nodal inhibitor treatment (see Figure 4.8B). In addition, the phenotype of the AxNodal-2 and -1 MOs combined is equivalent to the AxNodal-1 phenotype alone (Figure 4.9). qPCR analysis for *AxMix*, *AxBra*, *AxFGF8* and *AxSox17* expression suggests loss of these genes expression is a result of the knockdown of *AxNodal-1* but not *AxNodal-2*. All together, these results indicate that AxNodal-1 alone is required to initiate mesoderm development, a marked contrast to *Xenopus* embryos in which subfunctionalisation of gene family members prevents a requirement for any single *nodal-related* gene to produce mesoderm (Luxardi et al., 2010).

Figure 4.9 - AxNodal-1 and AxNodal-2 gene knockdown

(A) Schematic illustrating the action of the two splice morpholinos targeted to *AxNodal-1* and *AxNodal-2* (shown as M:A and M:B). Approximate location of PCR primers indicated by arrows. **(B)** PCR demonstrates effectiveness of the AxNodal-1 and AxNodal-2 morpholinos (MO:AxNodal-1 and MO:AxNodal-2). MO:Control = Control. 80 ng of each of M:A and M:B, 160 ng in total. 160 ng MO:Contol. **(C)** *AxNodal-1* and *AxNodal-2* morphant embryos. Vegetal views, except uninjected (iii,iv) and MO:AxNodal-2, stage 28 (xii), lateral view. AxNodal-2 morphants gastrulate, subsequent axial patterning is disrupted. AxNodal-1 morphants fail to gastrulate, remaining phenotypically at stage 9. Each morpholino combination is 80 ng of two splice morpholinos, 160 ng total. Dorsal lips indicated by arrows. (100%, *n*=3x20) **(D)** qPCR analysis of MO:AxNodal embryos at stage 12 and 15.

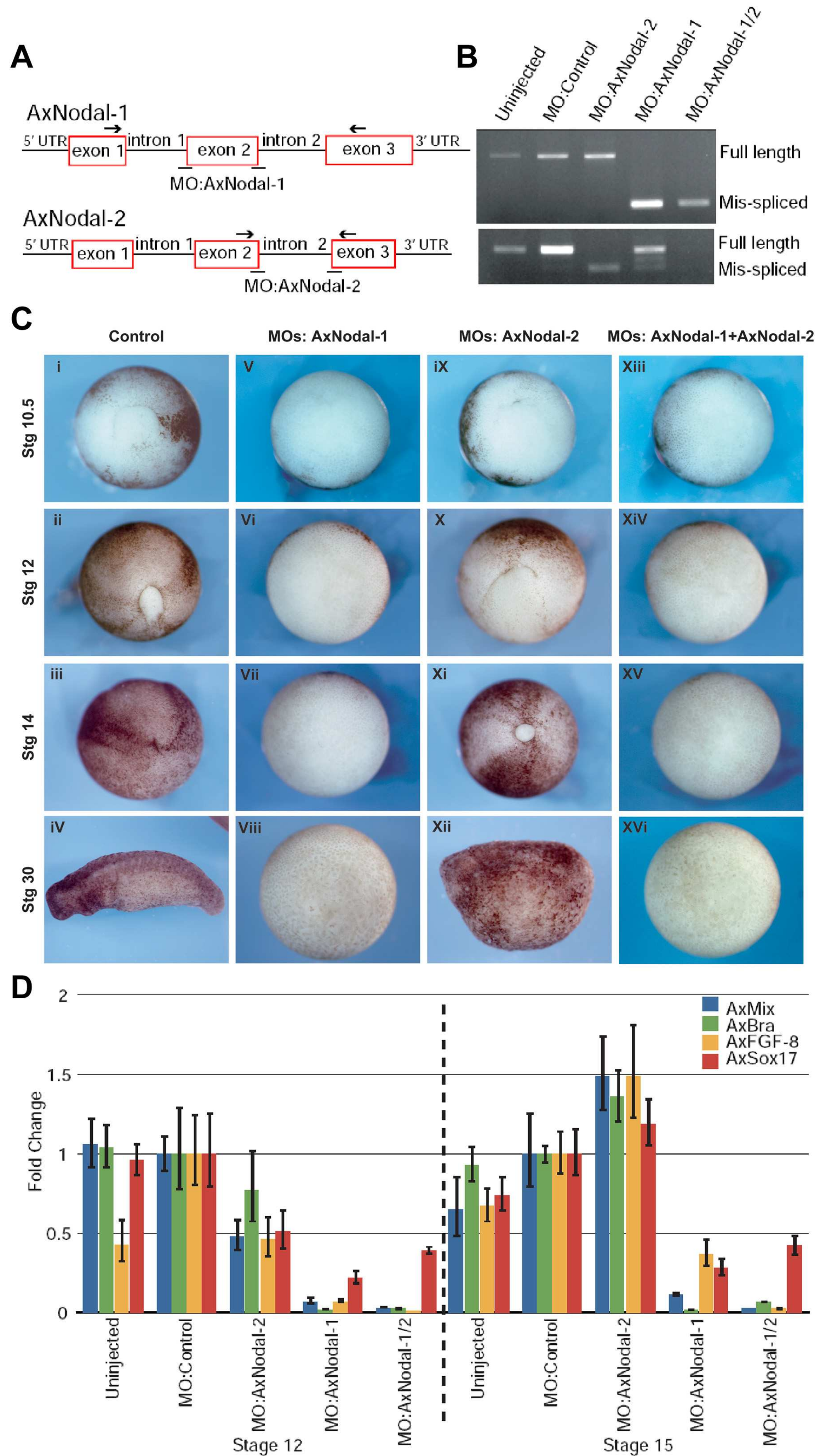


Figure 4.9 – AxNodal-1 and AxNodal-2 gene knockdown

4.5 AxMix and AxBrachyury in mesoderm specification

As already discussed, key molecules downstream of the Nodals in the mesoderm network include *Mix* and *Brachyury*. Notably, both these genes are downregulated in AxNodal morphants and we wished to see if the relationships between *Nodal*, *Mix* and *Brachyury* are conserved in the axolotl. In *Xenopus,* Mix.1 and Brachyury negatively regulate each other's expression and this is thought to drive the segregation of endoderm and mesoderm (Lemaire et al., 1998). By comparing the expression domains of *XlBra* and *XlMix.1* during gastrulation, Lemaire et al demonstrated the presence of a population of cells that initially appear to express both *XlMix.1* and *XlBra* (Lemaire et al., 1998). As gastrulation proceeds, *XlMix.1* expression is maintained in the deeper and more anterior territories of the organizer, and eventually restricted to the endoderm, with *XlBra* restricted to the mesoderm. The same progressive exclusion of *XlBra* and *XlMix.1* is found on the ventral side. Moreover, ectopic expression of *XlMix.1* or *XlBra* leads to the downregulation of *XlBra* and *XlMix.1* respectively. These observations drive the view that the *Mix* family members have a role in the specification of the endoderm.

Morphlinos have previously been used to knockdown the *Mix-like* genes in *Xenopus laevis* (Kofron et al., 2004; Trindade et al., 2003). However, knockdown of all 7 *Mix/Bix* family members is technically challenging and, to date, has not been achieved. Surprisingly, morpholinos targeted against *Xenopus laevis Mixer* suggest a role in negatively controlling of mesoderm

that forms in the embryo as judged by the upregulated expression of *Brachyury* in the morphants (Kofron et al., 2004). Mixer morphants also have increased levels of mesoderm inducing signals such as *FGF8*. The mouse *Mix* ortholog, *Mixl1* has been knocked out disrupting the morphogenesis of the mesoderm (Hart et al., 2002). Whilst an expansion of *T/Brachyury* is reported in these embryos, Hart et al. report that *T/Brachyury* is completely absent from the core of the primitive streak, even though the primitive streak can still be identified by the authors (Hart et al., 2002). Thus perhaps Mixl1 is required for *T/Brachyury* expression in the primitive streak, with other non-Mixl1 dependent pathways inducing *T/Brachyury* ectopically in the embryo. Together this data suggests the role of Mix family members in the induction of the mesoderm is more complex than the *Xenopus Mix* and *Brachyury* data suggest. Gemma Swiers tested the relationship between *AxMix* and *AxBrachyury* by knocking down AxMix activity in axolotl embryos (pers. Comm.). Surprisingly, this results in a failure to complete gastrulation and the loss of expression of *Brachyury* (Figure 4.10). These embryos also have upregulated levels of *AxSox17*, suggesting a failure of induction of the mesoderm from the endoderm. Control morphants show small changes in gene expression, which could probably be explained by the delay in development of the morphant embryos.

Comparing the expression patterns of the individual *Xenopus Mix* family members with *AxMix* suggests that *AxMix* is most like *Xenopus Mixer*. The evidence discussed in chapter 3 and the strength of the AxMix morpholino phenotype lead us to conclude that *AxMix* is likely to be the only representative of the *Mix* family in the Axolotl.

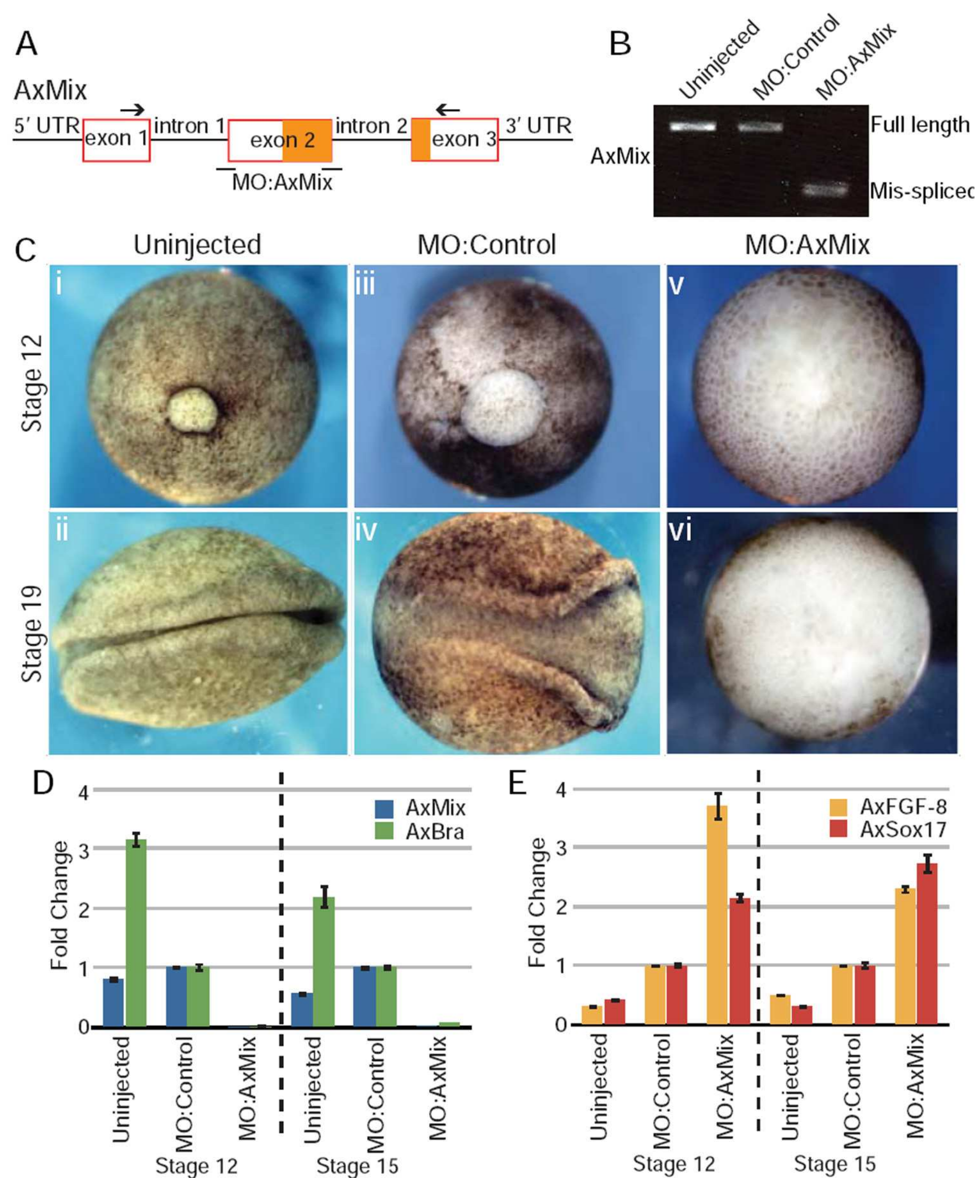


Figure 4.10 – AxMix gene knockdown

(A) Cartoon illustrating the morpholino design targeted to *AxMix*. The approximate location of PCR primers used to amplify the fragments in (B) are indicated by arrows. The homeodomain containing exons are marked in orange. **(B)** RT-PCR demonstrates effectiveness of MO:AxMix (80ng of each Sp1 and Sp2, 160 ng in total). MO:Control = Control, 160 ng. **(C)** AxMix morphants fail to gastrulate and do not form dorsal lips (97%, *n*=3x20). (i,iii,v and vi) vegetal view, (ii and iv) dorsal view. **(D and E)** qPCR analysis of MO:AxMix embryos, normalised to uninjected controls at each time point. (Data from G. Swiers)

4.6 Knock-down of AxBrachyury

To clarify the role of *AxBra* in mesoderm formation in the axolotl and the relationship between *AxMix* and *AxBra*, we designed splice disrupting morpholinos targeted to *AxBra*. Genomic PCR reactions were performed to identify intron sequence from *AxBra*. The DNA binding domain of AxBra extends through exon 4. Therefore splice MOs were designed to target the predicted intron3/exon4 boundary (Sp1) and exon4/intron5 boundary (Sp2) (Figure 4.11A). The effectiveness of the *AxBra* splice morpholinos is demonstrated by RT-PCR, demonstrating complete disruption to the T-box domain (Figure 4.11B).

To test whether *AxBra* is required for mesoderm formation, 80ng of each splice morpholino (160ng in total) were co-injected into the animal pole of 1 cell stage embryos. As with previous knockdown experiments, a mis-targeted control morpholino was used as a stage control compared with uninjected embryos. As with *AxNodal* and *AxMix*, *AxBra* splice morpholino injected embryos are severely affected by *AxBra* knockdown (Figure 4.11C). AxBra morphants exhibit complete failure of gastrulation and unusually large cells in the vegetal pole (Figure 4.11C). The phenotype, identical to that seen in AxMix and AxNodal morphants, suggest these embryos also fail to form mesoderm. As with previous morphants, we examined the expression of mesodermal and endodermal marker genes in AxBra morphants. Embryos were collected at stage 12 and 15 as judged by the control morpholino embryos. Gene expression was analyzed by qPCR. At stage 12, *AxMix* expression is down regulated. However, by stage 15 *AxMix* expression is

significantly upregulated (Figure 4.11D). This is consistent with the hypothesis that Brachyury negatively regulates *Mix* expression. In contrast with AxMix morphants, AxBra morphants lose *AxFGF8* expression (compare Figure 4.10D with Figure 4.11D). This is surprising as AxMix morphants lose *Brachyury* expression, but not *FGF*, and raises the possibility that AxMix itself may be a repressor of *AxFGF8* activity. This could further explain why *AxFGF8* is increased in AxMix morphants. Finally, *AxSox17* expression is up regulated in these embryos, suggesting that the failure to induce mesoderm results in a larger population of endoderm.

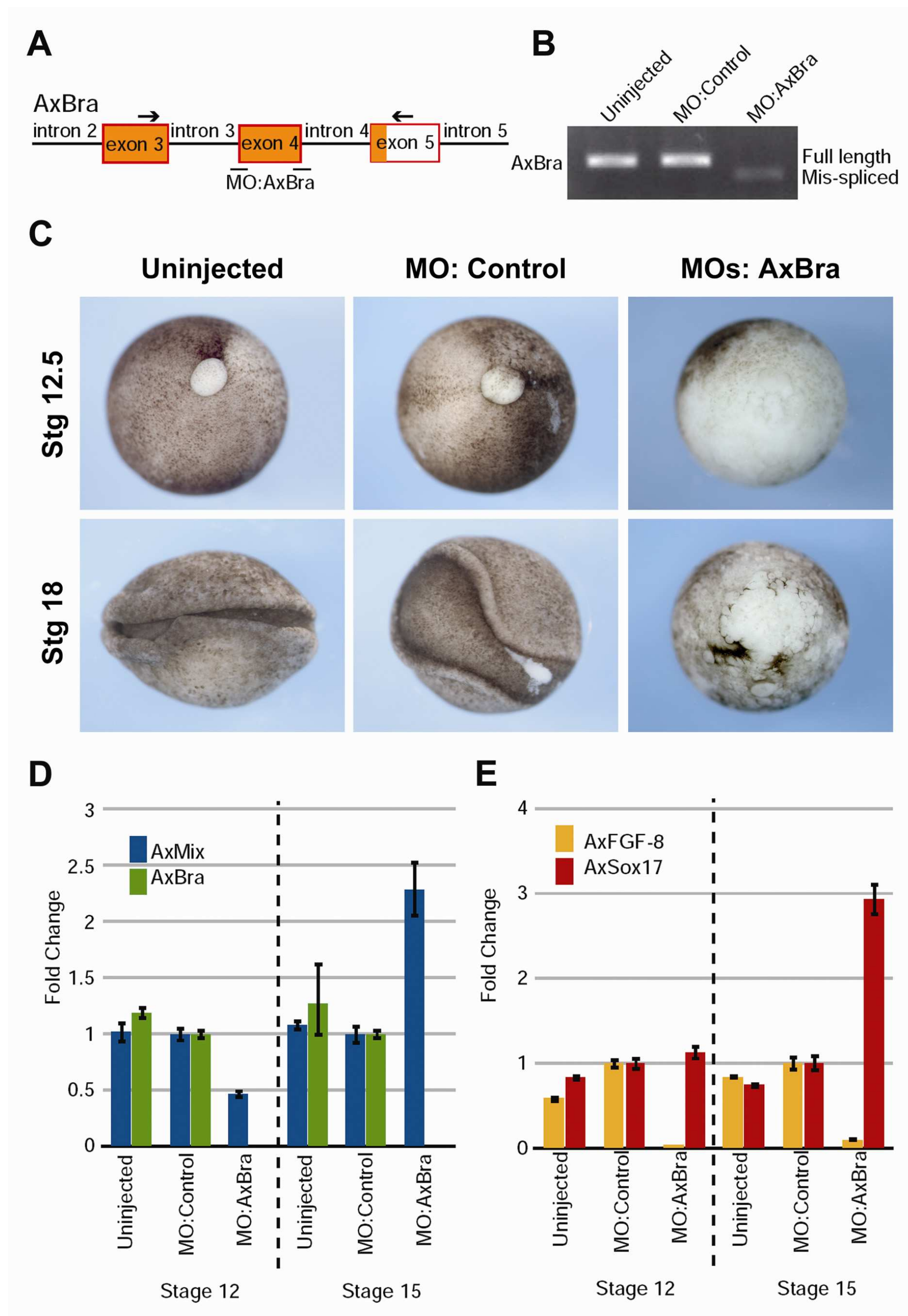


Figure 4.11 – AxBrachyury gene knockdown

(A) Cartoon illustrating the morpholino design targeted to *AxBra*. The approximate location of PCR primers used to amplify the fragments in (B) are indicated by arrows. Note that *AxBra* is predicted to have 8 exons. The T-box domain containing exons are marked in yellow. Exon 4, likely to be required for DNA binding was targeted for disruption. **(B)** RT-PCR demonstrates effectiveness of MO:AxBra (80ng of each Sp1 and Sp2, 160 ng in total). MO:Control = Control, 160 ng. **(C)** AxBra morphants fail to gastrulate and do not form dorsal lips (100%, *n*=3x20). **(D and E)** qPCR analysis of MO:AxBra embryos, normalised to uninjected controls at each time point.

4.7 The regulatory interactions between AxMix and AxBra

The data presented so far suggest that in the axolotl Mix activity is required for the activation of *Brachyury*, whilst Brachyury represses *Mix*. This is consistent with the expression patterns of *AxMix* and *AxBra* as described by G.Swiers (G.Swiers PhD thesis 2008 – see Figure 4.12). Unlike *Xenopus*, *AxMix* expression precedes that of *AxBrachyury*; *AxMix* expression begins at stage 9 whereas Ax*Bra* expression is later, starting around stage 10.75. In contrast to *Xenopus*, there is no co-expression of *AxMix* and *AxBra* during early gastrulation. By stage 10.75 *AxMix* expression is retained in the involuted dorsal mesoderm and at the leading edge of the involuting mesoderm in the blastopore lip. By this point, some co-expression of *AxMix* and *AxBra* can be seen in the ventral, but not dorsal, mesoderm. By stage 14 *AxMix* ventral expression is maintained, whereas *AxBra* transcripts are absent in ventral mesoderm and found only in the posterior mesoderm and dorsal mesoderm, which corresponds to the presumptive notochord. Taken together the lack of early *AxBra* expression and the perturbation experiments with *AxMix* and *AxBra* suggest a novel regulatory network between *AxMix* and *AxBra* in the axolotl. Unlike *Xenopus, AxMix* appears to be upstream of *AxBra* and*,* perhaps is required for its induction in the future mesoderm.

To further test this idea, we used three approaches. Firstly we analysed the expression patterns of *AxMix* and *AxBra* in early axolotl gastrulae whilst overexpressing either *AxBra* or *AxMix*. Secondly, we attempted to rescue the loss of mesoderm by overexpressing *AxMix*. Finally, we sought to test if any

requirement for Mix activity in the induction of the mesoderm was conserved in mammals by investigating the role of Mixl1 in the differentiation of murine embryoid bodies.

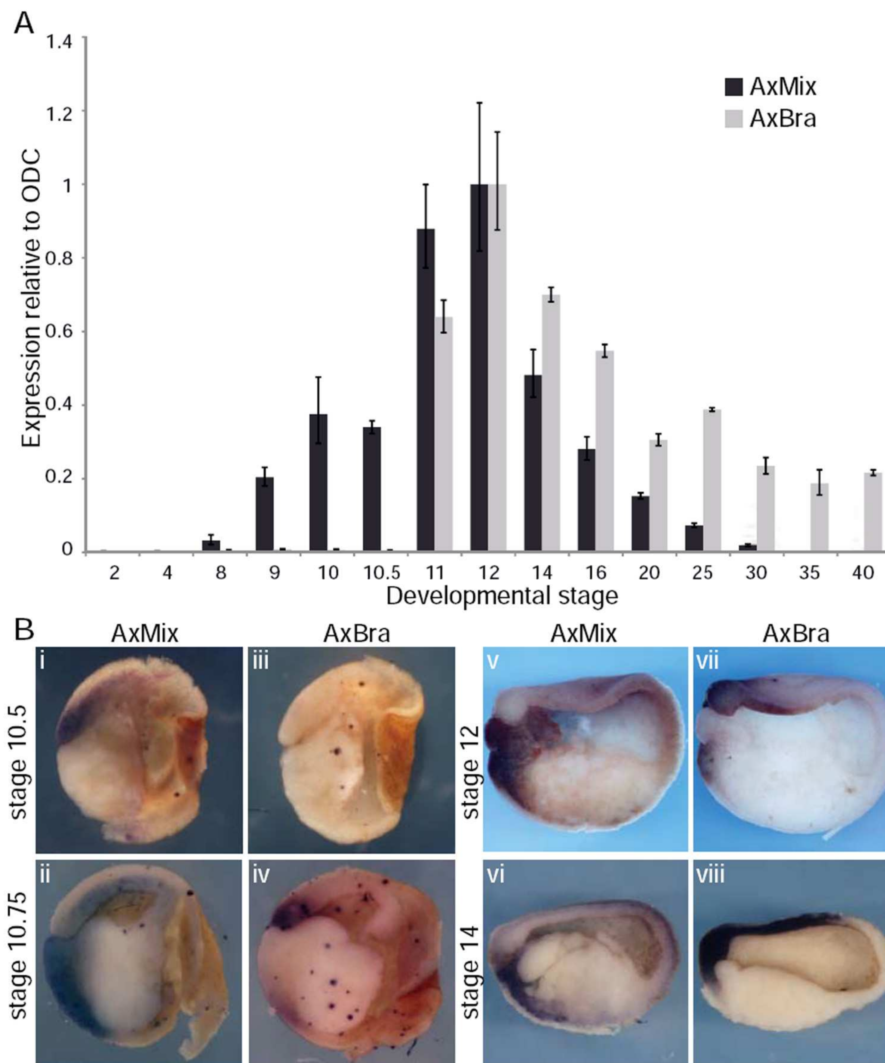


Figure 4.12 - Analysis of AxMix and AxBrachyury expression during axolotl early development

(A) qPCR of *AxMix* and *AxBra,* normalised to *ODC* and then to stage 12. **(B)** *In situ* hybridisation on hemi-sectioned embryos. Stage 10.5, 10.75 and 12 images are the same embryo – dorsal=top, vegetal=left. During early gastrulation (stage 10.5), *AxMix* expression is detectable throughout the dorsal mesoderm and in dorsal cells that have not yet gastrulated (i), whereas *AxBra* cannot be detected at stage 10.5 (iii). By stage 10.75, *AxMix* expression appears in the presumptive ventral mesoderm and endoderm (ii), with expression maintained in the endodermal yolk plug and ventral mesoderm at stage 12 (v). *AxMix* expression is retained in the posterior ventral mesoderm at the end of gastrulation, stage 14 (vi). By stage 10.75, *AxBra* expression is detected in the posterior dorsal mesoderm (iv). At stage 12 *AxBra* is expressed in the dorsal and ventral posterior mesoderm (vii). At stage 14 *AxBra* is solely expressed in the dorsal mesoderm (viii). (Data from G.Swiers)

4.7.1 Ectopic expression of *AxMix* and *AxBra*

Overexpression of *Brachyury* or *Mix* in *Xenopus laevis* results in the downregulation of *Mix* or *Brachyury* respectively (Lemaire et al., 1998). Our data suggests this relationship is not conserved in the axolotl. Specifically, overexpression of *AxMix* mRNA may not lead to downregulation of *AxBra*, whereas overexpression of *AxBra* may lead to downregulation of *AxMix*. To test this in the axolotl, 200pg *AxMix* or *AxBra* mRNA alongside a lineage tracer (mini-ruby) were injected into one blastomere at the 4 cell stage. For *AxMix,* dorsal blastomeres were targeted, for *AxBra,* ventral blastomeres. This corresponds with the known expression patterns of the genes in the axolotl. Injected embryos were collected at stage 12 and the endogenous gene transcripts were analyzed by WISH (Figure 4.13). As expected, and as with *Xenopus* (Lemaire et al., 1998), injection of *AxBra* mRNA inhibits *AxMix* expression at the site of injection indicating the negative regulation of *Mix* expression by *Brachyury* is conserved (compare Figure 4.13F and G). However, dorsal overexpression of *AxMix* mRNA induces ectopic *AxBra* expression, revealing a novel role for *AxMix* in the induction of *Brachyury* in the axolotl (compare Figure 4.13B and C). This *in-vivo* evidence places *AxMix* upstream of *AxBra* in the mesoderm GRN.

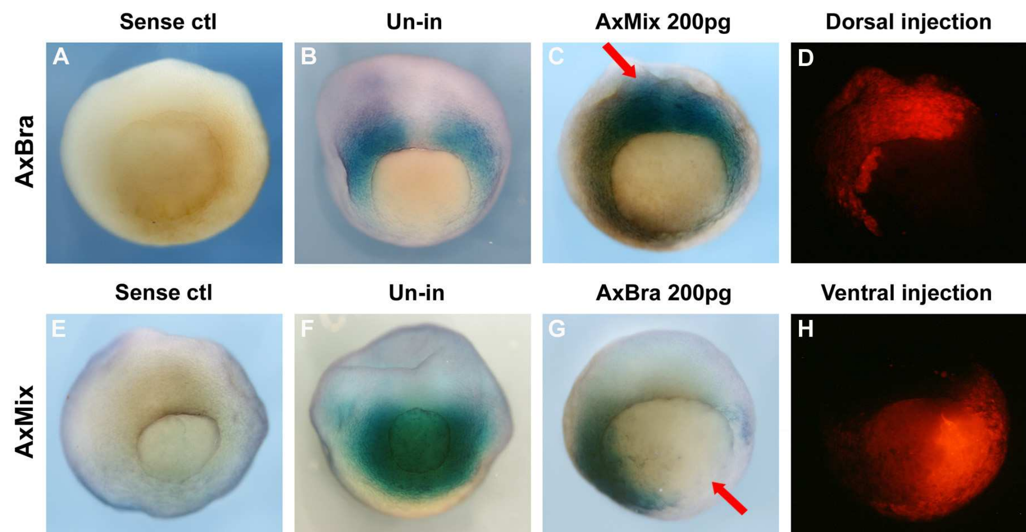


Figure 4.13 – Ectopic overexpression of AxMix or AxBrachyury in whole embryos

(A) *AxBra* sense control. **(B)** Uninjected embryo showing normal *AxBra* expression. **(C)** Overexpression of 200pg *AxMix* mRNA dorsally upregulates *AxBra* expression. **(D)** Mini-ruby (red fluorescence) marks the injection site. **(E)** *AxMix* sense control. **(F)** Uninjected embryo showing normal *AxMix* expression. **(G)** Overexpression of 200pg *AxBra* mRNA ventrally downregulates *AxMix* expression. **(H)** Mini-ruby (red fluorescence) marks the injection site. (Red arrow: the affected area)

4.7.2 Rescuing the loss of mesoderm in *AxMix* morphants

AxMix morphants do not form a blastopore or undergo gastrulation movements, indicative of a failure to induce mesoderm (see Figure 4.10). Furthermore, qPCR analysis of AxMix morphants reveals the unexpected loss of *AxBra* expression. Ectopic overexpression of *AxMix* and *AxBra* mRNA support the hypothesis that AxMix is upstream of AxBra and required for *AxBra* expression. Taken together, this suggests that AxMix is required for mesoderm induction in axolotl embryos. To test this hypothesis, we used an animal cap assay where we induce mesoderm directly in explants using *activin* mRNA. We identified the appropriate level of *activin* mRNA (1pg) to induce mesoderm by titration. At this level, caps elongate, an indicator for the induction of mesoderm in the animal cap explants (Green et al., 1992).

Combinations of *activin* mRNA, AxMix morpholinos and *AxMix* mRNA were injected into the animal pole of 1 cell stage axolotl embryos and animal cap explants were cut at stage 9 (Figure 4.14A). In addition, a mis-targeted control morpholino was used to test for off-target effects. Whole embryos injected with control morpholino were used as a stage control. Morpholino injected embryos develop more slowly than their uninjected siblings and so caps were cultured until stage 12.5 as judged by control morpholino injected siblings (Figure 4.14B). Control cap explants differentiate into atypical epidermis, whereas caps injected with 1pg *activin* mRNA elongate as expected indicative of mesoderm induction (Figure 4.14B). Co-injection of the control morpholino does not prevent this mesoderm induction. However, co-injection of morpholinos disrupting AxMix splicing (80ng) block the induction of mesoderm, resulting in caps that fail to elongate. This

recapitulates the failure to induce mesoderm in whole embryos when *AxMix* is blocked by morpholinos. To test if this effect is a direct consequence of the disruption of *AxMix*, we used *AxMix* mRNA, which is not disrupted by the splice targeted morpholinos, to rescue the induction of mesoderm. Initially, we were only able to rescue caps to an endodermal phenotype. However, by titrating down the levels of *AxMix* mRNA, we determined that 20pg of *AxMix* mRNA was sufficient to rescue elongation and mesoderm formation in these explants (Figure 4.14B).

To further characterize this phenotype, we extracted RNA from the explants and analysed the expression of *AxBra*, *AxFGF-8* and *AxSox17* (Figure 4.15). Caps treated with *activin* alone show 30 fold upregulation of *AxBra* and *AxFGF-8*, with a lesser upregulation of *AxSox17*, indicating these explants are mesoderm (Figure 4.15). In the presence of the control morpholino, explants express similar levels of all three genes. AxMix morphant explants show a loss of *AxBra* expression, with an upregulation of *AxFGF-8* and *AxSox17* as seen in AxMix morphant whole embryos (see Figure 4.10). *AxBra* expression, alongside mesoderm induction, is rescued by overexpression of low (20pg) levels of *AxMix* mRNA, whereas high levels induce *AxSox17* (Figure 4.15), presumably representing endoderm as expected (Green et al., 1992). Taken together, these data suggest that AxMix is required for the induction of mesoderm, but can also contribute to the specification of the endoderm.

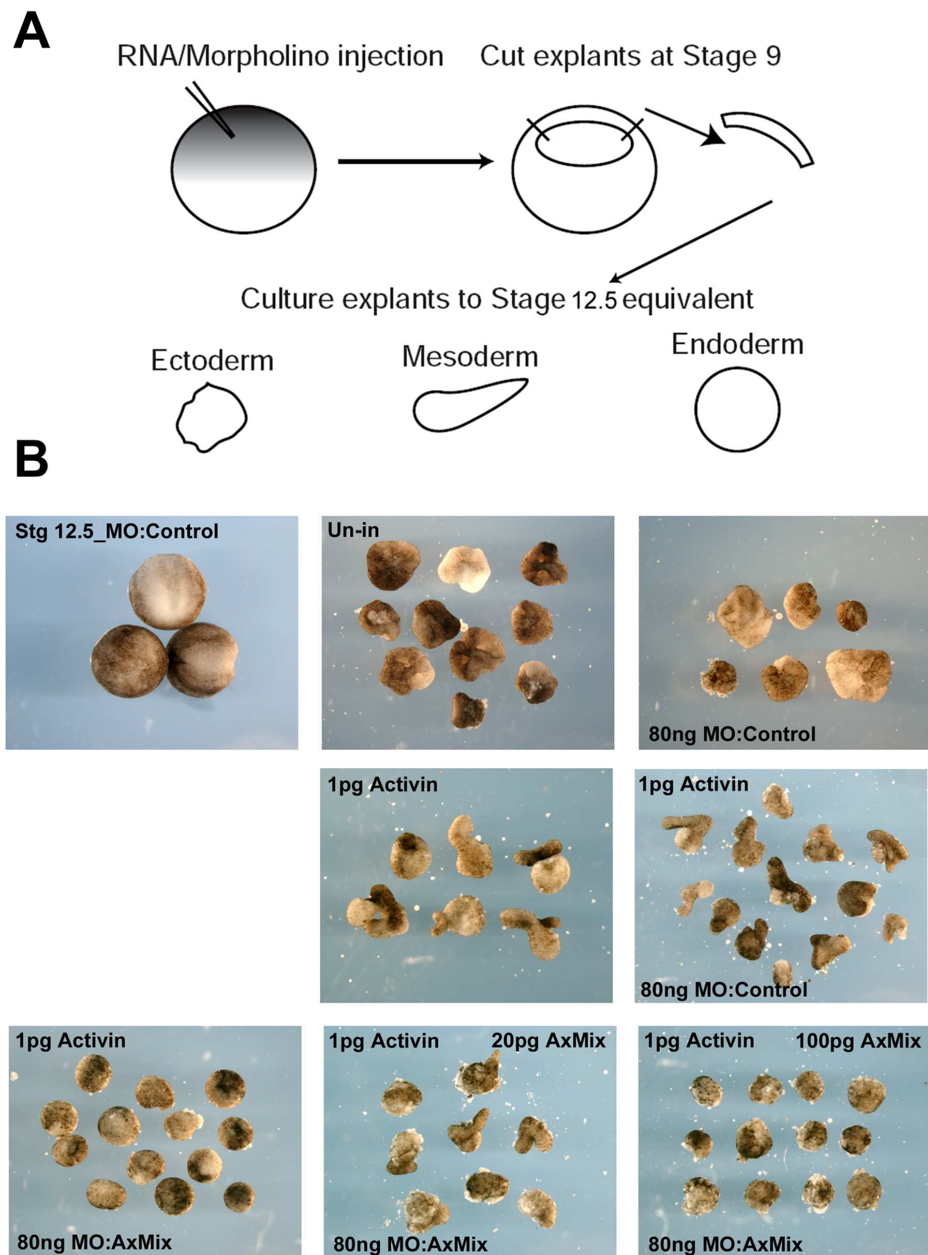


Figure 4.14 – AxMix RNA rescues mesoderm formation in animal cap explants

(A) Schematic illustrating animal cap explants. **(B)** Stage 12.5 embryos injected with control morpholino (80ng) are used as stage control. Animal caps with the injection of control morpholino also are regarded as control cap explants compared to no morpholino controls. Axolotl animal caps injected with 1pg *activin* mRNA to induce mesoderm in the presence or absence of Mo:AxMix. The Mo:AxMix can be rescued by overexpression of low levels of *AxMix* mRNA (20 pg). High levels of *AxMix* mRNA (100 pg) fail to rescue.

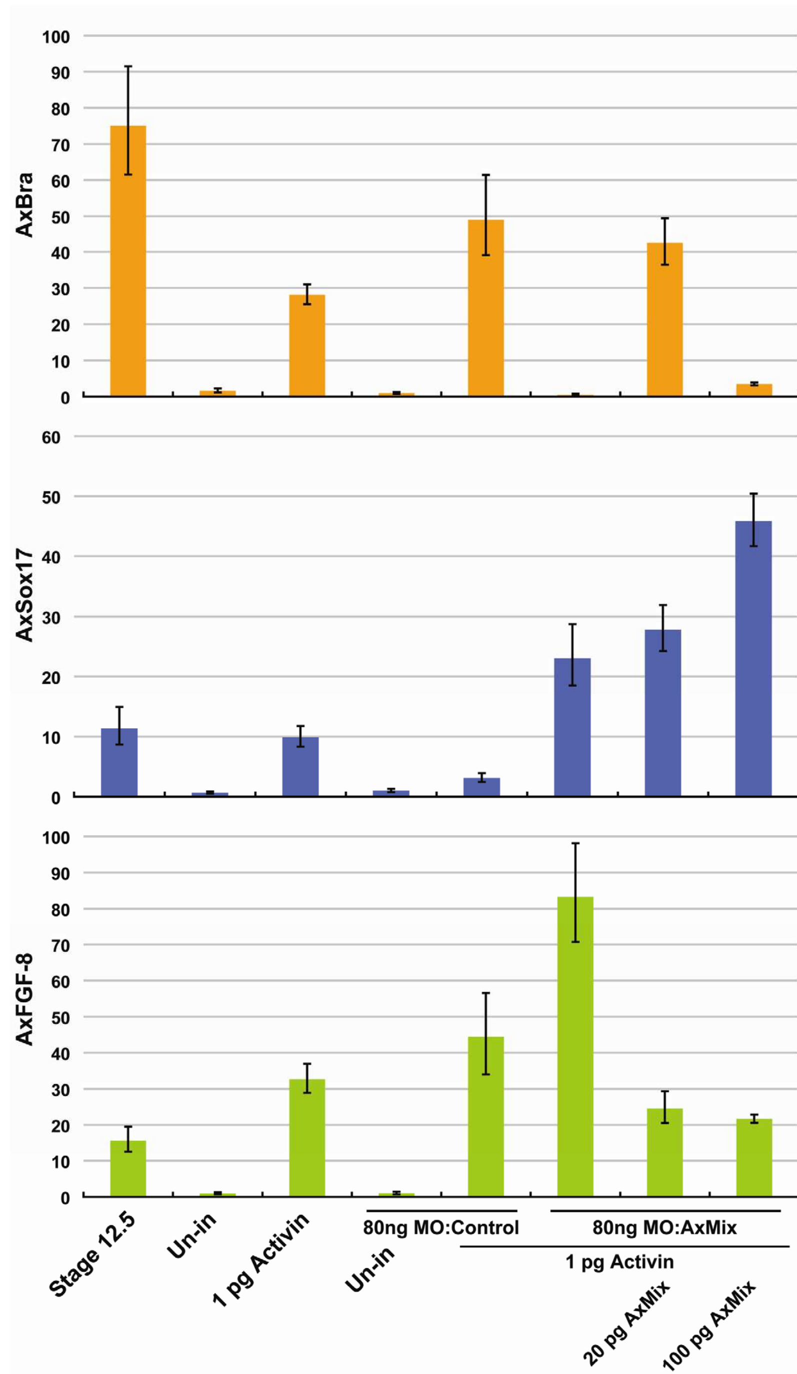


Figure 4.15 – qPCR analysis of the mesodermal and endodermal markers in mesoderm rescued caps with the injection of AxMix mRNA

Cap explants are collected at stage 12.5. Data is normalised to *ODC* and then MO:Control alone sample. Rescue of *AxBra* expression is seen at low levels of *AxMix* mRNA, whereas high *AxMix* mRNA levels lead to upregulation of *AxSox17*. The expression of *AxFGF-8* in the animal cap explants mirrors that seen in whole embryos.

4.7.3 Mixl1 in ES cell differentiation

These results indicate that the requirement for Mix activity in *Xenopus* and the axolotl is distinct. In *Xenopus,* Mix activity induces the endoderm at the expense of mesoderm, whilst in the axolotl Mix activity is required for the induction of mesoderm from the endoderm. We therefore sought to test which of these modes of development is conserved with mammals. Although a variety of studies have identified a critical role for mMixl1 in the specification of mesoderm and endoderm, the prevailing view is that *mMixl1* is an endodermal gene (Lim et al., 2009; Mohn et al., 2003; Tam et al., 2007). In particular, the exact relationship between *mMixl1* and *T/Brachyury* has not been clearly defined. For example, previous studies indicate that conditional induction of mMixl1 in embryonic stem cells results in the expression of early mesoderm markers, such as *T/Brachyury*, and acceleration of the mesoderm developmental program (Willey et al., 2006). In contrast, differentiation of mES cells in the absence of *mMixl1* suggests that mMixl1 acts as negative regulator of *T/Brachyury* expression (Izumi et al., 2007).

Genetic studies in *Mixl1*-null mice identify a role for Mixl1 in axial mesendoderm morphogenesis and endoderm formation during early gastrulation (Hart et al., 2002). Embryonic stem cells provide an *in vitro* approach for studying the induction and differentiation of the mesoderm, endoderm and ectoderm under appropriate conditions (Keller, 2005). The appearance of hematopoietic and endothelial progenitors in developing embryoid bodies (EBs) has been well characterized and recapitulates normal embryogenesis (Choi et al., 1998; Fehling et al., 2003). But the specification of blood from the mesoderm is poorly understood; however, recently some

data have accumulated to suggest that the *Mix* family is involved in the specification of blood from the mesoderm (Davis et al., 2008; Mead et al., 1996; Ng et al., 2005; Willey et al., 2006). Indeed, the hemangioblast, a common progenitor for hematopoietic and vascular cells, has been identified in the ES cell system (Choi et al., 1998; Kennedy et al., 1997). Recent studies use transgenic mice with green fluorescent protein (GFP) targeted to either the *Mixl1* or *T/Brachyury* locus to investigate their role in mesoderm patterning and hematopoiesis. These studies indicate that hemangioblasts arise from both $Mixl1^{+}FLK1^{+}$ and $Bra^{+}FLK1^{+}$ cells (Huber et al., 2004; Ng et al., 2005). Using a doxycycline (DOX) conditional induction system, *mMixl1* overexpression during the earliest stage of ES cell differentiation results in an increase in the number of mesodermal, hemangioblastic and hematopoietic progenitors (Willey et al., 2006). However, conflicting results indicate that overexpression of mMixl1 during ES cell differentiation will allocate cells to endoderm, supporting its role in endoderm induction (Lim et al., 2009). Indeed, Lim et al actually suggest that these different results may depend on the level of Mixl1 expression resembling the effect of Bix.1 on ventral mesoderm induction in *Xenopus* (Tada et al., 1998), with low levels inducing mesoderm and high levels promoting endoderm formation. In *Xenopus,* these different activities may have been adopted by differnt Mix-like gene family members following their formation through duplication of the ancestral Mix gene and subsequent subfunctionalization.

To clarify the relationship between mMixl1 and T/Brachyury, we used a drug inducible system to control the expression of small inhibitory hairpin RNAs (shRNA) targeted to *mMixl1*. The sequence for the Mixl1 shRNA was as

previously reported by Izumi et al (Izumi et al., 2007). The shRNA was cloned into a tet-on vector allowing a reversible drug controlled knockdown of Mixl1 activity in murine embryonic stem cells in the presence of doxycycline (1μg/ml) (Szulc et al., 2006). To generate stable cell lines, CGR8 ES cell lines were transfected with different linearized vectors including Mixl1 shRNA, a scrambled Mixl1 control, or the vector alone, and then selected for in FBS-ESCs medium with Zeocin (25ug/ml). Stable ES cell lines were maintained in KSR-ESCs medium with Zeocin (5ug/ml) and these were used to generate embryoid bodies to test the consequence of Mixl1 knockdown on the expression of *T/Brachyury*. To knockdown endogenous *mMixl1*, CGR8 ES cells were plated in gelatin-coated plates in KSR-ESC medium and DOX (0.5μg/ml) for three days before forming embryoid bodies. After this, the cells were trypsinised and embryoid bodies were formed using the hanging drop method. All cells were maintained in KSR differentiation medium plus or minus DOX (0.5μg/ml) as appropriate. EBs were harvested beginning at day 2 and then each day thereafter until day 5 (shown schematically in Figure 4.16A). Total cellular RNA was isolated from each sample and analyzed by qPCR for expression of *Mixl1*, *T/Brachyury*, *Sox17* and *FGF-4* (Figure 4.16B). All samples were normalised against an untransformed day 0 control. As expected, the Mixl1 shRNA leads to a substantial inhibition of Mixl1 compared with non transfected, scrambled or vector only lines (Figure 4.16B i). As seen in the axolotl, *T/Brachyury* expression is dramatically decreased by Mixl1 shRNA knockdown (Figure 4.14B ii). Mixl1 and Sox17 are both involved in the commitment of the definitive endoderm (Hart et al., 2002). Knockdown (Izumi et al., 2007) or overexpression (Lim et al., 2009) of *Mixl1* suggests *Mixl1* expression affects *Sox17* expression during ESCs differentiation. In

agreement with this, we find that *Sox17* expression is also markedly decreased by *Mixl1* knockdown (Figure 4.16B iii). In differentiating EBs, levels of *FGF4* mRNA decrease steadily as ES cells differentiate (Figure 4.16B iv) and this is not affected by *Mixl1* knockdown.

Figure 4.16 – Mixl1 knockdown on mouse ES cells

(A) General experimental protocol for shRNA induction and differentiation of EBs in culture medium with DOX. DOX (0.5 μg/ml) was added for three days prior to differentiating cells in hanging drops. ES cells and EBs were collected at five time points from day 0 – day 5. **(B)** ES cells aggregated into embryoid bodies by hanging drop go on to express *T/Brachyury*. qPCR demonstrating the Mixl1 knockdown obtained by shRNA knockdown (i). qPCR demonstrating the loss of *T/Brachyury* in Mixl1 shRNA, but not scramble or vector alone, differentiated embryoid bodies (ii). The level of *Sox17* is lower in Mixl1 knockdown samples compared to controls (iii). For *FGF4*, the expression levels stay no change (iv).

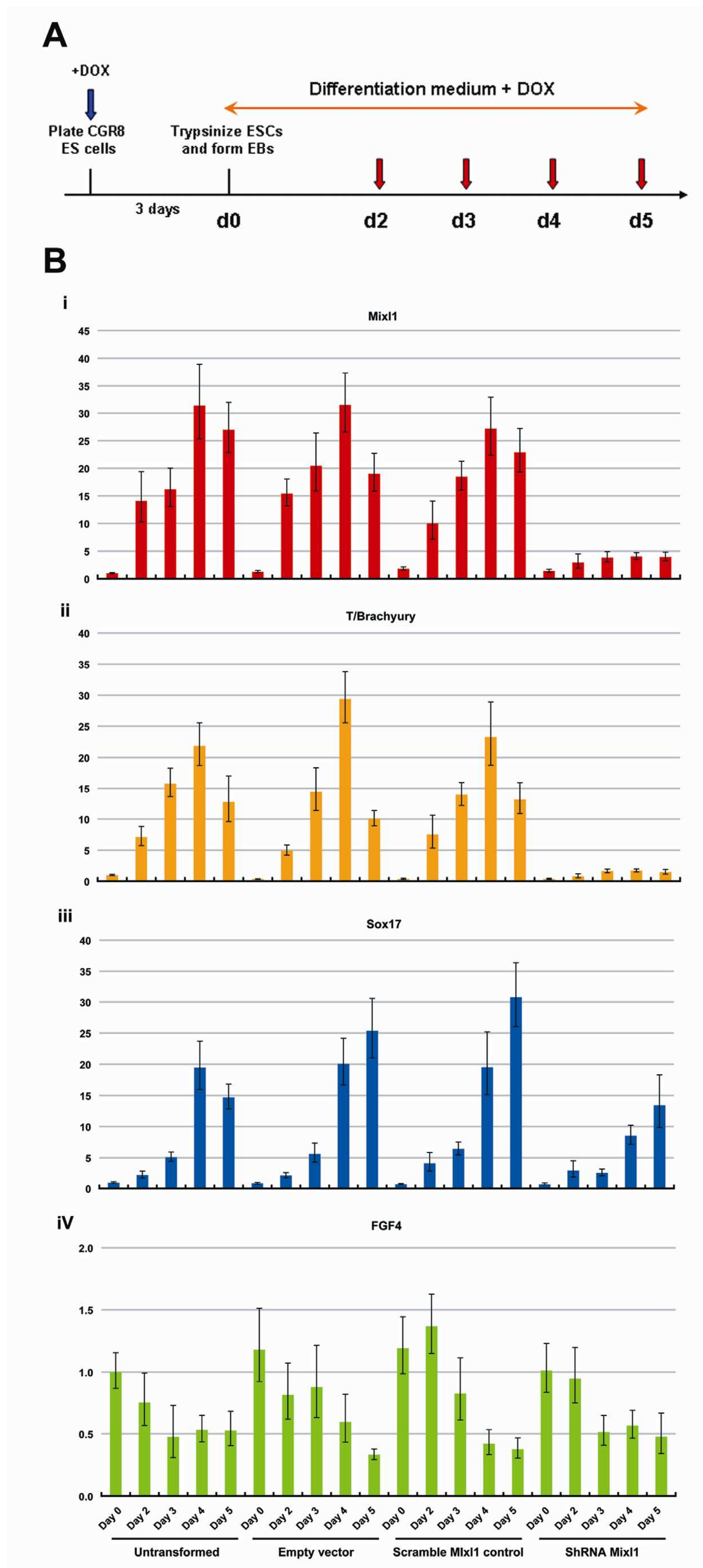


Figure 4.16 – Mixl1 knockdown on mouse ES cells

4.8 Discussion

In chapter 3 we reported the isolation of two axolotl *Nodal-related* genes, *AxNodal-1* and *AxNodal-2*. Here, we analyze their function during the earliest events of embryogenesis. As discussed in Chapter 3, the ancestor of *AxNodal-1* appears to have undergone extensive amplification in the anurans. RT-PCR and qPCR results show both *Nodal* genes commence expression at the MBT and are expressed in a similar pattern during gastrulation except the later asymmetrical expression of *AxNodal-1*. *In-situ* hybridization results reveal *AxNodal-1* but not *AxNodal-2* is detectable in the left lateral plate mesoderm, in a similar pattern to the well-characterised role for *Nodal* in left-right asymmetry. *AxNodal-2*, lacking the later asymmetrical expression, has an expression pattern similar to *Xnr4*, in agreement with the phylogenetic analysis (see Chapter 3). However, expression of *AxNodal-2* in the notochord has not been seen during neurula stages. *In situ* hybridysation of sectioned late neurala embryos may be able to reveal the expression of both Nodals in the notochord.

The different expression patterns of *AxNodal-1* and *AxNodal-2* suggest they may have different functions. Ectopic overepression of *AxNodal-1* and *AxNodal-2* in *Xenopus* animal caps identify different activities for the two Nodal genes in mesoderm and endoderm induction. Similar to *Xenopus Xnr1, 2, 5* and *6* (Engleka et al., 2001; Jones et al., 1995; Osada and Wright, 1999; Takahashi et al., 2000), injection of only 20pg *AxNodal-1* mRNA is sufficient to induce mesodermal (*XlBra* and *XlMyoD*) and endodermal (*XlSox17*) marker gene expression and results in elongation of the caps. However,

AxNodal-2 only weakly induces mesodermal (*XlBra* and *XlMyo*D) genes at high injection levels (200pg), similar to Xnr4 (Osada and Wright, 1999). We conclude that AxNodal-1 is the stronger mesoderm and endoderm inducer in the axolotl.

The inhibitor SB431542 has been shown to completely block nodal signalling in *Xenopus* embryos (Ho et al., 2006; Inman et al., 2002). We therefore used this inhibitor to block all Nodal activity in axolotl embryos. Embryos treated with SB431542 fail to form dorsal lips and do not gastrulate, phenocopying the effect of SB431542 treatment in *Xenopus* embryos. qPCR analysis on SB431542 treated embryos reveals downregulation of *AxMix, AxBra*, *AxFGF-8* and *AxSox17*, markers of mesoderm and endoderm. This phenotype represents a complete loss of nodal signalling and should be phenocopied by other methods to knockdown nodal activity.

Whilst we have carried out extensive analysis to determine if *AxNodal-1* and *AxNodal-2* represent the only *Nodal* family members in the axolotl, definitive proof can only be obtained by genome sequence which is not available at this time. We reasoned that *AxNodal* gene knockdown by morpholinos should phenocopy the SB431542 phenotype if *AxNodal-1* and *AxNodal-2* are the only nodal genes in the axolotl. We therefore used antisense morpholinos targeted to the splice junctions of *AxNodal-1* and *AxNodal-2* to unambiguously disrupt their expression. Surprisingly, knockdown of *AxNodal-1* alone is sufficient to block the induction of mesoderm and endoderm, phenocopying the effects of chemical inhibition (SB431542) of Nodal signalling at both a morphological and molecular level. In contrast,

AxNodal-2 morphants are able to gastrulate and do form mesoderm and endoderm. Later in development, AxNodal-2 morphants show abnormal axial patterning with disruption to anterior and posterior structures, and a failure in closing the neural plate. The AxNodal-2 morphants suggest that AxNodal-2 may play a role similar to *Xenopus* Xnr4 which shows restricted expression in notochord precursors and is unlikely to play a major role in general mesendoderm induction, although it may participate in patterning (Joseph and Melton, 1997). These results suggest that only one nodal gene, AxNodal-1, is required for the induction of the mesoderm in the axolotl and if any other nodal genes are found in the axolotl genome they are not sufficient to induce mesoderm.

Surprisingly, knockdown of *AxMix* also blocks the induction of mesoderm, demonstrating that Nodal and AxMix act together in a pathway for mesoderm specification. Notably, AxNodal and AxMix morphants result in the loss of *AxBra* expression. We therefore investigated the phenotype of AxBra morphants, showing they have a similar disruption to gastrulation, failing to form a dorsal lip. To further explore the requirement for AxMix in the induction of mesoderm, we used the animal cap assay, blocking mesoderm induction by activin with AxMix morpholinos. Here we show the rescue of mesoderm by the overexpression of *AxMix* mRNA, demonstrating a role for AxMix in mesoderm induction. It is also possible to induce some elongation in Activin/AxMix morphant caps by overexpression of *AxBra*, although a lack of downstream targets for AxBra in the axolotl prevented us from investigating this further (data not shown).

It is important to note that such experiments are practically impossible in *Xenopus* due to the amplifications that have occurred in the *Nodal* and *Mix* gene families. Indeed, morphants of individual *Xenopus Mix* genes gastrulate with no failure in mesoderm specification, although FGF signalling is upregulated (Colas et al., 2008; Kofron et al., 2004; Trindade et al., 2003). Similarly, although mesoderm specification in *Xenopus* can be prevented by chemical inhibition of Nodal signalling, there is no evidence that expression of any one *Nodal* gene family member is necessary for the production of mesoderm (Ho et al., 2006; Osada and Wright, 1999; Takahashi et al., 2006). Thus, amplification of the *Nodal* and *Mix* genes renders the mesodermal GRN of *Xenopus* resistant to perturbations that would be lethal in axolotl. The evolution of gene expansion within a GRN is likely to include the establishment of novel genetic interactions within the network, and we have identified critical differences in the role for *Mix* in axolotl and *Xenopus*.

In *Xenopus* embryos Nodal signalling induces co-expression of the *Mix* genes and *Brachyury* in the mesendoderm (Lemaire et al., 1998; Wardle and Smith, 2006). The negative regulatory loop between these factors causes Brachyury to segregate with the mesoderm and Mix-like genes to segregate with endoderm. However, previous genetic studies have shown limited co-expression of *AxBra* and *AxMix* in axolotl embryos, and even then only in the ventral mesoderm (work carried out by G.Swiers). Furthermore, our results place *AxFGF8*, A*xSox17*, *AxMix* and *AxBra* downstream of Nodal signalling, with the activation of *AxBra* dependent on AxMix activity. This suggests that mesoderm specification in the axolotl requires AxMix activity. In contrast, based on the available evidence from *Xenopus*, we would have

expected AxMix morphants to promote mesoderm and suppress endoderm (Kofron et al., 2004; Lemaire et al., 1998). In *Xenopus,* Mixer morphants result in reduced *XlSox17* expression suggesting a reduction in the endoderm (Kofron et al., 2004). In axolotl AxMix morphants, *AxSox17* expression is increased, suggesting a loss of mesoderm and a gain of endoderm. In this context, it is worth considering the AxMix rescue experiment. Here, low levels of exogenous AxMix can rescue mesoderm induction and *AxBra* expression. However, high levels of exogenous AxMix induce *AxSox17* expression and low levels of *AxBra*, indicative of endoderm induction. These data indicate that AxMix has a role in the induction of mesoderm and endoderm in axolotls, and the up-regulation of *AxSox17* expression in AxMix morphants is a secondary effect due to the loss of mesoderm. AxBra morphants confirm this idea as *AxBra* knockdown increases *AxSox17* expression indicating that the normal role of AxBra may be to negatively regulate *AxSox17* expression during mesoderm and endoderm specification. This identifies a requirement for AxMix in mesoderm induction prior to any role in its induction or suppression, and this is difficult to see in *Xenopus* due to multiple *Mix-like* genes in the mesendoderm GRN. In the axolotl we demonstrated this directly, showing that the *AxBra* domain is expanded in response to forced *AxMix* expression.

Studies of the role of Mix in mouse embryos have led to conflicting results. Some studies implicate Mix in mesoderm production, others in its repression (Lim et al., 2009; Willey et al., 2006). In *mMixl1*$^{-/-}$ mutant embryos, although mMixl1 is not required for the mesoderm induction as suggested by the observed expression of *T/Brachyury* (but absent in the core of the primitive

streak) and *Nodal*, deficient mesoderm development suggesting mMixl1 is required for normal development of node, notochord, axial mesendoerm and heart (Hart et al., 2002). However, it should be noted that the increased expression of *Brachyury* is ectopic and actually excluded from the core of the primitive streak compared to controls (Hart et al., 2002). Moreover, though *Mixer* depletion results in an expansion of mesoderm in *Xenopus* embryos, the expression of *Brachyury* is decreased (Kofron et al., 2004). Conditional activation of *mMixl1* is sufficient to accelerate the formation of mesoderm followed by inducing early activation of *T/Brachyury* (Willey et al., 2006). However, constitutive overexpression of mMixl1 in differentiating ES cells suppresses hematopoietic mesoderm and promotes endoderm formation (Lim et al., 2009), revealing mMixl1 is able to determine the formation of mesoderm and endoderm depending on different inductive activity. Previous genetic studies in *Xenopus* and zebrafish have established *Mix/Bix* genes as determining factors in endoderm formation (Henry and Melton, 1998; Kikuchi et al., 2000; Latinkic and Smith, 1999; Lemaire et al., 1998; Poulain and Lepage, 2002; Tada et al., 1998), but the evidence for the *Xenopus Mix* genes being involved in the specification of blood from the mesoderm is not as convincing (Willey et al., 2006). However, we knocked down *mMixl1* in EBs and showed a clear inhibition of *T/Brachyury* expression. This is consistent with the absence of *T/Brachyury* expression in the primitive streak (the site of nascent mesoderm production) of *Mixl1*$^{-/-}$ mouse embryos, suggesting that the role for Mix at the top of a hierarchy or transcription factors leading to mesoderm specification is conserved in vertebrates.

Therefore, similar to mMixl1, AxMix may have a non–cell-autonomous role in

mesoderm that serves to modulate endodermal differentiation, or it may function cell-autonomously within a transient population of mesendodermal progenitors. Nevertheless, our observations suggest a two-step process for mesoderm induction in the axolotl. Firstly, Nodal, via Mix, induces a population of mesendodermal cells, the bipotential precursors of the mesoderm and endoderm. In the second step, *Brachyury* expression, triggered by Mix, induces the mesoderm. The loss of mesoderm in the Nodal and Mix morphants reflects the loss of the bipotential mesendoderm which accounts for the mesodermal defects we observe.

Chapter 5. Maternal determinants and Wnt/β-catenin signalling in the induction of mesoderm in axolotl embryos

5.1 Introduction

The induction and patterning of the mesoderm and endoderm in the frog, *Xenopus laevis*, requires the presence of asymmetrically localised maternal determinants including VegT, Vg1 and Wnt11 (Horb and Thomsen, 1997; Ku and Melton, 1993; Rebagliati et al., 1985; Weeks and Melton, 1987). In *Xenopus* these maternal factors regulate Nodal signalling activity and subsequently the induction of the mesendoderm, in part through the activity of the Mix-like and Brachyury transcription factors (Agius et al., 2000; Clements et al., 1999; Joseph and Melton, 1998; Xanthos et al., 2002).

The axolotl has only a single *Mix* and two *Nodal* genes, representing a dramatically simplified regulatory network compared with *Xenopus*. We investigated the interactions downstream of AxNodal-1, revealing differences between *Xenopus* and axolotl in the formation of the mesoderm. We therefore asked if the upstream regulation of Nodal activity is conserved from *Xenopus* to axolotl. In particular, we investigated the role of VegT and β-catenin in mesoderm specification. Vg1 has long been considered a likely candidate for the TGF-β signal, however, failure to identify significant amounts of endogenous mature Vg1 protein in the embryo restrict its role to body axial patterning (Birsoy et al., 2006; Thomas and Moos, Jr., 2007). A recent report of a *Vg1* allele with improved proteolytic processing provides a

plausible mechanism for the local action of Vg1 in the developing body axis (Birsoy et al., 2006). However, conflicting rescue experiments and the processing of Vg1 required to generate its mature form means its role in establishing the germ layers in *Xenopus* remains elusive (Thomas and Moos, Jr., 2007; Wylie et al., 1996). We therefore chose to predominantly focus our efforts on the molecular function downstream of VegT and β-catenin during mesoderm formation in the axolotl.

The *VegT* ortholog (*AmVegT,* here referred to as *AxVegT*) has previously been cloned from the axolotl (Nath and Elinson, 2007). Unexpectedly, *in situ* hybridization for *AxVegT* in early axolotl embryos reveals no vegetal localisation of *AxVegT* transcripts. Rather, the transcripts are uniformly localised around the oocyte in the inner central cytoplasm. VegT (also known as Brat, Xombi or Antipodean in *Xenopus laevis*) is a T-box transcription factor supplied maternally in the oocyte and transcribed zygotically within the equatorial zone. It is so named as a consequence of its vegetal localisation in *Xenopus laevis* embryos (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). The change in localisation between the axolotl and *Xenopus* suggests differences in the molecular organization of the oocytes of these two amphibians.

Similarly, the axolotl *Vg1* ortholog, previously isolated by E. Richardson and A.D.Johnson*,* does not localise to the vegetal pole (Pers. Comm.). This suggests fundamental differences in the regulation of mesoderm induction between anurans (exemplified by *Xenopus laevis*) and urodeles (*Ambystoma*). To determine which of these two states, localised or

non-localised, is ancestral, we investigated the localisation of *VegT* and *Vg1* in two ancient fish, the lungfish and sturgeon. Subsequently, by injections in both *Xenopus* and axolotl embryos, we examined the role of AxVegT and β-catenin in mesoderm induction.

5.2 Expression of Vg1 and VegT in axolotl embryos

Firstly, we re-examined the expression patterns of *AxVegT* and *AxVg1* mRNA in axolotl oocyte sections (Figure 5.1A ii and vi) confirming previous studies and revealing no evidence for *AxVegT* or *AxVg1* mRNA localisation in any region of the embryo. In early vitellogenic axolotl oocytes (stage III-IV), staining for *AxVegT* and *AxVg1* is present throughout the inner central cytoplasm around the nucleus in the oocyte. A similar expression pattern was previously described for *AxDazl* mRNA (Johnson et al., 2001). The localised expression of *XlVegT* and *XlVg1* was re-confirmed in *Xenopus laevis* oocytes (Figure 5.1A i and v). To demonstrate the existence of localisation machinery in the axolotl the expression pattern of *Hermes*, an RNA binding protein vegetally localised in *Xenopus laevis* oocytes (Song et al., 2007), was determined. *In-situ* hybridization with *AxHermes* in axolotl oocytes demonstrates localisation to the vegetal hemisphere (Figure 5.1B ix - work carried out by E.Richardson), confirming that axolotl oocytes do contain RNA localisation machinery, but mRNAs encoding germ line or mesendodermal determinants are not localised.

To determine if the localisation of RNAs encoding deteminants of the mesendoderm represents a derived trait in anurans, we investigated the

localisation of *Vg1* and *VegT* in extant fish with primitive characteristics. In the absence of EST or genomic sequences, we used a degenerate PCR-based approach designed against the conserved regions of *VegT* and *Vg1* in *Xenopus* and axolotl. Both *VegT* and *Vg1* were cloned from *Neoceratodus forsteri*, the Australian lungfish, and *Acipenser oxyrinchus*, the gulf sturgeon, oocytes (see Appendix). These sequences were confirmed as *VegT* and *Vg1* orthologs by NCBI BLAST. We investigated the expression of *VegT* and *Vg1* orthologs in lungfish and sturgeon ovary sections by *in-situ* hybridization.

Sections from lungfish ovaries hybridized to *VegT* or *Vg1* show strong expression throughout the oocyte cytoplasm (Figure 5.1A iii and vii). Similarly, sturgeon oocytes show strong maternal expression of *VegT* and *Vg1* mRNAs throughout the central cytoplasm (Figure 5.1A iv and viii). These results most closely resemble the distribution of *VegT* and *Vg1* mRNAs in the axolotl. This suggests that the absence of asymmetric localisation of transcripts for mesendodermal determinants is the ancestral vertebrate trait. No staining is seen in different oocytes hybridized to the sense probe (Figure 5.1B).

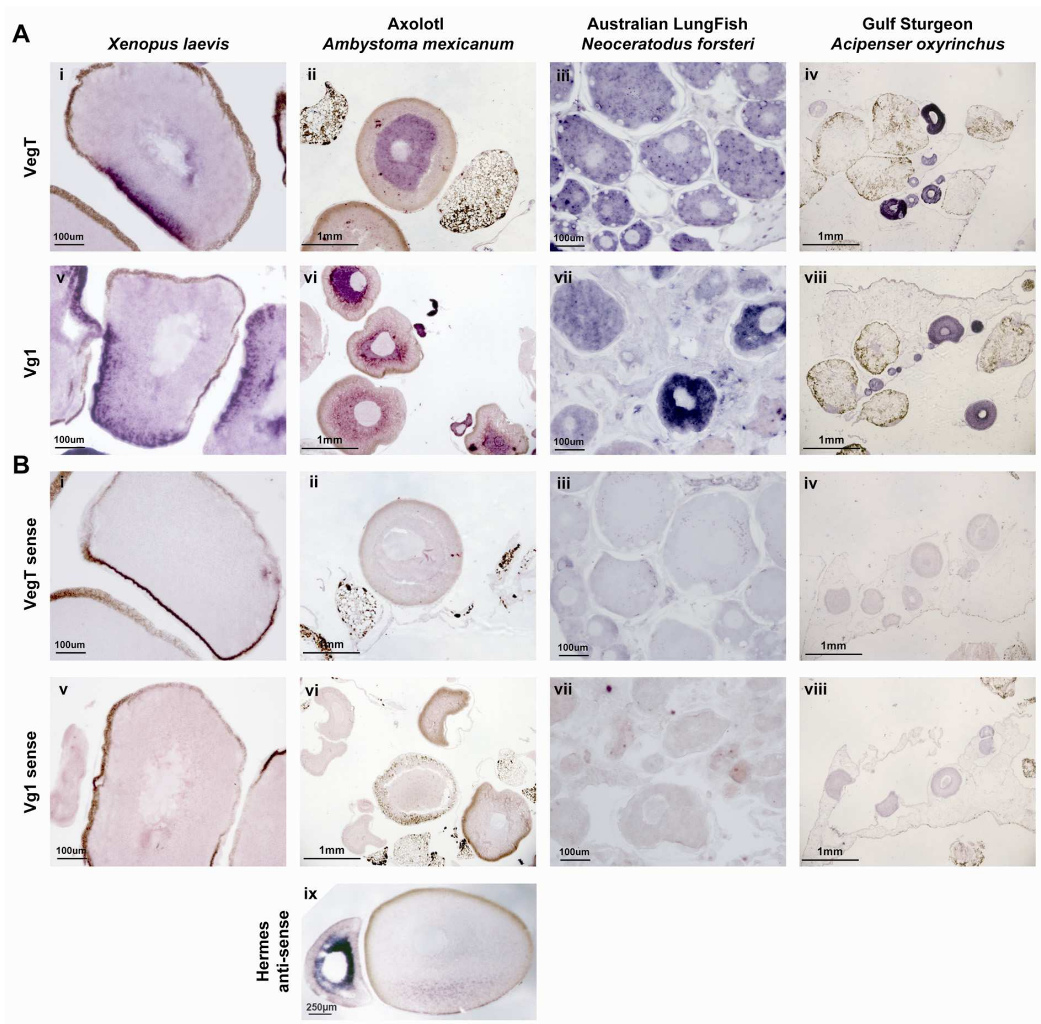


Figure 5.1 - Expression of VegT and Vg1 orthologs in ovarian oocytes
Early vitellogenic oocytes from *Xenopus laevis*, *Ambystoma mexicanum*, *Neoceratodus forsteri* and *Acipenser oxyrinchus* hybridized with an antisense *VegT* or *Vg1* probe. The clear area in the center of the oocytes is the nucleus surrounded by central cytoplasm. **(A)** Staining reveals the asymmetric localisation of *XlVegT* and *XlVg1* (i and v). In contrast to *Xenopus*, sections from the axolotl, lungfish and sturgeon show strong stained cytoplasm (purple) surrounding the pale nucleus. **(B)** Adjacent sections hybridized with a sense probe show little staining. Scale bar: 100 µm (i, v, iii and vii); 1 mm (ii, vi, iv and viii).

5.3 The effects of VegT, Vg1 and β-catenin

Given the absence of localisation of *AxVegT* and *AxVg1* in axolotl oocytes and the observation that this appears to be the ancestral state, we sought to identify the roles of VegT and Vg1 in early axolotl development. Alongside this we investigated the role of β-catenin as this molecule is also maternally deposited and localised with cortical rotation (Moon and Kimelman, 1998). Firstly, we confirmed the role of these molecules in *Xenopus laevis*.

5.3.1 Over-expression of VegT, Vg1 and β-catenin in *Xenopus laevis* embryos

After the mid-blastula transition, VegT activates the expression of a large number of zygotic genes, many of them transcription factors which themselves regulate the formation of the endoderm, including Sox17, GATA factors, Mix.1 and Mixer (Clements et al., 1999; Kofron et al., 2004; Xanthos et al., 2001). For mesoderm induction, VegT activates the expression of TGF-β family signals, including Xnr1, Xnr2, Xnr4 and derriere (Clements et al., 1999; Kofron et al., 1999), themselves inducers of mesoderm fates (Kofron et al., 1999; Lee et al., 2001).

In *Xenopus*, VegT and Wnt/β-catenin signalling are involved in specifying the organizer and axial mesoderm (Agius et al., 2000). Rescue experiments in VegT and β-catenin depleted embryos indicate that VegT and its targets are essential for mesoderm and axis formation, whilst β-catenin is required for normal axial mesoderm development (Katsumoto et al., 2004; Xanthos et al., 2002). In *Xenopus* embryos, β-catenin depleted equators express general mesodermal genes, but not dorsal mesodermal markers demonstrating that

β-catenin is the dorsal determinant (Xanthos et al., 2002). The Wnt pathway acts with VegT to de-repress XTcf3-inhibited genes in the equator and vegetal mass and to modulate the level and timing of signalling downstream of VegT (Xanthos et al., 2002).

The effects of XlVegT, XlVg1 and β-catenin on *Xenopus laevis* development have previously been determined (Katsumoto et al., 2004; White and Heasman, 2008; Xanthos et al., 2002). We repeated these experiments in *Xenopus* and axolotl embryos to compare them with one another. First, we injected each *Xenopus* ortholog (50pg, 200pg and 1ng) into the animal pole of *Xenopus laevis* embryos at the one or two cells stage, alongside a lineage tracer (100pg GFP mRNA). Animal caps were dissected at stage 9 and collected when sibling embryos reached stage 20 in order to observe the animal caps undergoing convergent extension (Figure 5.2). Uninjected control caps remain rounded and differentiate into atypical epidermis. XlVegT is unable to induce elongation (characteristic of mesoderm induction) at any level; however, cap explants do show some endodermal tissue phenotypes (Figure 5.2 i-iii). Overexpression of *XlVg1* mRNA has no effect in *Xenopus laevis* animal caps (Figure 5.2 iv-vi) (Tannahill and Melton, 1989). *β-catenin* mRNA injected caps do not elongate, but do form vesicle-like shapes indicating the possible presence of ventral mesoderm (Figure 5.2 ix-xi) (Domingos et al., 2001; Guger and Gumbiner, 1995).

These cap explants were assayed for expression of the mesoderm and endoderm markers; *Bra, MyoD*, *Mix.1*, *Mixer* and *Sox17,* and compared with uninjected caps that differentiate into epidermal or neural fates (Figure 5.3).

As expected, XlVegT induces a full spectrum of early markers representing dorsal mesoderm and endoderm. All doses of XlVegT induce *XlBra* (Figure 5.3 i) in agreement with previous observations (Clements et al., 1999; Horb and Thomsen, 1997). Over-expression of XlVegT only weakly induces the paraxial mesoderm marker, *MyoD,* at the highest dose (1ng) (Figure 5.3 ii). XlVegT also induces endodermal tissues as shown by the expression of *Mix.1*, *Mixer* and *Sox17* (Figure 5.3 iii-v). qPCR analysis of *XlVg1* injected animal caps shows no effect on mesoderm and endoderm formation. In *β-catenin* injected cap explants, *XlBra* is not induced above control caps at any level (Figure 5.3 i). Intermediate (200pg) and high (1ng) *β-catenin* mRNA doses induce *MyoD* compared to controls (Figure 5.3 ii). No dose of β-catenin has any effect on endoderm formation (Figure 5.3 iii-v).

Although these caps do not elongate, high doses of *β-catenin* alone can induce a 50-fold induction of *MyoD* expression. Previous studies indicate that Wnt/β-catenin signalling may be required for the expression of *MyoD* and myogenesis (Borello et al., 1999; Wang et al., 1997). The Wnt/β-catenin pathway is required for regulating myogenic gene expression in the presumptive mesoderm by inducing *Myf5* expression (Shi et al., 2002). Myf5 can activate *cardiac actin* and *MyoD,* but MyoD can not induce *Myf5* placing *Myf5* upstream of *MyoD* (Hopwood et al., 1991). Although β-catenin is important for the expression of *MyoD,* it is not sufficient to induce the full mesodermal gene expression program.

Our results demonstrate that *Xenopus* VegT acts as a maternally localised determinant for mesoderm and endoderm differentiation. However, in the

absence of synergy (explored further below), β-catenin alone is not sufficient to induce mesoderm (Clements et al., 1999; Domingos et al., 2001; Guger and Gumbiner, 1995; Wylie et al., 1996).

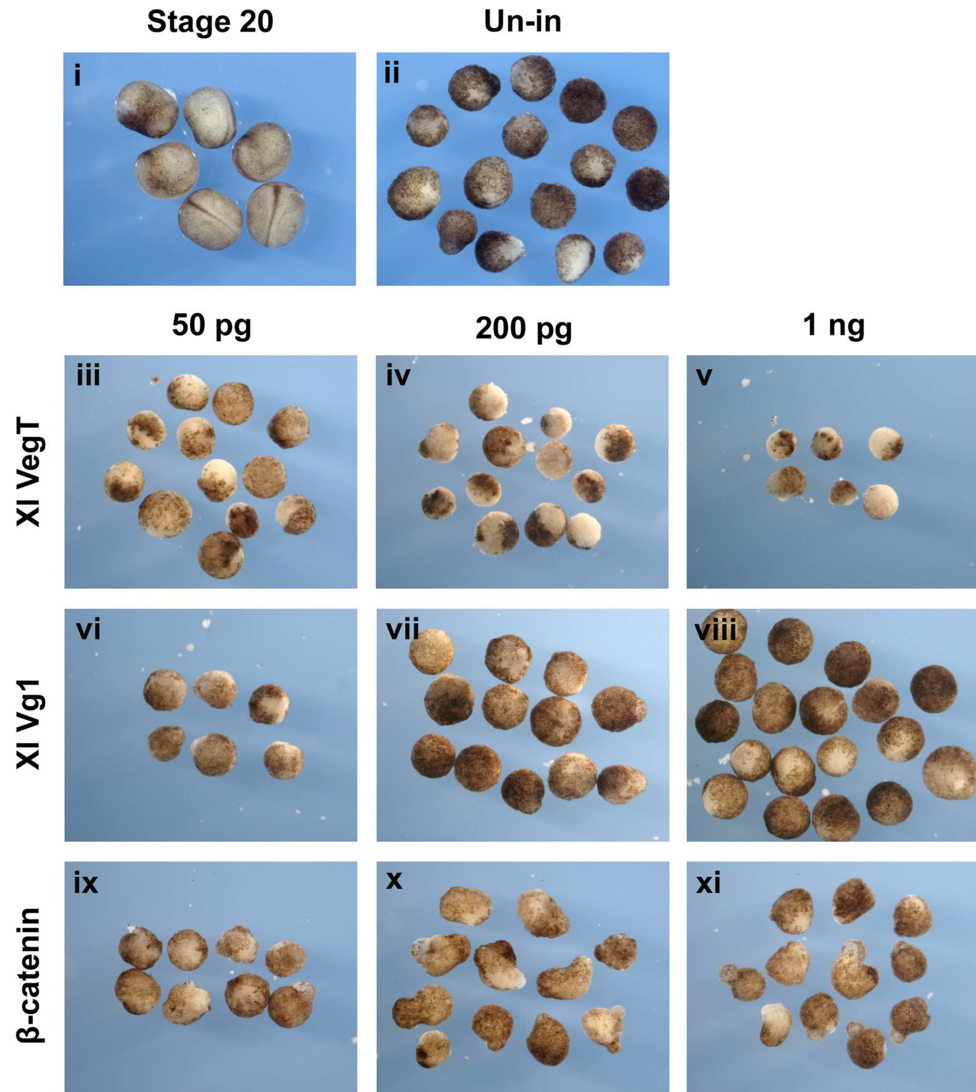


Figure 5.2 – *Xenopus* animal caps overexpressing XlVegT, XlVg1 and β-catenin

Animal cap explants are collected at stage 20 compared with control embryos. Animal caps were cut from stage 9 embryos injected with synthesized RNA; *XlVegT, XlVg1* or *β-catenin*, into the animal pole at the one or two-cell stage with coinjection of 100pg GFP RNA. Amounts of RNA injected (per embryo) are indicated on the top side of the panels. Animal caps developed endoderm-like tissue as a result of injection of *AxVegT* RNA at all levels compared to control caps. Wild type *XlVg1* injected cap explants are indistinguishable from controls. Similarly, in *β-catenin* injected samples, explants showed no elongation under all conditions but showed the vesicle-like shape at high doses (200pg and 1ng) of *β-catenin* RNA.

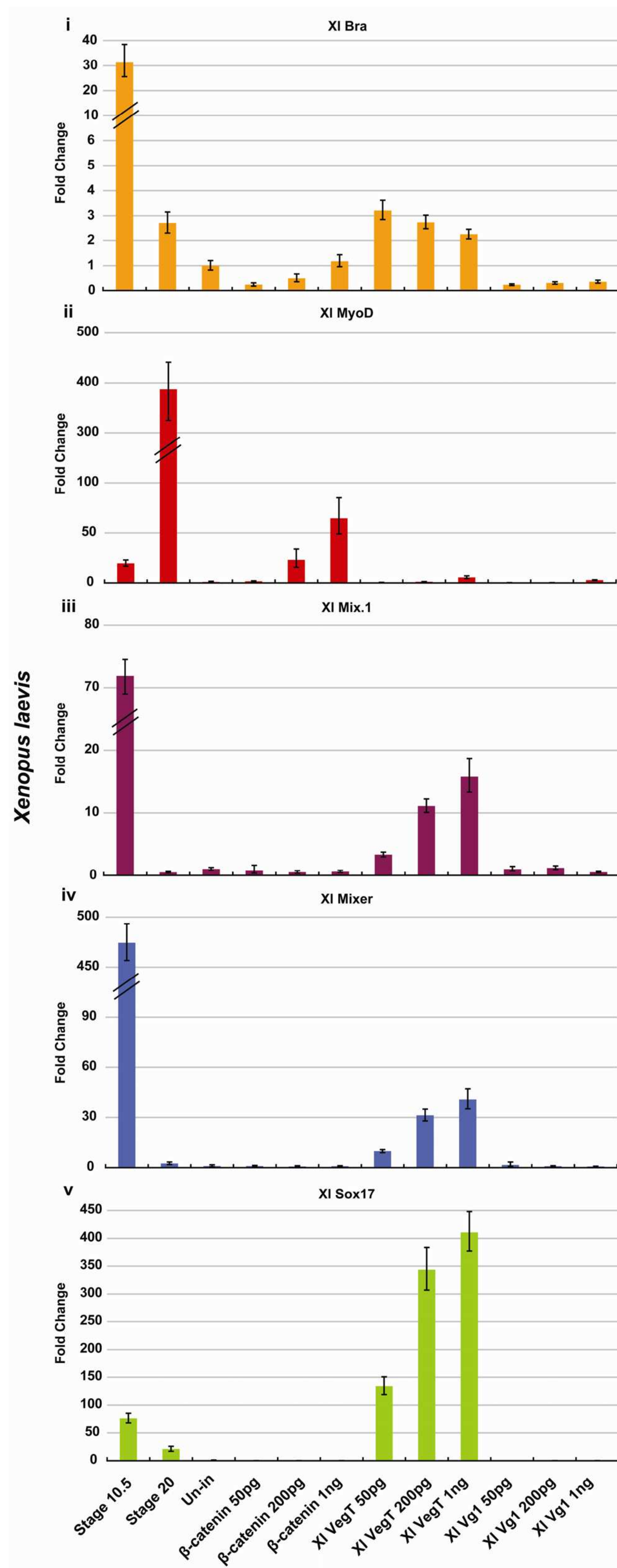


Figure 5.3 – qPCR analysis of mesoderm and endoderm induction by XlVegT, XlVg1 and β-catenin

Cap explants were collected at stage 20 and gene expression levels are relative to *ODC*, and then normalised to the uninjected samples. Primers and probes as described in the materials and methods.

5.3.2 Mesoderm induction in *Xenopus* animal caps

Ectopic overexpression of β-catenin in animal caps alone is incapable of inducing significant expression of mesodermal markers (this work and (Guger and Gumbiner, 1995), but can induce a second axis in whole embryos (Guger and Gumbiner, 1995). Later studies demonstrated that the canonical *β-catenin*-dependent Wnt pathway is required for early *Brachyury* expression (Vonica and Gumbiner, 2002). However, β-catenin mis-expression does not alter ectoderm cell fate but behaves synergistically with other factors in *Xenopus* dorsal-ventral patterning (Guger and Gumbiner, 1995; Katsumoto et al., 2004; Wylie et al., 1996).

Confirming this, we injected *Xenopus* embryos at the one or two cell stage with mRNAs encoding *activin*, *XlVegT* and *β-catenin* mRNAs alone and in combination (Figure 5.4). Caps isolated from embryos injected with 0.25 pg of *activin*, mimicking TGF-β signalling, elongate dramatically. *β-catenin* alone (200pg and 1ng) injected caps are indistinguishable from controls. Similarly, low levels of *XlVegT* alone (10pg and 50pg) fail to induce mesoderm formation. Co-injection of *β-catenin* and *XlVegT* mRNA causes an exaggerated dorsal mesoderm response in animal caps, measured both by elongation and gene expression (Figure 5.5).

Using qPCR analysis we examined the same panel of mesoderm and endoderm marker genes in these caps (Figure 5.5). Animal caps injected with 0.25 pg *activin* mRNA efficiently induce mesoderm (*XlBra* and *XlMyoD*) and endoderm (*XlMix.1*, *XlMixer* and *XlSox17*) marker genes. Low doses of *β-catenin* (200pg) fail to activate dorsal mesoderm and endoderm markers,

and only weakly induce *XlMyoD*. High doses (1ng) of *β-catenin* weakly induce *XlBra* and *XlMyoD* expression but not endodermal marker genes. 10pg *XlVegT* mRNA weakly induces *XlBra* and *XlSox17* expression but not *XlMyoD*, *XlMix.1* and *XlMixer*. At higher doses (50pg) *XlVegT* weakly induces both mesoderm and endoderm marker genes. Co-injection of *β-catenin* (200pg and 1ng) and *XlVegT* (10pg) mRNA synergise to significantly induce mesoderm markers. Co-injection of *β-catenin* and 50pg *XlVegT* mRNA synergise to induce *XlMyoD* expression and also the endodermal marker genes (*Mix.1*, *Mixer* and *Sox17*). All together, these results confirm *VegT* acts as an endomesodermal determinant in *Xenopus* embryos with β-catenin potentiating the response to VegT.

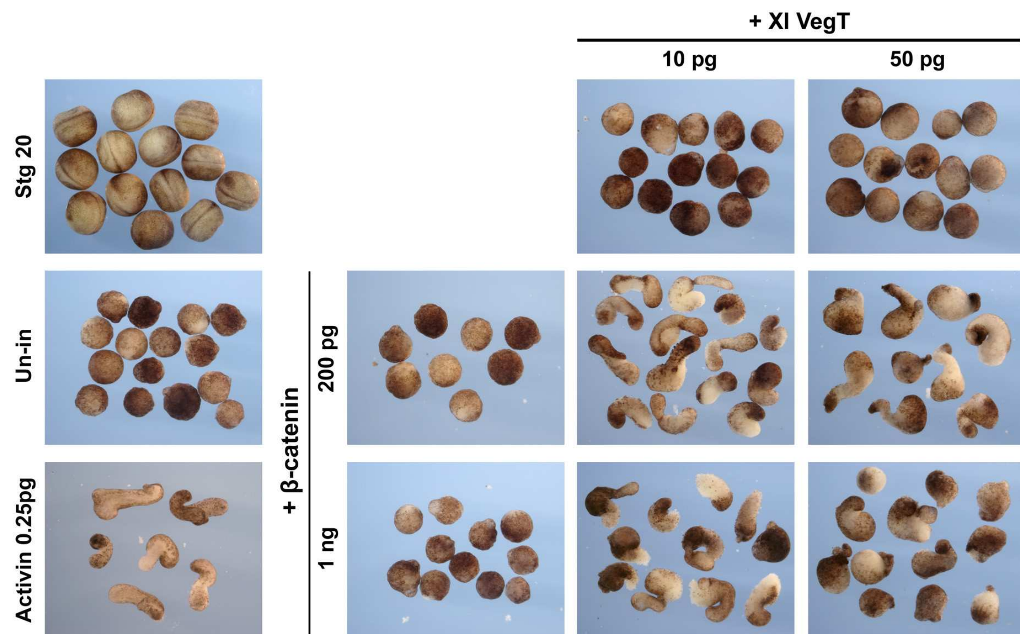


Figure 5.4 – *Xenopus* animal caps overexpressing XlVegT, β-catenin and in combination

Animal caps were cut from stage 9 embryos injected with various combinations of *activin*, *XlVegT* and *β-catenin.* The amounts of RNA injected are indicated. Animal cap explants were collected at stage 20. Animal caps treated with activin elongate. Dramatic elongatation is caused by coinjection of *XlVegT* and *β-catenin* mRNAs. *XlVegT* or *β-catenin* alone injected samples show no elongation.

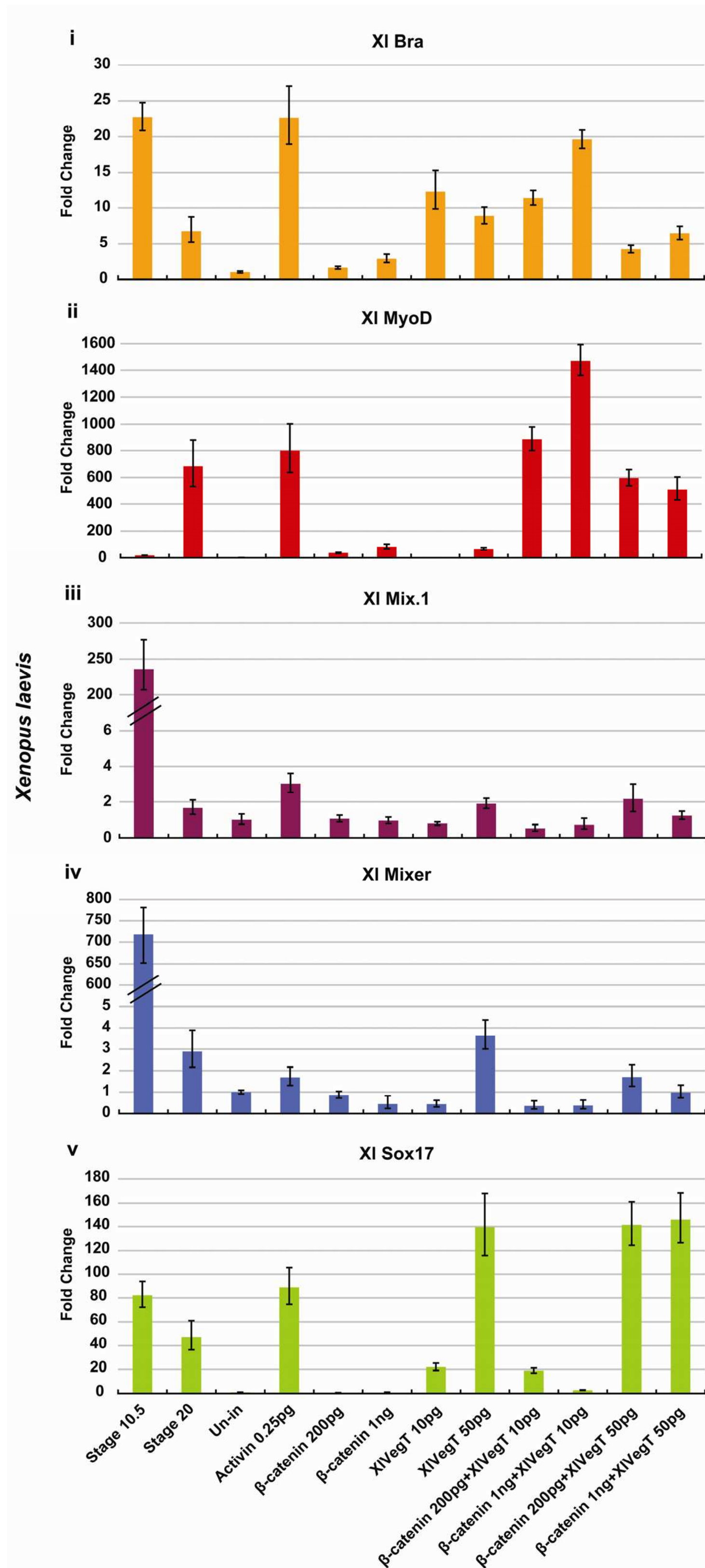


Figure 5.5 – qPCR analysis of mesoderm and endoderm induction by XlVegT and β-catenin RNAs

qPCR analysis shows gene expression levels relative to ODC and then normalised to uninjected samples (Un-in). The samples correspond to those shown in figure 5.4.

5.3.3 Over-expression of VegT and β-catenin in the axolotl embryos

We investigated the mesoderm-inducing potential of VegT and β-catenin in axolotl animal caps. Three different levels (200pg, 500pg and 1ng) were injected into one or two cell stage axolotl embryos, and caps were dissected at stage 9. Unlike *Xenopus,* caps were collected at stage 12 and subjected to further qPCR analysis, reflecting the fact that elongation can be seen much earlier in axolotl embryo explants. In controls, low dose (1pg) activin induces ectoderm to form mesoderm, whilst high levels (25pg) induce endoderm (Figure 5.6). In contrast to *Xenopus*, axolotl animal cap explants injected with *AxVegT* mRNAs alone do not show endoderm differentiation. Rather, *AxVegT* overexpressing caps have the same appearance as uninjected controls (compare Figure 5.2 and 5.6). More surprisingly, we find that *β-catenin* alone injected caps show a dose-dependent induction of mesoderm and endoderm. At low (200pg) and intermediate (500g) levels of *β-catenin*, animal caps undergo a change in shape and elongate as a result of convergence extension movements (Keller and Danilchik, 1988; Keller and Tibbetts, 1989). At higher doses (1ng) of *β-catenin*, some caps develop endodermal tissue similar to that seen with 25pg *activin*. Taken together, these observations suggest that VegT may not be sufficient for mesoderm and endoderm induction in the axolotl, as β-catenin alone seems capable of induction of both mesoderm and endoderm.

qPCR was carried out to study the effects of VegT and β-catenin on mesoderm and endoderm markers, comparing expression of the mesodermal markers *Brachyury*, *FGF8* and *Goosecoid* (Figure 5.7B), and

endodermal markers *Mix* and *Sox17* (Figure 5.7C) as well as the two axolotl *Nodal* genes (Figure 5.7A). In our overexpression experiments, low-dose *activin* induces both *Nodal* genes, the mesodermal genes *Brachyury*, *FGF8* and *Goosecoid* and lower levels of enodermal genes (Figure 5.7B). High-dose *activin* drives endoderm, inducing *AxNodal-1,* but not *AxNodal-2,* and high amounts of the endodermal genes *Mix* and *Sox17* (Figure 5.7C)*.* AxVegT (500pg and 1ng) induces both *Nodal* genes, but only weakly induces *AxBra*, *AxFGF8*, *AxGsc*, *AxMix* and *AxSox17*, whilst low-doses (200pg) of *AxVegT* have no effect on any of these genes (Figure 5.7A,B and C). All doses of *β-catenin* that strongly induce *AxNodal-1* (but not *AxNodal-2*) also induce *AxBra, AxFGF8* and *AxGsc.* Notably *AxGsc* expression is significantly induced at 1ng *β-catenin* (Figure 5.7B). At higher doses (500pg and 1ng) *β-catenin* also induces endoderm as judged by the expression of *AxMix* and *AxSox17*. As the dose of *β-catenin* is increased, the expression of *AxBra* declines as endodermal gene expression increases reflecting a shift in the proportion of mesoderm and endoderm induced by β-catenin. Thus β-catenin appears to be acting as a classical morphogen.

AxVegT and β-catenin can both induce *AxNodal-1* and *AxNodal-2*. However β-catenin shows a significantly stronger induction of *AxNodal-1* than *AxVegT* and vice-versa. All together, these two factors show distinct differences in their activities compared with *Xenopus.* However, one possible explanation for the ability of β-catenin to induce mesoderm in axolotl caps is the presence of endogenous AxVegT in the animal cap.

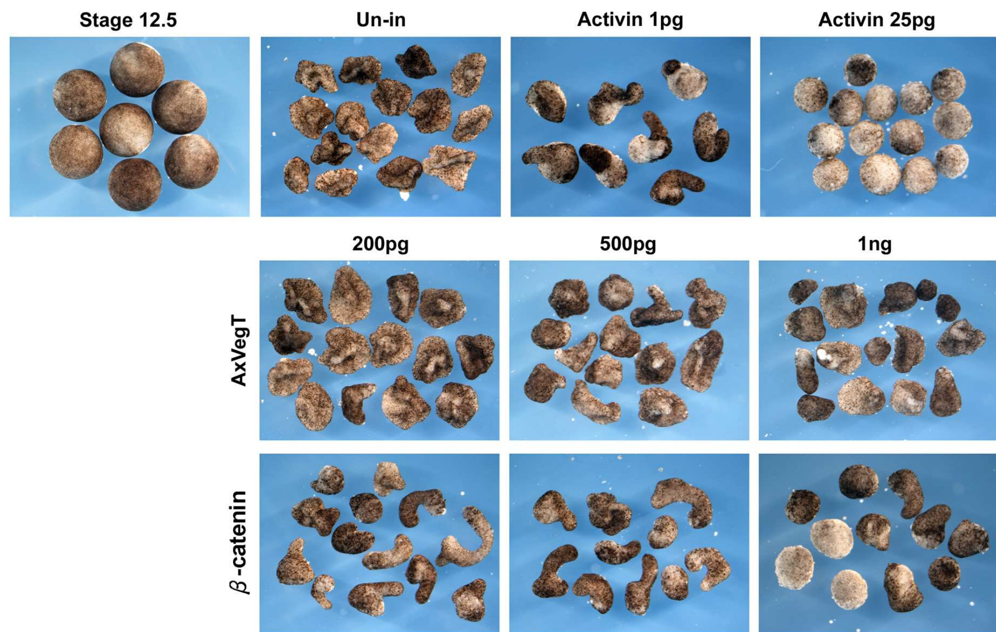


Figure 5.6 – Axolotl animal caps overexpressing AxVegT and β-catenin

Animal caps were dissected from stage 9 embryos injected with *activin*, *AxVegT* or *β-catenin* mRNA into the animal pole at the one or two-cell stage alongside 200pg GFP RNA. Amounts of RNA injected (per embryo) are indicated. Animal cap explants were collected at stage 12.5. 1pg activin mRNA causes cap explants to form mesoderm (as judged by elongation), whilst high amounts (25pg) induce endoderm (white tissue). Animal caps injected with *AxVegT* mRNA are indistinguishable from uninjected controls. *β-catenin* injected samples show elongation under all conditions, with some cap explants appearing to differentiate towards endoderm at high doses (1ng).

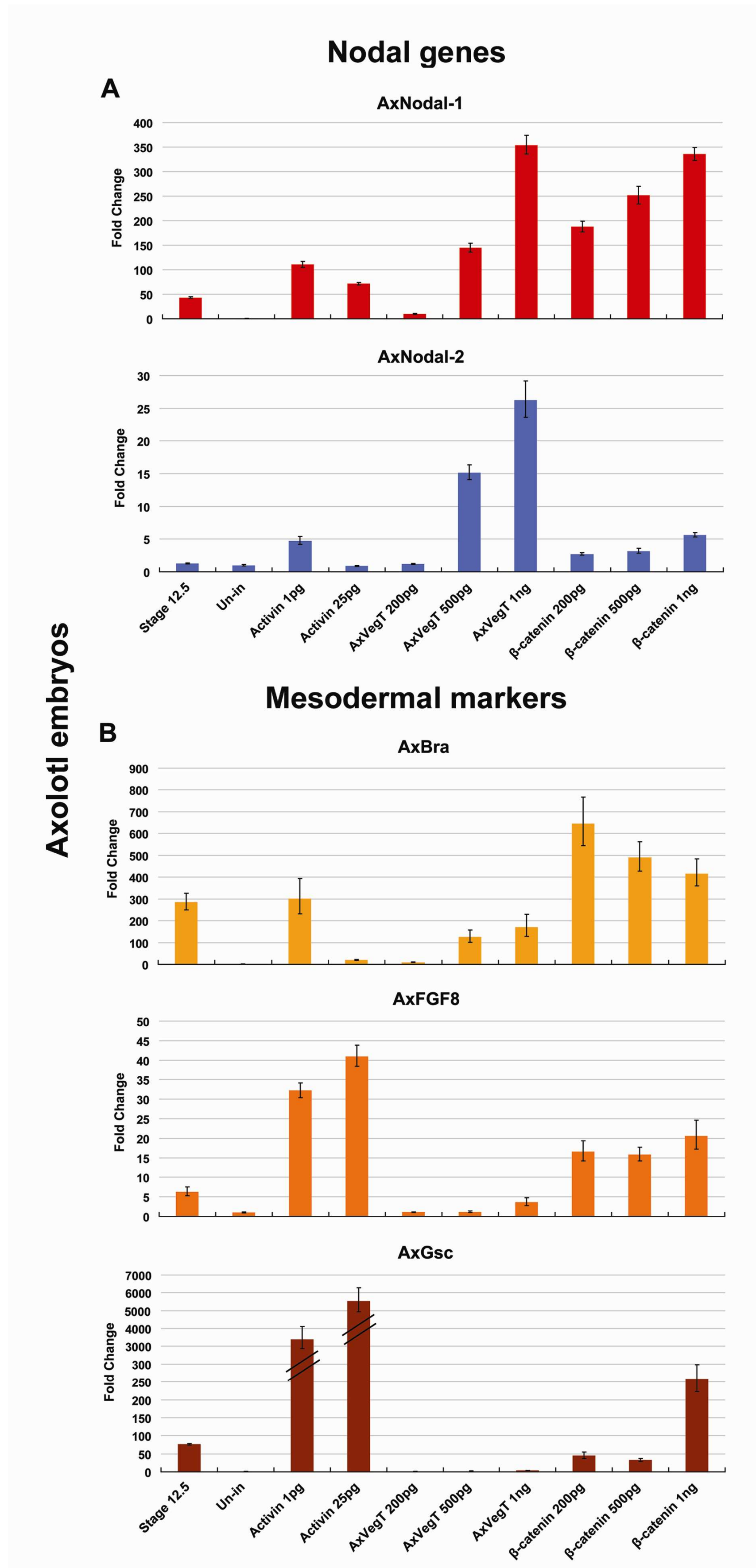


Figure 5.7A and B - qPCR analysis of mesoderm and endoderm induction by AxVegT and β-catenin

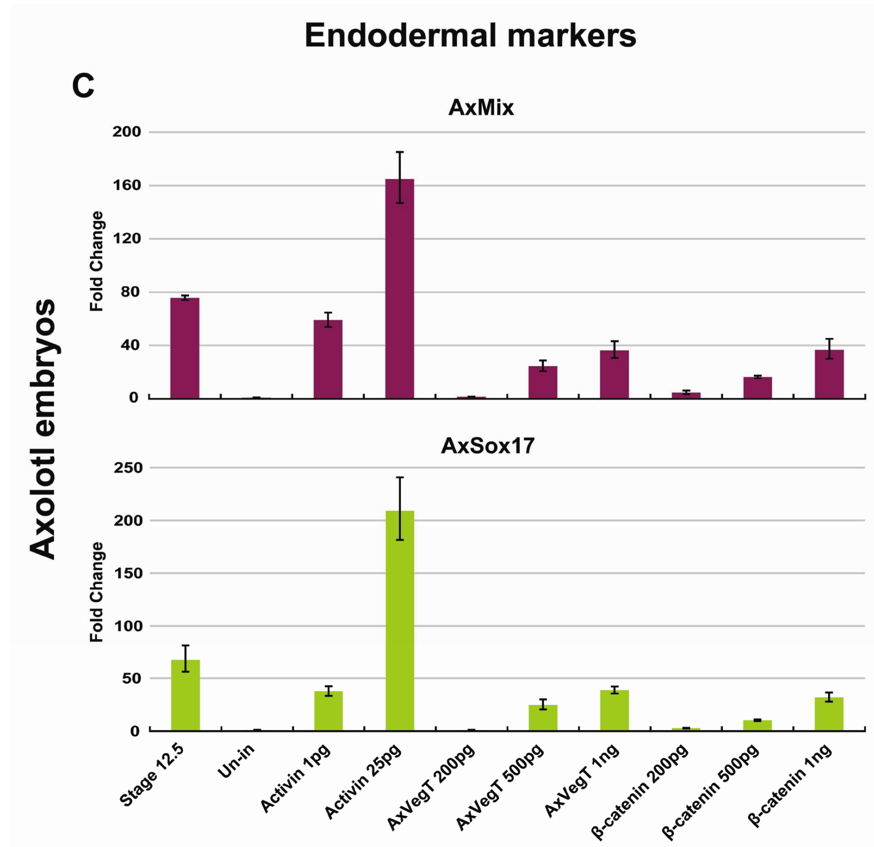


Figure 5.7C - qPCR analysis of mesoderm and endoderm induction by AxVegT and β-catenin

mRNA obtained from the caps described in figure 5.6 are analaysed for marker expression. All samples are relative to *ODC* and then normalised to the uninjected cap samples.

5.3.4 Quantification of AxVegT mRNA

To address this, we first determined the amount of *AxVegT* mRNA in the animal cap of the axolotl embryo. We used quantitative PCR to determine the amount of mRNA in various embryo explants relative to a known standard. Ten stage 10 embryos were dissected into five different parts; animal cap, vegetal mass, dorsal, ventral and lateral marginal regions (Figure 5.8). Total RNAs were prepared from each pool and 1 pg *in-vitro* transcribed *AxVegT* mRNA was added into each RNA sample (500pg) as an internal control. CDNA synthesis was carried out as normal. For a standard control, 1pg *AxVegT* mRNA was added to 500pg total RNA from *Xenopus laevis* animal caps and cDNA was synthesized alongside the axolotl dissected samples. The levels of *AxVegT* transcript present in each region of the embryo can be calculated by reference to the known standard (1 pg *AxVegT* mRNA). Serial dilution of samples from the standard control; 1X, 1/100X, 1/1000X and 1/10000X, were used to define a standard curve. **Table 5.1** shows the quantitation of *AxVegT* mRNA distribution in different parts of the axolotl embryo in a picogram scale. Previous *in-situ* hybridization results (Nath and Elinson, 2007) show the expression pattern of *AxVegT* in the axolotl embryo at late blastula stages. The *AxVegT* expression pattern indicates a lack of cortical localisation of *AxVegT* RNA and shows strong staining in the marginal zone. Our measurements support this observation, with a comparable level of *AxVegT* mRNA in the animal cap (0.6 pg) and vegetal region (0.46 pg). This level of *AxVegT* mRNA (0.6 pg) present in the cap region may be sufficient to synergise with β-catenin to induce the elongation and mesodermal gene expression we observed in axolotl animal caps (Figure 5.6 and 5.7).

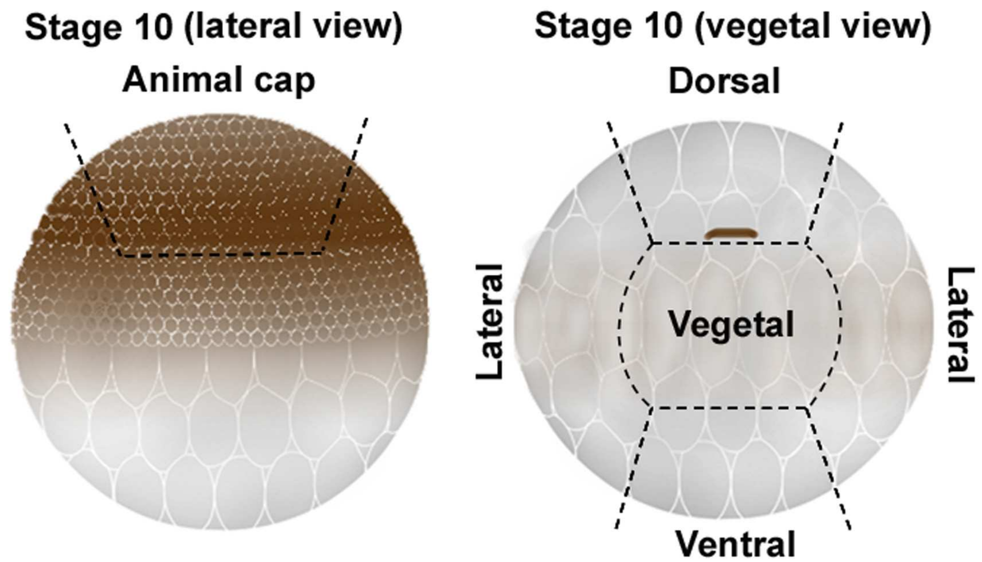


Figure 5.8 - Dissections of the axolotl embryos at stage 10
Animal, equatorial (dorsal, ventral and lateral marginal regions) and vegetal parts were excised from stage 10 embryos and subjected to real-time qPCR analysis.

Table 5.1 - Quantification of AxVegT mRNA in each dissection of an axolotl embryo

Quantitation of *AxVegT* RNA in one axolotl embryo					
	Animal cap	Dorsal region	Ventral region	Lateral marginal (2 parts)	Vegetal region
Concentration (pg)	0.618	0.982	1.281	2.133	0.462

Quantification of *AxVegT* expression level in each part was compared to the yield of standard 1pg *AxVegT* mRNA and represented the respective expression level in a whole embryo.

5.4 Knock-down of VegT, Vg1 and β-catenin

To futher explore the roles of these molecules, and in particular the role of endogenous AxVegT in the animal pole, we investigated the consequences of loss of function. Because VegT is a maternally expressed molecule, loss of function requires antisense oligonucleotides to deplete the maternal *VegT* mRNA (Zhang et al., 1998). This approach is technically not feasible with the axolotl. An alternative is to fuse VegT with the engrailed repressor domain to engineer a fusion (VegT-ENR) that represses target genes normally activated by VegT (Horb and Thomsen, 1997). The antisense oligonucleotide strategy has also been used to deplete *Xenopus* maternal *Vg1* mRNA (Zuck et al., 1998) although it has been argued that high levels of maternal protein still remain (Joseph and Melton, 1998). We therefore engineered an axolotl mutant Vg1 ligand to block Vg1 signalling based on previous work in *Xenopus* (Joseph and Melton, 1998). One of these mutants, B109111V, alters a cysteine residue of the BVg1 construct, which is thought to be involved in a disulfide knot structural motif important for mature Vg1 function. *Xenopus* embryos injected with *B10911V* develop without forming dorsal mesoderm or axial structures (Joseph and Melton, 1998). Wnt/β-catenin signalling can be disrupted by engineering a dominant negative form of the downstream transcription factor XTcf-3 (Molenaar et al., 1996). N-terminal deletion of XTcf-3 (ΔN-Tcf-3) abrogates interactions with β-catenin as well as the consequent transcriptional activation (Molenaar et al., 1996).

To clarify the requirement of VegT, Vg1 and β-catenin function in early axolotl embryo development, the knock-down phenotypes of VegT-ENR, Vg1 mutant

and ΔN-Tcf-3 injected whole embryos were compared to those in *Xenopus laevis* (Figure 5.9A and B). The expression of the *nodal* genes, and mesodermal and endodermal gene markers were analyzed by qPCR (Figure 5.10A,B and C).

Whole *Xenopus* or axolotl embryos were injected radially at the 4 cell stage either into all four blastomeres, or the two dorsal or ventral blastomeres only with mutant mRNAs encoding *XlVegT-ENR*/*AxVegT-ENR* mRNA (500pg or 1ng), mutant *AxVg1* mRNA (AxB109111V) (1ng and 2ng) and ΔN-Tcf-3 mRNA (250pg in each blastomere). In *Xenopus*, inhibition of *VegT* function disrupts body patterning (Figure 5.9A *Xenopus*-ii,iii,vii and viii) (Horb and Thomsen, 1997; Kofron et al., 1999). Similarly in the axolotl, overexpression of AxVegT-ENR inhibits the formation of the dorsal lip and embryos fail to gastrulate (Figure 5.9A Axolotl-ii and iii). At tadpole stages, embryos injected with *AxVegT-ENR* mRNA have severe embryonic body patterning defects and only have an animal/vegetal axis as a consequence of incomplete gastrulation (Figure 5.9A Axolotl-vii and viii).

In *Xenopus*, embryos injected with mutant *AxVg1* mRNA are phenotypically abnormal compared with controls and resemble embryos in which *Vg1* has been depleted by anti-sense oligonucleotides (Birsoy et al., 2006). In *Xenopus* embryos, 1ng of mutant *AxVg1* mRNA causes the blastopore to remain enlarged at late gastrula. Higher levels (2ng) delay the timing of blastopore formation (Figure 5.9A *Xenopus*-iv and v). At tailbud stages, Vg1-knockdown embryos have different degrees of anteroposterior and dorsoventral axis abnormalities. Lower doses result in stunted embryos,

whereas high doses cause a loss of head structures (Figure 5.9A Axolotl-ix and x). In contrast to *Xenopus*, axolotl embryos injected with 1ng of mutant *AxVg1* mRNA show no discernible effect on either gastrulation or development at the tailbud stage. Higher doses (2ng) cause a slight delay to normal development (Figure 5.9A compare *Xenopus* and Axolotl v and x). These results suggest that Vg1 does not affect germ layer formation in axolotls.

Injection of *ΔN-Tcf-3* RNA into either all four, or the two dorsal blastomeres of *Xenopus* leads to ventralised embryos with reduced or absent dorsal axial tissue indicating the effect is strictly localised to the prospective dorsal side (Figure 5.9B *Xenopus*-vi and vii). Notably injection into the two ventral blastomeres of *Xenopus* embryos has no effect on development (Figure 5.9B *Xenopus*-viii) (Roel et al., 2002). ΔN-Tcf-3 has no effect on *Xenopus* embryos at early gastrula stages, with embryos forming normal dorsal lips and able to commence gastrulation (Figure 5.9B *Xenopus*-ii,iii and iv). In contrast, axolotl embryos injected with *ΔN-Tcf-3* into either all four blastomeres, or just the two dorsal blastomeres, show a loss of blastopore formation and severe gastrulation abnormalities (Figure 5.9B Axolotl-ii and iii). Axolotl embryos injected into the two ventral blastomeres only are able to start gastrulation (Figure 5.9B Axolotl-iv). Axolotl embryos injected with *ΔN-Tcf-3* into all four blastomeres show no dorsal axis formation and have severe embryonic body patterning defects at tailbud stages (Figure 5.9B Axolotl-vi). Embryos injected into the dorsal blastomeres only develop with reduced axial tissue and a defect in neural plate closure (Figure 5.9B Axolotl-vii). Ventral injection of *ΔN-Tcf-3* disrupts posterior mesoderm development, but anterior

and dorsal development is not affected (Figure 5.9B Axolotl-viii).

The results of ΔN-Tcf-3 overexpression in axolotl embryos suggest that β-catenin is required for gastrulation and ventral mesoderm development in axolotl embryos. We reason that β-catenin is required for mesoderm induction as well as patterning. Thus in embryos injected radially or dorsally, a complete failure of mesoderm induction is seen (reminiscent of Nodal morphant embryos). Embryos injected ventrally only have defects in ventral mesoderm induction. Notably this is not so in *Xenopus.* Gastrulation can initiate in the absence of β-catenin and ventrally injected embryos are indistinguishable from wild-type.

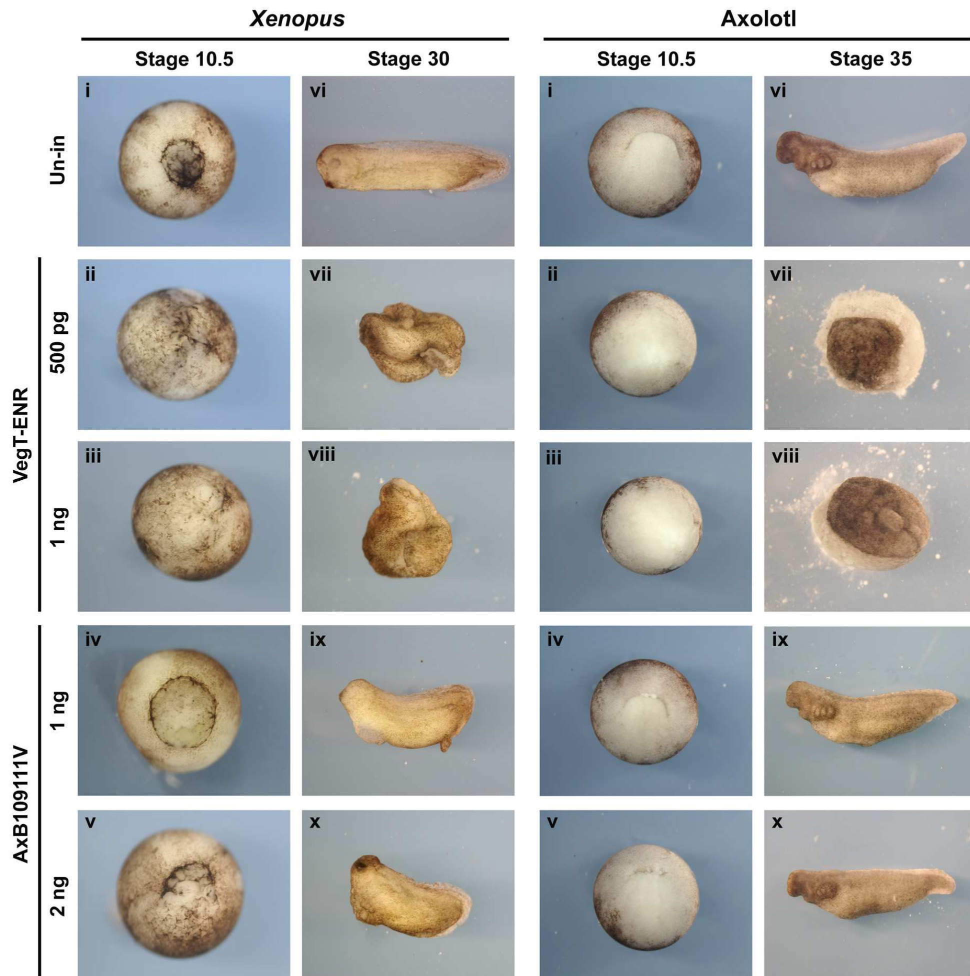


Figure 5.9A – Knockdown of VegT and Vg1 in *Xenopus* and axolotl embryos.

***Xenopus*:** (i) Vegetal view of a stage 10.5 wild-type embryo (vi) the tailbud stage *Xenopus* embryo. Embryos injected with *XlVegT-ENR* 500pg (ii) and 1ng (iii) fail to gastrulate and form a blastopore. The tailbud embryos (vii and viii) lack dorsal axial structures and have disrupted anterior and posterior development. Embryos injected with mutant *AxVg1* 1ng (iv) and 2ng (v) form a blastopore although its formation is delayed and it remains large even in late gastrulation. The tailbud embryos (ix and x) are stunted and lack head structures.

Axolotl: (i) Vegetal view stage 10.5 embryo, positioned with the dorsal blastopore at the top. Embryos injected with *AxVegT-ENR* 500pg (ii) and 1ng (iii) lack a blastopore at gastrulation. The tailbud embryos do not develop anterior and posterior structures, and have no dorsal axial structures. Embryos injected with mutant *AxVg1* 1ng (iv) and 2ng (v) develop normally as controls (vi), although development is slightly delayed at higher doses.

Figure 5.9B – Knockdown of Wnt/β-catenin signalling in *Xenopus* and axolotl embryos

Xenopus**:** (i) Vegetal view of an uninjected control stage 10.5 embryo. Embryos injected with *ΔN-Tcf-3* at the 4 cell stage into all four blastomeres (ii), dorsal only (iii) and ventral only (iv). *ΔN-Tcf-3* injected embryos develop normally and complete gastrulation. Subsequently, the embryos (vi and vii) fail to form neural folds and lack head, tail and dorsal ventral axes. Ventrally injected embryos develop normally (viii). Co-injection of a lineage tracer GFP fluorescent indicates correct targeting.

Axolotl: (i) Vegetal view of an uninjected control stage 10.5 embryo. Embryos were injected with *ΔN-Tcf-3* at the 4 cell stage into all four blastomeres (ii), dorsal only (iii) or ventral only (iv). All *ΔN-Tcf-3* injected embryos (ii, iii and vi) fail to gastrulate and do not form the blastopore. Subsequently, the embryos (vi and vii) failed to form, or close, the neural folds, and lack head, tail and dorsal ventral axes (v). ΔN-Tcf-3 also interferes with ventral development when expressed in ventral tissue but affects development of axial tissue only when expressed in all blastomeres and on the future dorsal side. Co-injection of a lineage tracer GFP fluorescent indicates correct targeting.

B

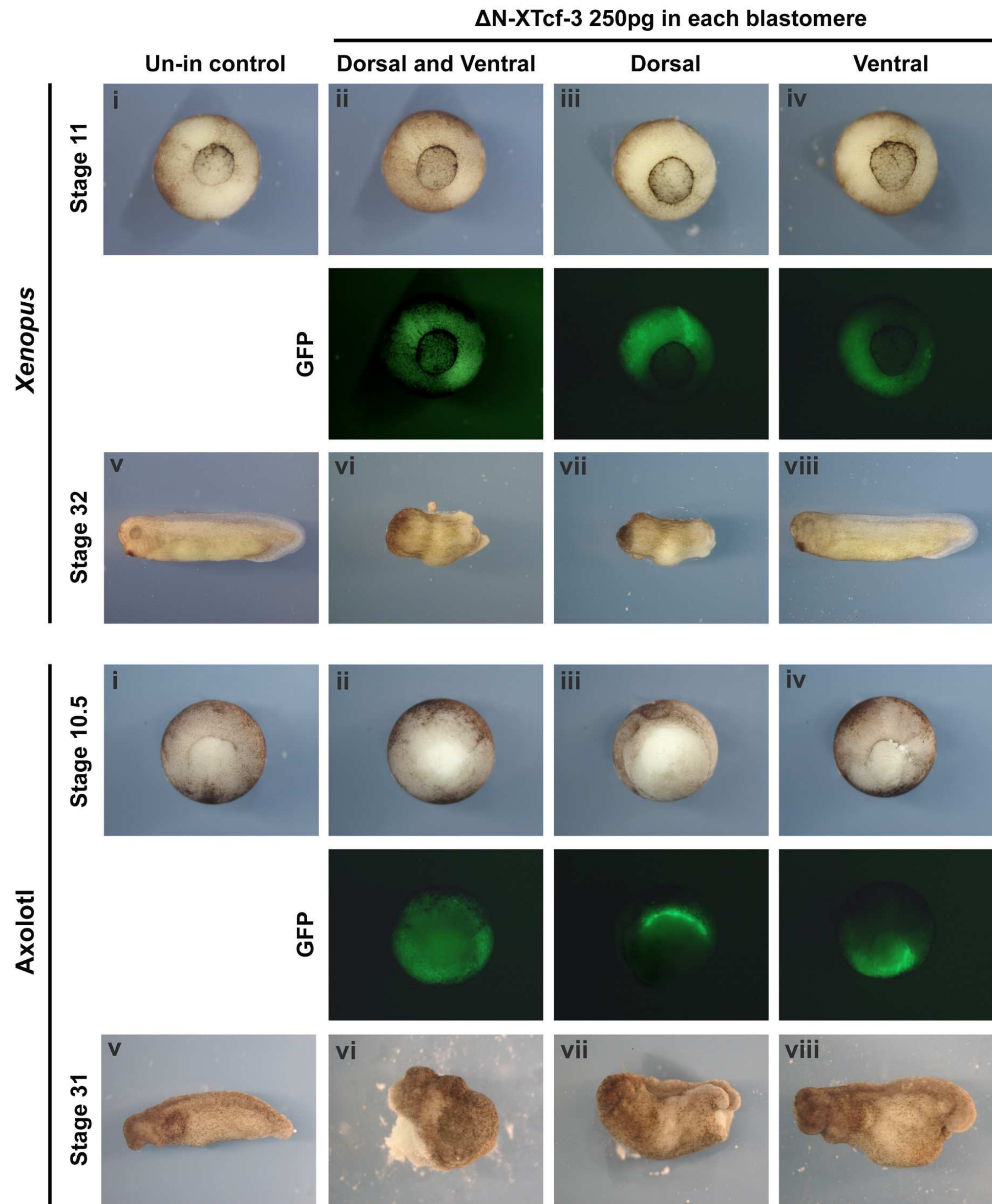


Figure 5.9B – Knockdown of Wnt/β-catenin signalling in *Xenopus* and axolotl embryos

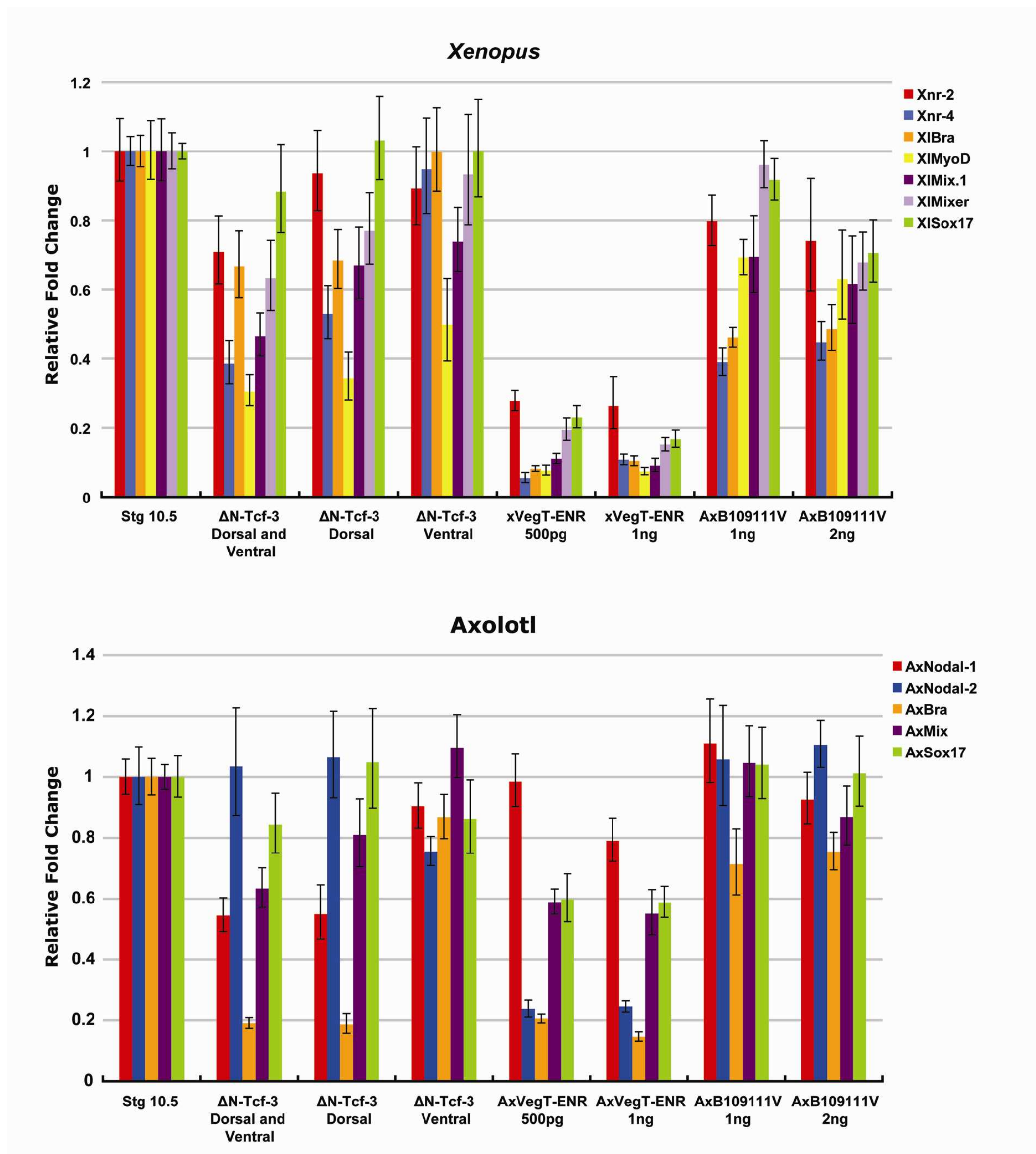


Figure 5.10 - qPCR analysis of gene expression in VegT, Vg1 and β-catenin knockdown embryos in *Xenopus* and the axolotl

Embryos were collected when sibling embryos reached stage 10.5 and qPCR analysis was performed to examine gene expression levels.

As previously reported (Kofron et al., 1999; Xanthos et al., 2001; Xanthos et al., 2002), qPCR analysis in *Xenopus* shows that the *nodal-related* genes, and mesoderm and endoderm marker genes depend on VegT for expression. However, β-catenin and Vg1 do affect the levels of gene expression. In *Xenopus* injected radially with *ΔN-Tcf-3* to knockdown β-catenin*,* the expression of *Xnr2* is slightly reduced (30%) compared with controls. In contrast, the expression of *Xnr4* is reduced by about 50-60% when ΔN-Tcf-3 is expressed in prospective dorsal, but not prospective ventral tissue (Figure 5.10 *Xenopus*). Thus β-catenin knockdown embryos may not form dorsal mesoderm (judged by the low expression of *XlBra* and *XlMyoD*) as the early peak of *Xnr* expression is lost. *Xenopus* Vg1 knockdown embryos have reduced *Xnr* expression as well as mesoderm and endoderm marker genes, particularly at higher doses.

In the axolotl, qPCR analysis from knockdown experiments reveals differential gene expression by β-catenin, AxVegT and AxVg1. *AxNodal-2* expression is significantly down-regulated in *AxVegT-ENR* embryos, whereas *AxNodal-1* expression is less affected. *AxBra* expression is reduced by approximately 80% in both *AxVegT-ENR* and *ΔN-Tcf-3* injected embryos, suggesting a failure of mesoderm induction. Endodermal genes are much more sensitive to AxVegT-ENR than ΔN-Tcf-3. Unexpectedly, β-catenin inhibition (ΔN-Tcf-3 overexpression) down-regulates *AxNodal-1* more than AxVegT-ENR. It should be noted that injection of *ΔN-Tcf-3* RNA in axolotl embryos interferes with normal anterior, dorsal axial and ventral development (Figure 5.9B vi-viii), although reduced gene expression is only apparent as a consequence of dorsal injection.

Ventral injection of *ΔN-Tcf-3* RNA interferes with ventral development, phenocopying ventral-specific inhibition of β-catenin in *Xenopus* (Hamilton et al., 2001; Roel et al., 2002). In *Xenopus*, two different mutant X-Tcf-3 constructs, ΔN-Tcf-3 and N-XTcf-3, demonstrate the importance of β-catenin function in dorsal and ventral mesoderm (Hamilton et al., 2001). Maternal Wnt/β-catenin signalling in the dorsal mesoderm is dependent on XTcf-3 function, whereas ventral mesoderm patterning requires zygotic Wnt signalling via XLef-1 (Roel et al., 2002). Unlike *Xenopus,* in the axolotl embryo, Tcf-3 mediated Wnt/β-catenin signalling is required for both dorsal and ventral mesoderm patterning via XTcf-3. Ventral mesoderm markers could be used to verify the role of Wnt/β-catenin signalling in ventral mesoderm induction in the future.

Our data suggest that inhibition of AxVegT does not affect *AxNodal-1* levels, which itself plays a critical role in mesoderm specification in the axolotl (see Chapter 4). This suggests that non-localised AxVegT may function in another pathway rather than mesoderm specification in axolotls.

5.5 The function of VegT and β-catenin in patterning mesoderm in the axolotl

We have performed gain and loss of function analyses on VegT and β-catenin in *Xenopus* and axolotl embryos (section 5.3 and 5.4). In *Xenopus* animal cap explants, overexpression results suggest mesoderm induction requires synergism between VegT and β-catenin. In contrast in the axolotl overexpression of β-catenin alone is sufficient to induce expression of mesoderm and endoderm genes. To further investigate the requirement for

AxVegT and β-catenin in mesoderm induction, experiments were carried out in *Xenopus* animal caps to test whether AxVegT functions as XlVegT. We also examined the ability of β-catenin to induce mesoderm in the presence of the various dominant-negative mutant mRNAs (*AxVegT*, *AxVg1* and *ΔN-Tcf-3*).

5.5.1 The action of XlVegT, AxVegT and β-catenin in mesoderm induction in *Xenopus*

We first investigated the effects of AxVegT on the induction of mesoderm markers in *Xenopus* embryos. Embryos were injected at the one or two cell stage with mRNAs encoding *XlVegT*, *AxVegT*, *β-catenin* and AxVegT-ENR, and ectoderm cap explants were dissected at stage 9 and harvested when sibling embryos reached stage 20 (Figure 5.11A and B). Animal cap explants were analyzed for expression of the mesodermal markers, *XlBra* and *XlMyoD* by qPCR (Figure 5.12).

As a positive control we used 0.25 pg *activin* mRNA to induce mesoderm, judged by elongation and *XlBra* and *XlMyoD* expression. 10 pg *XlVegT* mRNA alone activates *XlBra* expression, whilst 10 pg *AxVegT* RNA alone only weakly induces *XlBra* (about 3 fold) above background (Figure 5.12). As expected, 200 pg *β-catenin* mRNA alone results in caps that are indistinguishable from controls. The *β-catenin* injected caps do not express dorsal or general mesoderm markers such as *XlBra* and *XlMyoD*. Co-injection of either *XlVegT* or *AxVegT* with *β-catenin* causes animal caps to undergo convergent extension movements typical of axial mesoderm and express the mesodermal markers *XlBra* and *XlMyoD.* Taken together, these data demonstrate that AxVegT can mimic XlVegT to induce *XlBra* expression in

Xenopus animal cap explants. Ectopic overexpression of *VegT* or *β-catenin* mRNA alone does not induce *XlMyoD*; *XlMyoD* is dramatically upregulated only when embryos are co-injected with either *AxVegT* or *XlVegT* and *β-catenin* mRNAs*.* Similar to XlVegT, AxVegT therefore can act synergistically with β-catenin to induce axial mesoderm patterning, elongation and expression of *XlBra* and *XlMyoD* in *Xenopus* animal cap explants.

To further investigate the requirement for VegT in mesoderm formation, we co-injected *Xenopus* embryos with *AxVegT-ENR*, *AxVegT* and *β-catenin* or *XlVegT-ENR, XlVegT and β-catenin* mRNA. Both *Xenopus* and axolotl VegT-ENR constructs are sufficient to block elongation in *Xenopus* animal cap explants (Figure 5.11A and B). This demonstrates the absolute requirement for VegT in normal axial mesoderm induction in *Xenopus* embryos. In *Xenopus*, vegetally localised cytoplasmic determinants such as VegT and Vg1 generate a gradient of expression of mesoderm-inducing molecules in the blastula endoderm by synergising with the dorsal determinant β-catenin (Agius et al., 2000). Clearly this process does not happen in the axolotl as AxVegT is not localised, nor can it induce mesoderm in the axolotl. However, injection of *AxVegT* into *Xenopus* embryos (Figure 5.12) does weakly induce *XlBra* in animal caps and the induction of *XlMyoD* expression is potentiated by co-injection with *β-catenin*. This suggests that axolotl VegT can mimic *Xenopus* VegT in the specification and development of the axial mesoderm in *Xenopus* embryos; but the inductive activity of AxVegT is weaker than XlVegT.

Figure 5.11 –Ectopically express XlVegT, AxVegT and β-catenin with VegT-ENR in *Xenopus* animal caps

Both *XlVegT* **(A)** and *AxVegT* **(B)** were injected into *Xenopus* embryos and the effect on mesoderm induction analyzed. Embryos were injected with synthesized RNA; *activin*, *XlVegT* or *AxVegT*, *β-catenin* and *VegT-ENR*, into the animal pole at the one or two-cell stage alongside 100pg GFP RNA. Animal cap explants were cut from stage 9 and collected at stage 20 as control embryos. Animal caps treated with 0.25pg *activin* elongated and also dramatically elongated by co-injection of *XlVegT* or *AxVegT* with *β-catenin* mRNAs compared to control caps. However, in *XlVegT, AxVegT* or *β-catenin* singly injected samples; explants showed no elongation at all conditions. VegT-ENR was able to inhibit the mesoderm induction and cap elongation in the presence of *VegT* and *β-catenin* mRNAs.

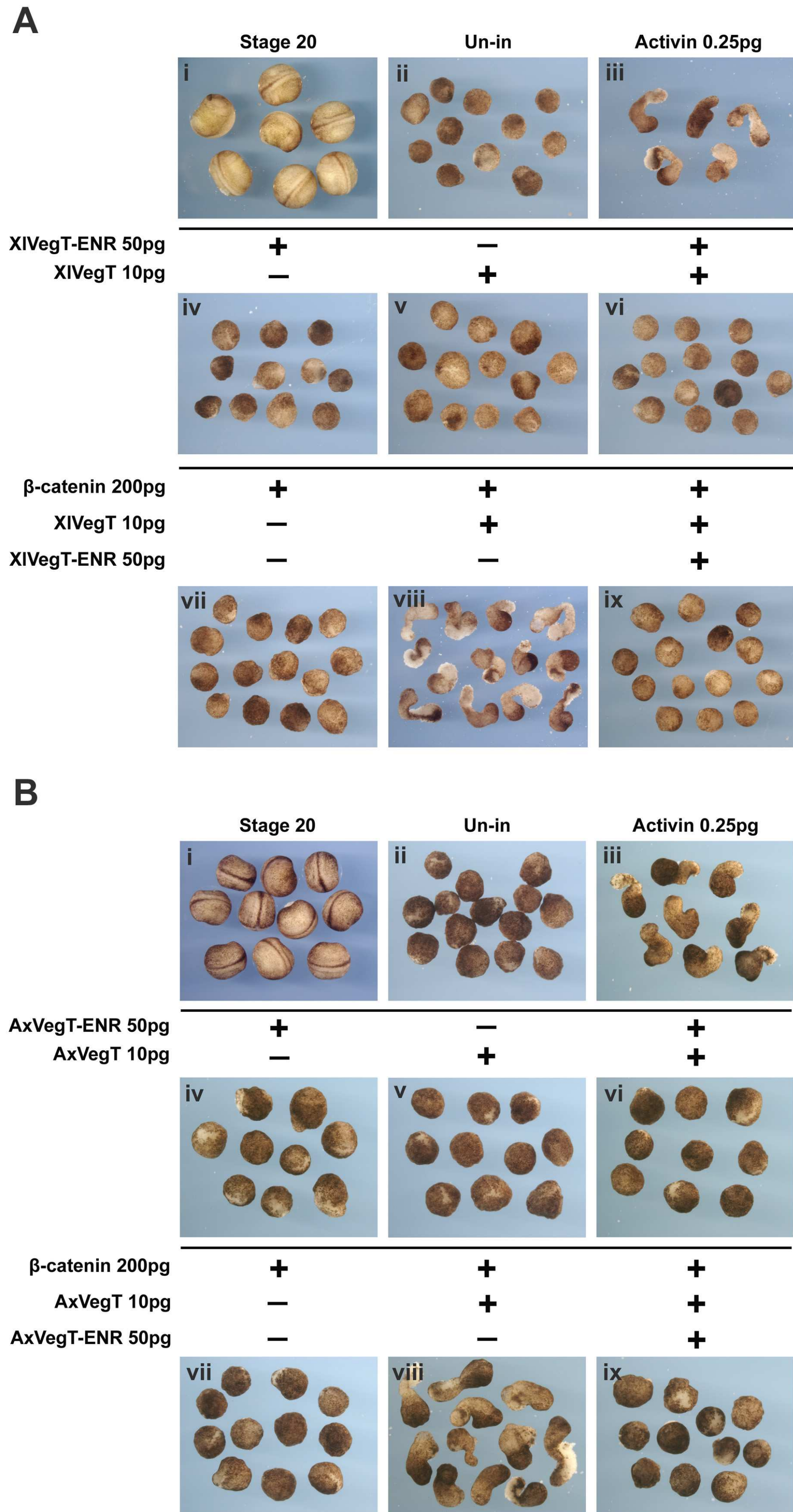


Figure 5.11 –Ectopically express XlVegT, AxVegT and β-catenin with VegT-ENR in *Xenopus* animal caps

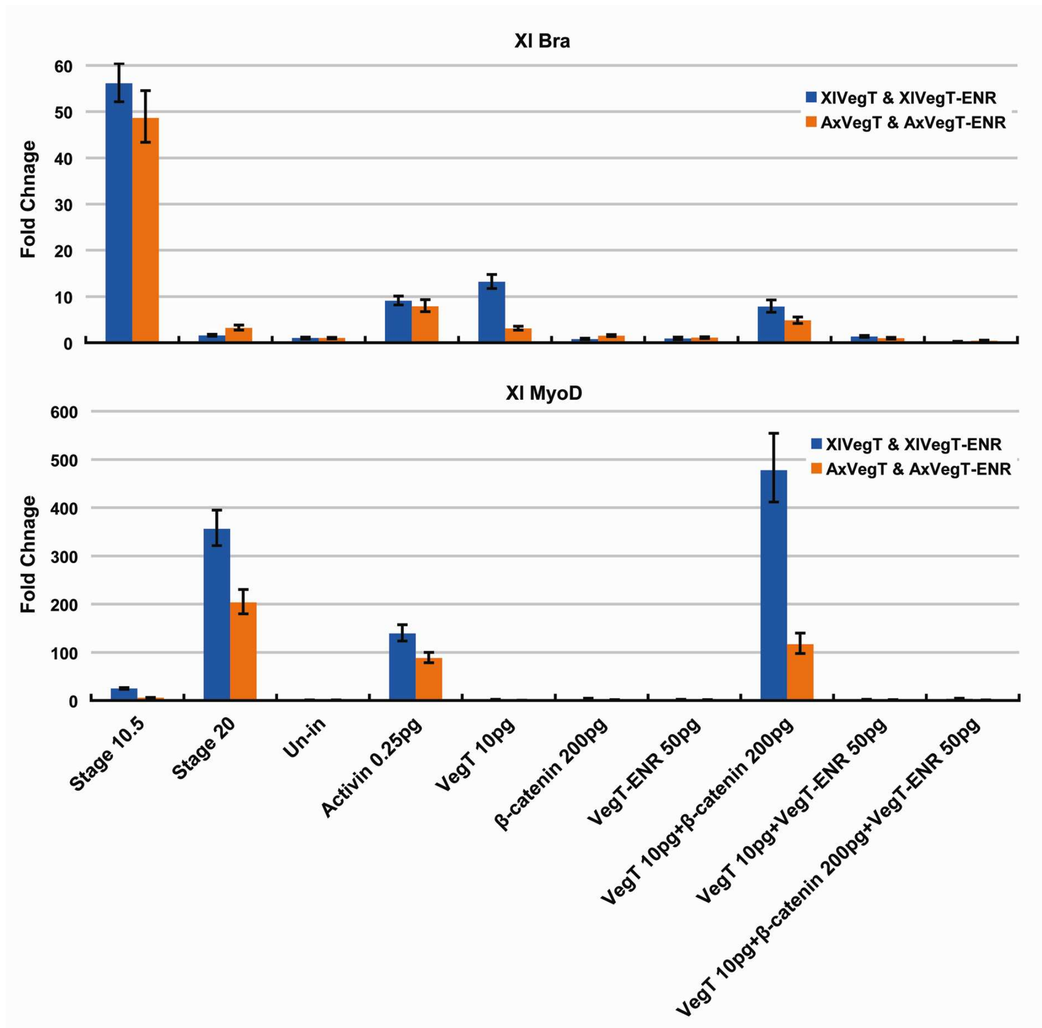


Figure 5.12 - qPCR analysis of mesoderm induction by XlVegT or AxVegT and β-catenin in *Xenopus* animal caps

Cap explants were collected when sibling embryos reached stage 20. Blue: *XlVegT* and *XlVegT-ENR* RNAs   Orange: *AxVegT* and *AxVegT-ENR* RNAs

5.5.2 The action of AxVegT in patterning axolotl mesoderm

In *Xenopus*, it is generally accepted that the mesoderm forms in the equatorial region as a result of signals released from the vegetal mass (Nieuwkoop.P.D., 1969a; Smith, 1989). Maternal *VegT* mRNA is localised in the vegetal hemisphere of *Xenopus laevis* embryos generating endodermal and mesodermal signals at various levels (Clements et al., 1999). XlVegT induces a signal that forms axial mesoderm at the dorsal equator by acting as a co-factor of dorsal determinans to activate the Wnt-dorsalizing cascade and then cooperates with β-catenin pathway to generate the high nodal-related concentrations which is required to form the Spemann organizer and pattern the normal dorsal mesoderm (Agius et al., 2000; Clements et al., 1999; Katsumoto et al., 2004). However, *AxVegT* and *AxVg1* are not localised in axolotl occytes. Further, in contrast to *Xenopus*, our results demonstrate that injection of *β-catenin* alone is sufficient to induce mesoderm in axolotl animal cap explants. Perhaps the *VegT* mRNA (0.6 pg) present in the animal pole of axolotl embryos is sufficient to synergise with β-catenin in the induction of mesoderm?

To clarify the requirement for VegT function in early axolotl embryos, animal cap assay was carried out to further investigate. *AxVegT-ENR* mRNA was injected alone or in combination into the animal pole of axolotl embryos at the one or two cell stage. Animal caps were dissected at stage 9 and collected when sibling embryos reached stage 12.5. 1 pg *activin* causes elongation of animal cap explants and induces expression of both *Nodal* genes (*AxNodal-1* and *AxNodal-2*) as well as *AxMix*, *AxGsc* and *AxMix*. *β-catenin* mRNA alone

induces cap explants to elongate and express *AxNodal-1*, *AxNodal-2*, *AxBra*, *AxGsc* and *AxMix*, while low doses of *AxVegT* RNA (50pg) do not strongly induce mesodermal genes but weakly induce *AxNodal-1*, *AxNodal-2* and *AxMix* (see Figure 5.14A and B). VegT-ENR (50 pg) can completely block the activity of either XlVegT or AxVegT in *Xenopus* animal caps (Figure 5.12). To block endogenous AxVegT activity in axolotl animal caps, we co-injected 50pg *AxVegT-ENR* with *β-catenin* mRNA. Surprisingly, elongation of cap explants in response to β-catenin is not prevented by blocking the AxVegT pathway (compare Figure 5.13 vii and vii). β-catenin still activates *AxNodal-1*, *AxBra*, *AxGsc* and *AxMix* in the absence of AxVegT (Figure 5.14). *AxNodal-2* induction is slightly reduced suggesting AxVegT may be involved in driving *AxNodal-2* expression. All together, we conclude that AxVegT signalling is not required for dorsal mesoderm formation and mesodermal gene activation in axolotl animal caps.

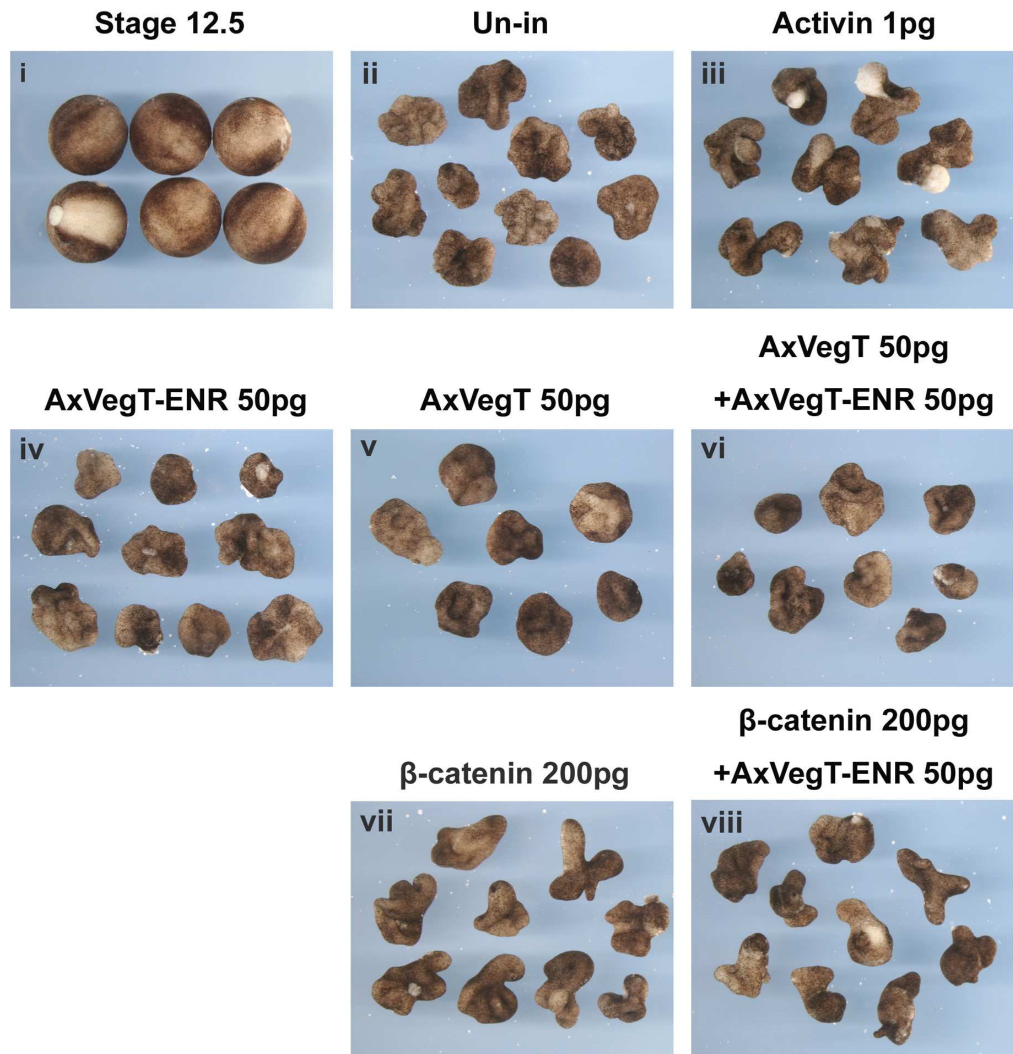


Figure 5.13 - Axolotl animal caps overexpressing AxVegT and β-catenin with or without AxVegT-ENR

Animal cap explants are collected at stage 12.5. Animal caps were dissected from stage 9 embryos injected with synthesized mRNAs; *activin*, *AxVegT* or *β-catenin*, into the animal pole at the one or two-cell stage alongside 200pg GFP RNA. Amounts of RNA injected (per embryo) are as indicated. Animal caps injected with *AxVegT* or *AxVegT-ENR* RNA resemble uninjected control caps. In *β-catenin* injected samples, explants elongate and this is not blocked by VegT-ENR.

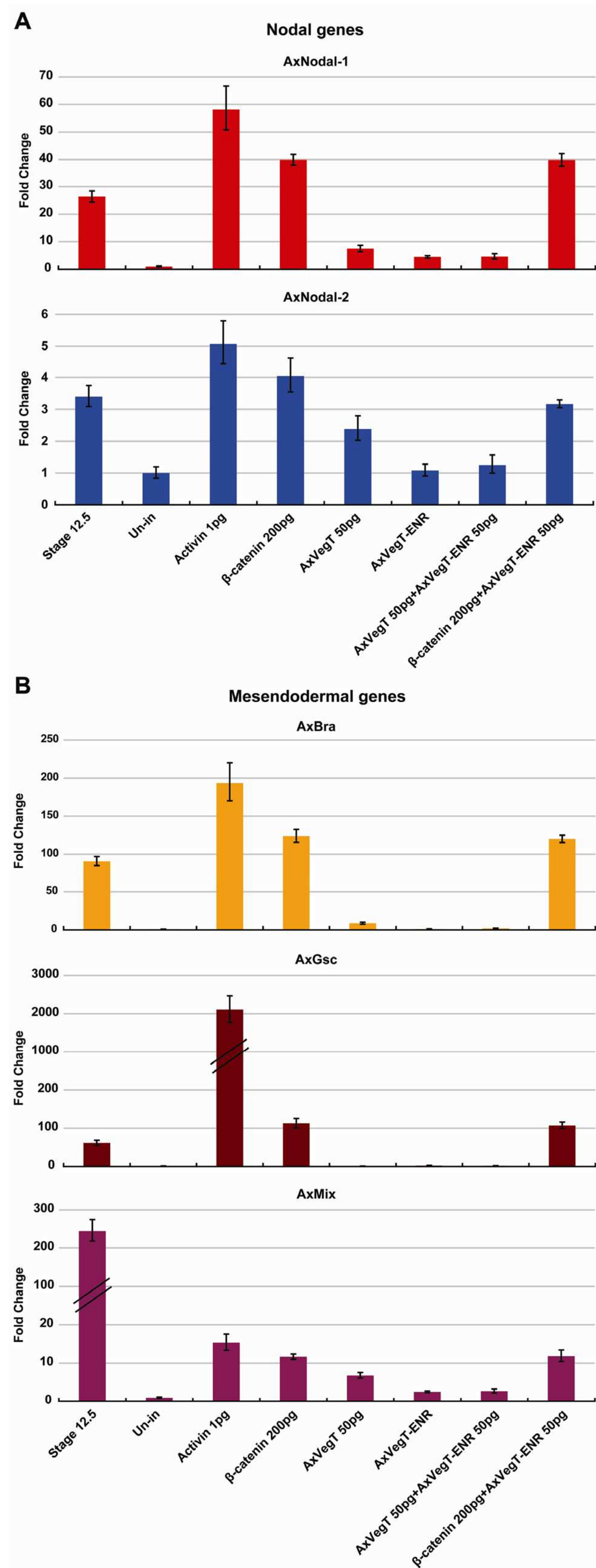


Figure 5.14 - qPCR analysis of gene expression induced by AxVegT and β-catenin with or without AxVegT-ENR in axolotl animal caps

Cap explants were collected when sibling embryos reached stage 12.5 and qPCR analysis was performed to examine gene expression levels.

The results discussed above demonstrate that β-catenin induces expression of mesodermal and endodermal genes in animal cap explants in the absence of VegT signalling. To further define the role of β-catenin in the activation of mesodermal and endodermal gene expression, *ΔN-XTcf-3* and *AxB109111V* mRNA were co-injected with *β-catenin* into one-cell stage embryos. Injection of mRNAs encoding wild type XlVg1 has no effect in either *Xenopus laevis* animal caps (Figure 5.2 and 5.3) (Tannahill and Melton, 1989). In part this reflects a specific processing requirement for Vg1 that restricts its action to body axis patterning (Kessler and Melton, 1995; Thomas and Moos, Jr., 2007; Thomsen and Melton, 1993). The function of mature Vg1 protein is revealed by the chimeric BMP2-Vg1 construct (BVg1) (Thomsen and Melton, 1993). In *Xenopus*, ectopic expression of BVg1 demonstrates that Vg1 is able to induce both endodermal and mesodermal markers (Henry et al., 1996; Thomsen and Melton, 1993). *Vg1* mutants have been designed which act as either dominant negative proteins or competitive antagonists of Vg1 signalling (Joseph and Melton, 1998). We therefore engineered axolotl variants of these fusions, AxBMP2-Vg1 and AxB109111V to clarify the requirement of AxVg1 in dorsal mesoderm formation of axolotl embryos.

Our previous results show that AxB109111V (dominant negative) can phenocopy Vg1 depletion in *Xenopus* embryos (Birsoy et al., 2006) (see Figure 5.9A). However, AxB109111V does not cause defects in axolotl embryos other than a delay in development compared to controls. Gene expression was analyzed by real time qPCR in animal explants injected with dominant-negative mutants, *β-catenin* mRNA or in combination (Figure 5.16). We further tested these dominant-negative mutants (ΔN-XTcf-3 and

AxB109111V) by using the animal cap assay. Synthesized RNAs were injected into the animal pole of one-cell stage axolotl embryos. Animal caps were cut from stage 9 at late blastulae and observed when sibling embryos reached stage 12.5 (Figure 5.15). Real time qPCR was performed to detect expression of *AxBra* and both *Nodal* genes (*AxNodal-1* and *AxNodal-2*) at late gastrula stages (stage 12.5). ΔN-XTcf-3 RNA inhibited the elongation of animal caps and the expression of *AxBra* and both *Nodal* genes induced by *β-catenin* 200pg RNA injection. As expected, wild type *AxVg1* mRNA (AxVg1 Wt) does not induce *AxBra* or *Nodal* gene expression. Similar to previous reports (Onuma et al., 2002; Thomas and Moos, Jr., 2007), 10pg chimeric *AxBMP2-Vg1* RNA (AxBVg1) results in the activation of *AxBra* and both *Nodal* genes, although the induction is less than that seen in *β-catenin* injections. A 50-fold excess of *AxVg1* mutant RNA (*AxB109111V*) inhibits the induction of marker gene expression after injection of *AxBVg1* indicating AxB109111V could block the signalling by mature AxVg1 *in vivo*. Conversely, mesoderm induction by β-catenin is not affected by the AxVg1 dominant negative mutant.

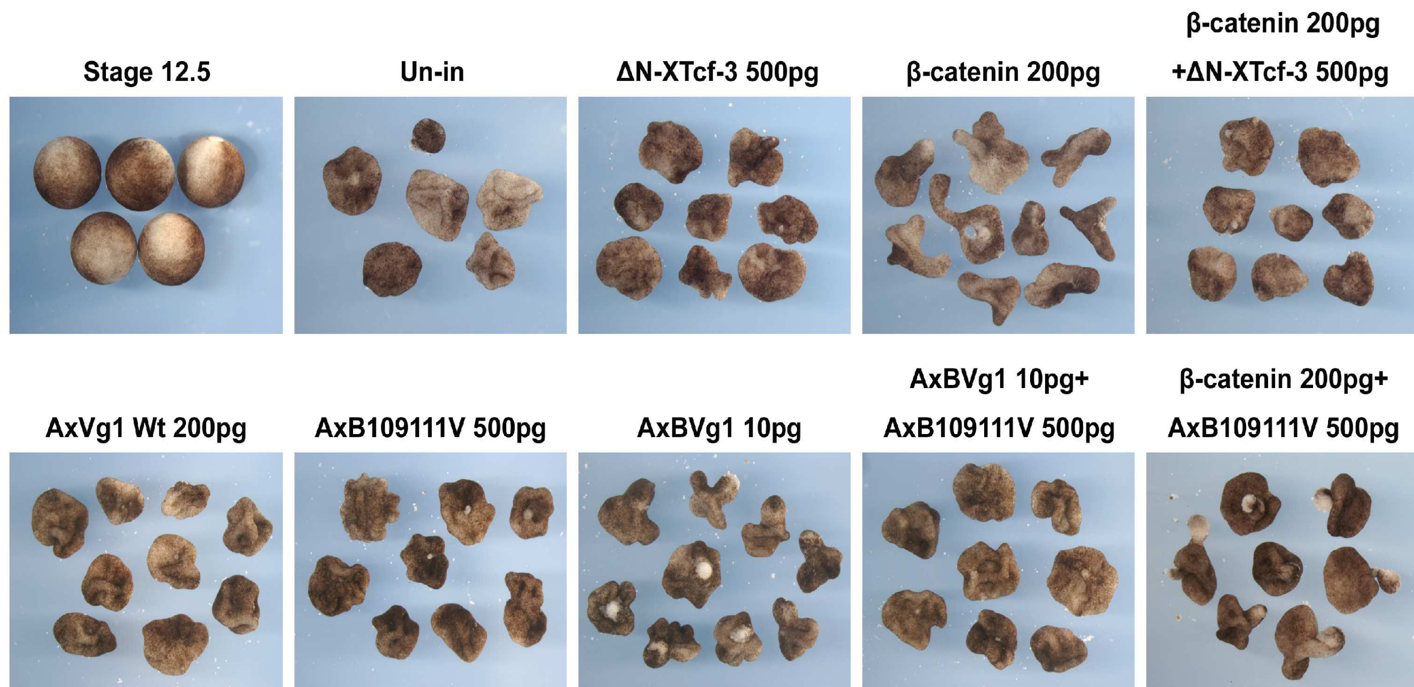


Figure 5.15 –Axolotl animal caps overexpressing β-catenin and AxBVg1 with or without ΔN-Tcf3 or AxB109111V RNA

Animal caps were dissected from stage 9 and observed when the sibling embryos were at stage 12.5. Embryos were injected with synthesized RNAs; *ΔN-XTcf-3*, *β-catenin* and wild type (Wt), active (*AxBVg1*) or mutant AxVg1 (*AxB109111V*), into the animal pole at the one or two-cell stage alongside 200pg GFP RNA. 200pg *β-catenin* and 10pg *AxBVg1* caused cap explants forming mesoderm (elongation). Animal caps with mutant RNAs; *ΔN-Tcf3* and *AxB109111V*, stayed as the uninjected control caps. In *β-catenin* injected samples, explants showed cap elongation, whileΔN-XTcf-3 blocked the mesoderm induction by inhibiting β-catenin signalling. However, cap explants coinjected *β-catenin* and *AxB109111V* RNAs still showed the mesoderm elongation as *β-catenin* RNA injection alone.

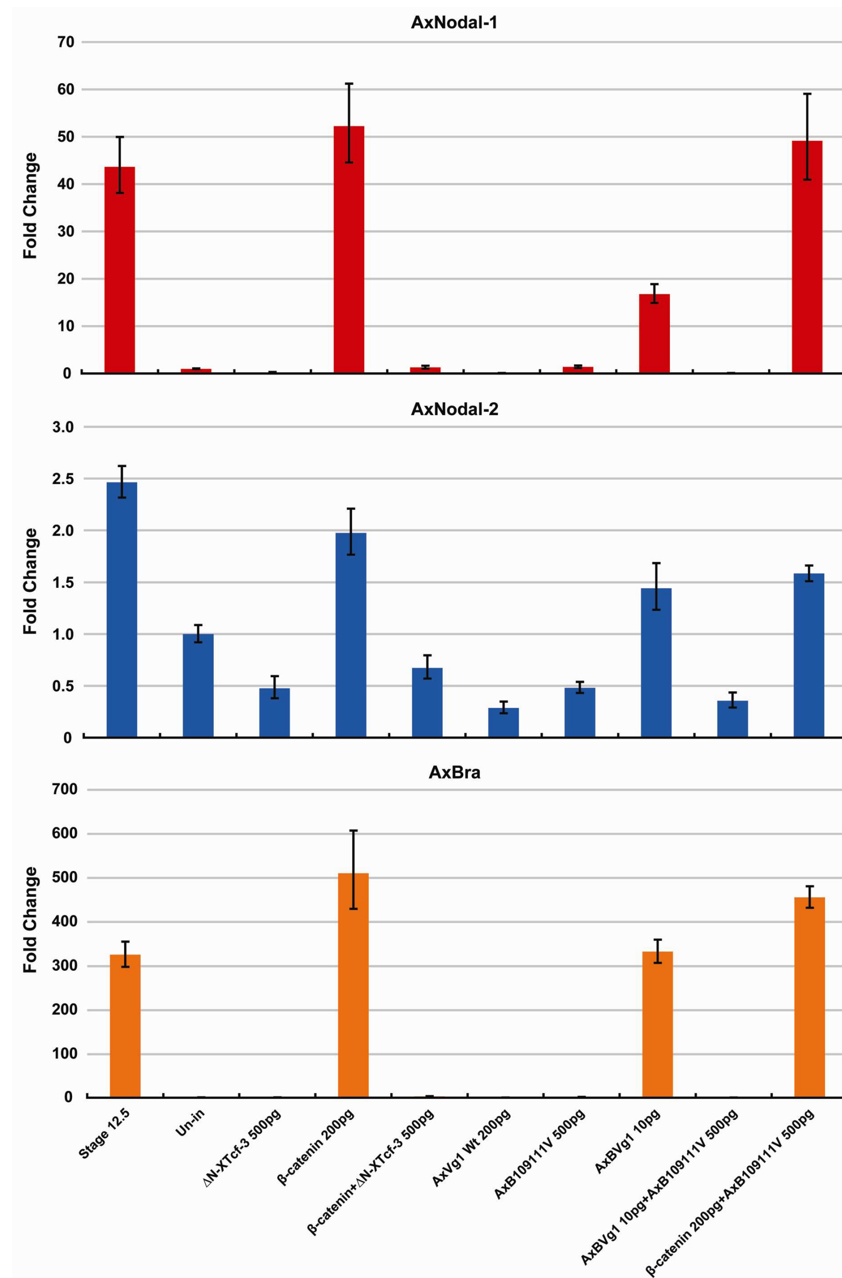


Figure 5.16 - qPCR analysis of gene expression induced by β-catenin and AxBVg1 with or without ΔN-Tcf3 or AxB109111V in axolotl animal caps

Cap explants were collected when sibling embryos reached stage 12.5 and qPCR analysis was performed to examine gene expression levels. The cDNA prepared from these samples was tested sequentially using specific Nodal genes (*AxNodal-1* and *AxNodal-2*) and mesedodermal gene (*AxBra*) qPCR primers and probes. The X-axis indicates the sequential cDNA samples, and the Y-axis indicates the relative gene expression levels which are relative to *ODC*, and normalised to the uninjected (Un-in) cap explants. △N-XTcf-3 had an inhibitory effect on β-catenin pathway, whereas AxVg1 mutant (AxB109111V) had no effect on β-catenin pathway.

5.5.3 Target genes of AxVegT and Wnt/β-catenin signalling in axolotls

Our previous results have shown that expression of *AxNodal-1*, *AxNodal-2* and *AxBra* can be induced by overexpression of *AxVegT* and *β-catenin* mRNAs in axolotl animal caps, but the inductive ability of these two factors differ. Overexpression of *AxVegT* RNA dramatically induces *AxNodal-2* compared to *β-catenin* injection (Figure 5.7A). However, co-injection of *β-catenin* and *AxVegT-ENR* does not inhibit the activation of marker gene expression by β-catenin except *AxNodal-2* (Figure 5.14A) suggesting that AxVegT may be a direct activator of *AxNodal-2* but not *AxNodal-1* or *AxBra*. In the mouse embryo, *Brachyury (T)* has been shown to be a direct target gene of the Wnt/β-catenin pathway and involved in mesoderm formation (Arnold et al., 2000; Morkel et al., 2003; Yamaguchi et al., 1999). In *Xenopus* inhibition of the Wnt pathway has no effect on *XlBra* expression (Zorn et al., 1999) although promoter assays suggest *XlBra* is under the control of the Wnt/β-catenin signalling pathway (Vonica and Gumbiner, 2002). To determine if *AxNodal-2* is a direct target of AxVegT, and *AxNodal-1* and *AxBra* are direct targets of β-catenin, the ability of AxVegT and β-catenin to activate *AxNodal-1*, *AxNodal-2* and *AxBra* in the presence or absence of cycloheximide (CHX) was tested. Axolotl embryos were injected at the one or two cell stage with two levels (500pg and 1ng) of *AxVegT* and *β-catenin* RNAs. The cycloheximide pretreatment was carried out once sibling embryos reached stage 7 by incubating these embryos in 0.2X MBS, 10μg/ml CHX. Animal cap explants were dissected from stage 9 embryos, incubated in the presence or absence of CHX, and analyzed by real time qPCR for the expression of *AxNodal-1*, *AxNodal-2, AxBra* and *AxMix* when sibling embryos

reached stage 10.5 (Figure 5.17). In the absence of CHX, AxVegT activates the expression of *AxNodal-2* (7-9 fold) and *AxMix* (8-12 fold), but only weakly induces the expression of *AxNodal-1* (5 fold) and *AxBra* (4 fold) compared to uninjected caps. In the presence of CHX, only *AxNodal-2* expression is induced by VegT, identifying *AxNodal-2* to be a direct target of VegT. In the absence of CHX, β-catenin induces strong expression of *AxNodal-1*, *AxBra* and *AxMix* but only weakly induces *AxNodal-2* (4 fold). The addition of CHX blocks the induction of *AxNodal-2* and *AxMix*, suggesting that β-catenin directly activates *AxNodal-1* and *AxBra* expression. In this experiment, treatment with CHX alone resulted in a low level of *AxNodal-2* and *AxBra* expression. Certain markers were sometimes weakly induced by CHX and the induction of makers by CHX has previously been reported in the *Xenopus* embryos (Tadano et al., 1993). However, quantitation confirms that *AxNodal-2* levels were significantly higher in response to AxVegT plus CHX over CHX alone. We note, however, that the level of activation of *AxNodal-2* by AxVegT and *AxNodal-1* by β-catenin is reduced by CHX, and this does not occur with the induction of *AxBra*. The possible explanation of this is that optimal activation of *AxNodal-2* by AxVegT or *AxNodal-1* by β-catenin involves some indirect effects. These results indicate that *AxNodal-1* and *AxBra* are both immediate-early targets of β-catenin, whilst *AxNodal-2* is a direct early target of AxVegT.

Figure 5.17 - Immediate-early targets of AxVegT signalling and β-catenin pathway in axolotls

At the one or two-cell stage, the animal pole was injected with 500 pg and 1ng of *AxVegT* or *β-catenin* mRNA. By stage 7 embryos were cultured with or without cycloheximide (CHX, 10 μg/ml) and animal explants prepared at stage 9 were harvested at stage 10.5 for real time qPCR analysis. *ODC* served as a control for RNA recovery and loading. Whole embryos served as positive control (stage 10.5). CHX treatment alone induces the expression of *AxNodal-2* and *AXBra*. Notice that CHX and AxVegT additively induce *AxNodal-2* expression, while both *AxNodal-1* and *AxBra* are induced by β-catenin in the presence of CHX.

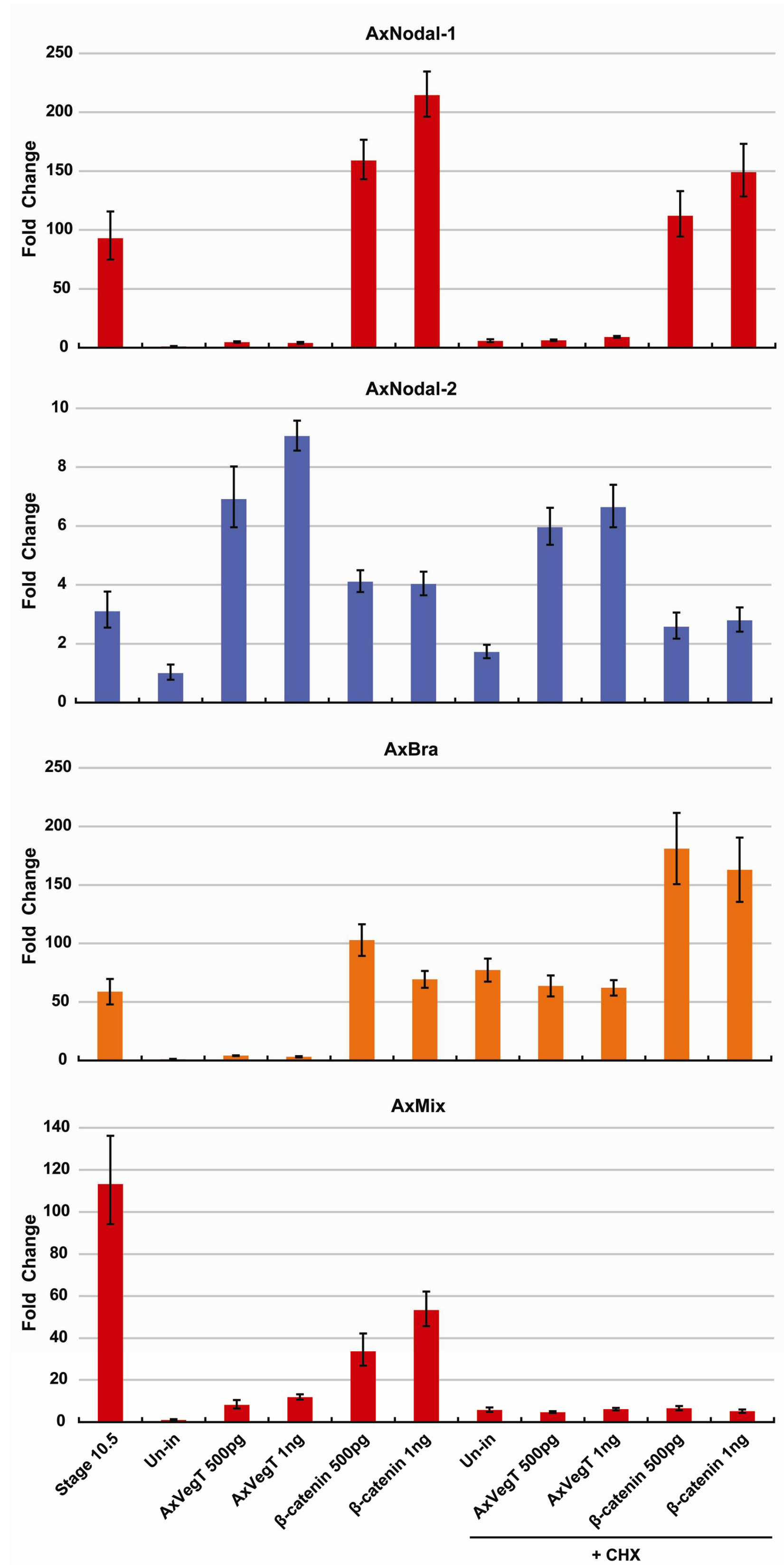


Figure 5.17 - Immediate-early targets of AxVegT signalling and β-catenin pathway in axolotls

In mouse embryos, evidence suggests that *Nodal* can be regulated by Wnt/β-catenin signalling. For example, *Cripto*, which encodes a Nodal co-receptor, has been identified as a primary target of *β-catenin* (Morkel et al., 2003). In addition, Wnt3 can stimulate *Nodal* expression via conserved Tcf binding sites in the mouse PEE (Proximal Epiblast Enhancer) within the 5' promoter of the *Nodal* gene (Ben-Haim et al., 2006). This suggests a molecular interaction between Nodal and β-catenin signalling in the mouse embryo. In *Xenopus* previous data shows that the *Xnrs*, including *Xnr1,2,4,5* and *6*, but not *Xnr3,* are regulated by VegT (Clements et al., 1999; Hyde and Old, 2000; Takahashi et al., 2000). Moreover, VegT activates the expression of *Xnr1* via T-box binding sites within the *Xnr1* promoter (Kofron et al., 1999). In contrast, *Xnr3* (McKendry et al., 1997) and *siamois* (Brannon et al., 1997; Carnac et al., 1996) are known target genes of Wnt/β-catenin signalling in *Xenopus*.

To explore these relationships further in the axolotl, we cloned the promoter regions for both *Nodal* genes, *AxBra* and *AxMix,* and looked for potential TCF/LEF (WWCAAAG) (van de et al., 1991) and T-box (VegT) (CACACCY) (Conlon et al., 2001) binding sites. The results (see Appendix) demonstrate that the *AxNodal-1* promoter has two putative TCF/LEF binding sites, whilst *AxNodal-2* has one putative TCF/LEF and two T-box (VegT) binding sites. The *AxBra* and *AxMix* promoters have two putative TCF/LEF and one T-box (VegT) binding sites each. To test these promoters response to β-catenin and AxVegT, luciferase reporter assays were performed (Figure 5.18). To remove complications from endogenous non-localised *AxVegT* in axolotl embryos, reporter assays were performed in *Xenopus* embryos. Promoter-luciferase

constructs were injected either alone or with *β-catenin* and *VegT* mRNAs in various combinations. β-catenin strongly activates the reporter gene from the *AxNodal-1* and *AxBra* promoters, whilst only weakly activating the *AxNodal-2* promoter. In contrast, the *AxNodal-1* and *AxBra* promoters are not responsive to AxVegT whilst *AxNodal-2* is. Although TCF/LEF and T-box binding sites were found in the *AxMix* promoter, we could only observe weak reporter activity, suggesting that β-catenin and AxVegT are not strong activators of *AxMix*. All together, the results from the CHX treatments and reporter assays indicate that *AxNodal-1* and *AxBra* are directly regulated by β-catenin whilst *AxNodal-2* is regulated by AxVegT in the axolotl.

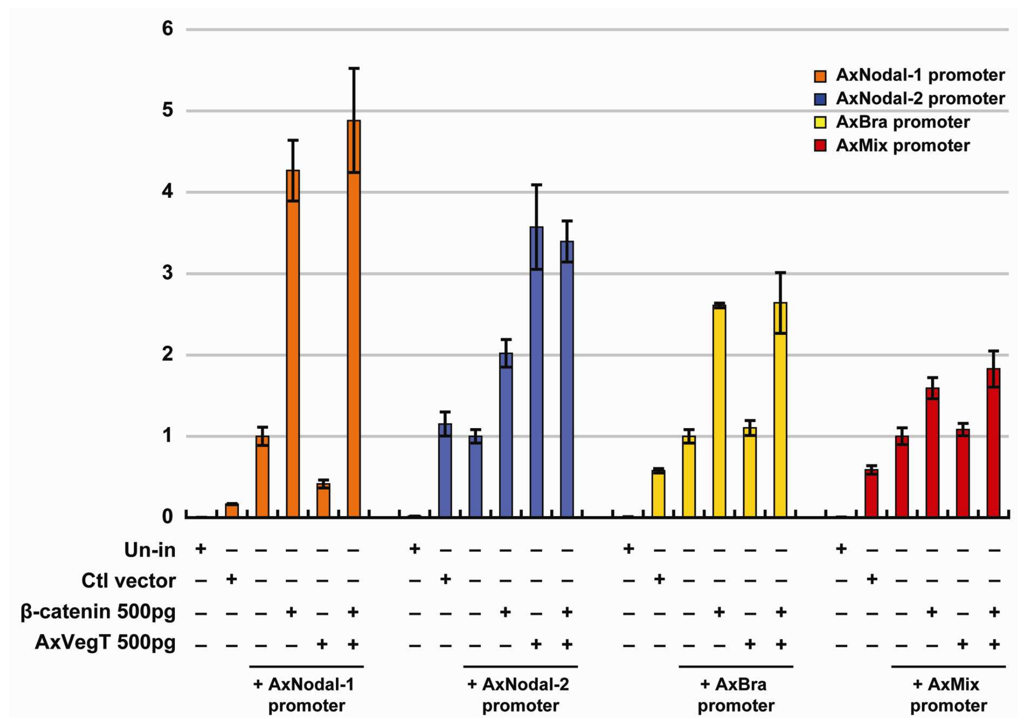


Figure 5.18 – Regulation of AxNodal-1, AxNodal-2, AxBra and AxMix promoter activity by β-catenin and AxVegT signals

AxNodal-1 and *AxBra* promoters were significantly stimulated by overexpression of *β-catenin* but not *AxVegT*. AxVegT induces the *AxNodal-2* promoter more strongly than β-catenin does. The *AxMix* promoter is only weakly activated by overexpression of *β-catenin* and *AxVegT*. Synergistic induction was not seen in response to co-injection of *β-catenin* and *AxVegT.* Un-in: uninjected stage 10.5 embryos. Ctl vector: embryos injected with control pGL3 reporter vector. The Y-axis indicates the relative luciferase activity which is relative to renilla luciferase, and normalised to individual promoter injected embryos.

5.6 Discussion

Previous studies in *Drosophila*, *C.elegans*, zebrafish and *Xenopus* demonstrate that the formation of germ cells in these species is linked with the presence of a specialized cytoplasmic domain called the germ plasm. Localised maternal RNAs are involved in the assembly of germinal granules and formation, proliferation, migration, and survival of PGCs (Wylie, 2000; Zhou and King, 2004). Alongside the germ plasm, key maternal determinants of cell fate, including *VegT* and Vg1, are also localised in *Xenopus* embryos and those of other anurans. We find that, as with the absence of germ plasm in urodeles, *VegT* and *Vg1* are not asymmetrically localised in axolotl oocytes. Importantly, orthologs of these determinants are not localised in the gulf sturgeon and the Australian lungfish, suggesting that the localisation of maternal determinants is a derived trait. Therefore the asymmetric localisation of *VegT* and *Vg1* RNAs in the oocytes of frogs represents an evolutionary innovation of anurans.

In the previous chapter, we demonstrated that AxNodal-1 and AxMix are critical for mesoderm induction in the axolotl embryo; moreover, the results also support the idea of mesendoderm sGRN comprising one *Nodal* and one *Mix* gene as that of mammals. Here, we investigated the signalling upstream of the *Nodal* genes in the axolotl. In *Xenopus*, mesoderm and endoderm are induced by signalling networks triggered by maternal VegT and then subsequently dorsalized by β-catenin. Note that high levels of VegT induce endoderm whereas low levels induce mesoderm (Kavka and Green, 2000). Our *Xenopus* animal cap assays confirm that low levels (50pg) of *VegT* are

sufficient to induce mesodermal marker genes whilst high levels (200pg and 1ng) dramatically activate endodermal genes (Figure 5.3). In comparison, overexpression of high levels of *AxVegT* mRNA (500pg and 1ng) in axolotl animal caps weakly induce both *Nodal* genes, *AxBra*, *AxMix* and *AxSox17* but 200pg *AxVegT* mRNA fails to induce any significant gene expression (Figure 5.7) revealing that AxVegT is a relatively weak inducer of mesoderm and endoderm in axolotls.

In *Xenopus* results from overexpression experiments show that ectopic injection of *β-catenin* into the ventral vegetal region causes duplication of the embryonic axis (Molenaar et al., 1996). In fact, β-catenin alone is not sufficient for the induction of mesoderm, rather it must be co-expressed with VegT or noggin in *Xenopus* embryos (Agius et al., 2000; Domingos et al., 2001; Wylie et al., 1996; Xanthos et al., 2002). Here, we re-examined the effect of overexpression of *β-catenin* in *Xenopus* animal caps (Figure 5.3) supporting this finding. β-catenin alone is unable to induce expression of mesodermal and endodermal marker genes except *MyoD*. Previous work has shown that the Wnt/β-catenin pathway is required for regulating myogenic gene expression in the presumptive mesoderm (Shi et al., 2002). In contrast, we have demonstrated that naïve axolotl animal caps can be induced to form mesoderm and endoderm in response to *β-catenin* injection alone (Figure 5.7).

Instead of using overexpression in animal caps, we re-investigated the knockdown effects of VegT, Vg1 and β-catenin in *Xenopus* and axolotl whole embryos. Our knockdown results for VegT, Vg1 and β-catenin in *Xenopus* are

consistent with previous studies (Birsoy et al., 2006; Horb and Thomsen, 1997; Kofron et al., 1999; Roel et al., 2002); however knockdown of AxVg1 and β-catenin in the axolotl show different phenotypes compared to those seen in *Xenopus* (Figure 5.9 and 5.10). VegT-depleted embryos suggested that mesoderm and endoderm formation rely on inducing signals downstream of VegT in *Xenopus* embryos (Kofron et al., 1999; Zhang et al., 1998). The AxVegT knockdown shows the same phenotype as *Xenopus* embryos at the early gastrulae stage with a loss of blastopore formation and no gastrulation movements; however, the AxVegT knockdown embryos show different phenotypes at the tailbud stage. These embryos typically have two separate regions; animal pole and vegetal mass, and the ectodermal territory of the embryo is not expanded like VegT-knockdown *Xenopus* embryos (Zhang et al., 1998). These embryos have normal expression of *AxNodal-1*; therefore, they are not identical to the *AxNodal-1* knockdown embryos previously reported. Rather, inhibition of AxVegT leads to downregulation of *AxNodal-2* expression, a molecule we have shown is not required for mesoderm formation. We therefore conclude that AxVegT regulates *AxNodal-2* in an alternative pathway which is not directly related to mesoderm induction.

In *Xenopus*, the depletion of maternal β-catenin causes the upregulation of BMP signalling across the embryo, allowing epidermal fates to predominate in the ectoderm germ layer, and blocking somite, notochord and head formation (Heasman et al., 1994; Heasman et al., 2000; Wylie et al., 1996). In our knockdown results, inhibition of β-catenin with ΔN-Tcf-3 reveals that the effect of knockdown of Wnt/β-catenin signalling is localised to the

prospective dorsal side; ΔN-Tcf-3 has no discernible effect on the ventral side (Figure 5.10) as well as previous work (Roel et al., 2002). These data highlight the role of β-catenin for dorsal axis formation in *Xenopus* embryos. However, in axolotls, dorsal and ventral injection of *ΔN-Tcf-3* causes embryos fail to form the blastopore and leads to development with no anterior-posterior pattern and no dorsal axis tissue. Similarly, *ΔN-Tcf-3* injections on the dorsal side result in ventralized embryos with a reduced dorsal axis and defects in closing the neural plate. Surprisingly, *ΔN-Tcf-3* injection on the ventral side shows interference with ventral and posterior mesoderm induction, whereas it has no defect on ventral mesoderm development in *Xenopus* embryos (Figure 5.9B). In summary, β-catenin is not only required for dorsal body axis formation, but also ventral and posterior patterning in axolotl embryos.

Inhibition of AxVg1 does not disrupt axolotl embryo development except for a timing delay compared to controls. Again, this suggests that AxVg1 is not required for early patterning of the germ layers in axolotl embryos.

The fact that AxVegT knockdown embryos continue to express *AxNodal-1, AxNodal-2, AxMix, AxBra, AxSox17* and *AxFGF8* shows that the axolotl does not use the same pathways as *Xenopus* for mesoderm induction. Is this a difference in the mechanisms in the axolotl, or a difference in the VegT molecule itself? We compared the mesoderm inducing ability of XlVegT and AxVegT with or without β-catenin in *Xenopus* animal cap explants. We demonstrate that AxVegT and β-catenin synergistically activate mesodermal gene (*XlBra* and *XlMyoD*) expression and elongation. Thus AxVegT is a true

VegT. However, AxVegT is a weaker mesendoderm inducer than XlVegT. The synergistic effect observed for AxVegT and β-catenin in *Xenopus* caps suggested that the β-catenin alone induced mesoderm induction in axolotl animal caps may be because of the presence of endogenous AxVegT in the cap. Using *AxVegT-ENR* mRNA to investigate this, we find that AxVegT is not required for mesoderm induction and β-catenin alone is sufficient to induce mesoderm formation in presence of *AxVegT-ENR* (50 pg) (Figure 5.13 viii), even though there is approximately 0.6 pg *AxVegT* mRNA endogenous to the animal cap. 100 pg AxVegT-ENR also does not block elongation, but does induce cell death in animal caps (data not shown). Therefore, the specific role of AxVegT needs to be further examined in the future. The AxVg1 mutant also showed no significant effect on the activation of *AxNodal-1, AxNodal-2* and *AxBra* by β-catenin (Figure 5.16) indicating mature AxVg1 is not required for mesoderm induction by Wnt/β-catenin in the axolotl.

In *Xenopus*, the importance of maternal VegT signalling in germ layer induction has been discussed previously (see Chapter 1.3.1). In comparison, inhibition of VegT signalling in the axolotl embryo shows no defects in germ layer formation, and mesoderm (*AxBra, AxFGF8*) and endoderm (*AxMix, AxSox17*) genes are still expressed. Moreover, high doses (1 ng) of AxVegT-ENR only slightly reduced the expression of *AxNodal-1*, which we have shown to be required for mesoderm specification in the axolotl, whereas AxVegT-ENR results in a dramatic reduction of *AxNodal-2* expression at all levels. Surprisingly, inhibition of β-catenin reduced the expression of *AxNodal-1* but not *AxNodal-2*, and mesodermal genes (*AxBra, AxGsc* and *AxFGF8*) were expressed at lower levels. These data indicate that maternal

AxVegT and β-catenin might be involved in two different regulatory pathways ending in either *AxNodal-1* or *AxNodal-2*. Our cycloheximide treatment results demonstrate that β-catenin directly activates *AxNodal-1* and *AxBra,* and that *AxNodal-2* is a direct target of AxVegT.

Previous work has shown that mesoderm induction (rather than subsequent patterning) is dependent on a functional Wnt pathway in the mouse and the sea urchin (Angerer and Angerer, 2000; Haegel et al., 1995; Huelsken et al., 2000; Liu et al., 1999), but this has not been considered to be the case in *Xenopus* (Harland and Gerhart, 1997). *β-catenin* depleted *Xenopus* embryos show no significant decrease in *XlBra* expression (Heasman et al., 1994). However, *T/Brachyury* has been shown to be a direct transcriptional target of β-catenin in the mouse embryos (Arnold et al., 2000; Galceran et al., 2001; Yamaguchi et al., 1999) and mouse *β-catenin*$^{-/-}$ embryos show down-regulation of *T/Brachyury* and other genes which have known functions in mesoderm development (Morkel et al., 2003). Although TCF sites have been found in the *XlBra* promoter, there is no direct evidence showing *XlBra* to be an immediate-early target for β-catenin or that the activation of *XlBra* is solely via Wnt/β-catenin pathway. Our results demonstrate that VegT is not required for mesoderm induction in the axolotl. The molecular function of AxVegT obviously needs further investigation, but the localisation of VegT to the vegetal pole and its role in mesoderm specification would appear to be a derived function. In contrast, β-catenin serves as a mesoderm inducer in the axolotl, a pathway that is conserved with the mouse, further highlighting the importance of studying these pathways in embryos with true ancestral characteristics.

Chapter 6. Discussion

The two major amphibian lineages diverged from a common ancestor over 350 million years ago (Anderson et al., 2008; Cannatella DC and Hillis DM, 1993). Fossil evidence and comparative embryology clearly indicate that urodeles have retained primitive amphibian traits and that these were conserved as amniotes, including mammals, evolved (Anderson et al., 2008; Bachvarova et al., 2009a; Bachvarova et al., 2009b). Here we show that the mGRN of axolotls is simplified compared to that of *Xenopus,* and resembles that of mammals. As with the germ line, initial specification of the mesendoderm in *Xenopus* embryos is controlled by vegetally localised molecules; VegT and Vg1. We have demonstrated that *VegT* and *Vg1* are not asymmetrically localised in axolotl oocytes and also showed VegT is not required for mesoderm induction in the axolotl. Moreover, in contrast to *Xenopus*, we demonstrate that naïve axolotl animal caps can be induced to form mesoderm and endoderm in response to β-catenin alone.

A single Mix and Nodal gene in a simplified mesoderm network in the axolotl

A GRN for mesendoderm specification in *Xenopus laevis* had been constructed and applied as a useful tool for comparisons between species during the early embryogenesis (Loose and Patient, 2004). Analysis of the network highlighted the complexity of mesoderm and endoderm formation in *Xenopus* in part a consequence of gene duplication and subfunctionalisation of key genes such as the *Mix-like* and *nodal-related* families in *Xenopus*.

Considering more primitive protochordates, ascidians have been thought to be the most primitive model of dorsoventral patterning, and *Nodal* is a single copy gene in ascidians (Dehal et al., 2002; Duboc et al., 2004; Morokuma et al., 2002). In addition the genome of amphioxus, representing primitive chordates, also contains only a single *Nodal* (Yu et al., 2002). Similarly, only a single *Nodal* gene is found in mouse, human and chick (Schier, 2003). Even though a *Mix* gene has not yet been identified in ascidians and amphioxus, only one *Mix* gene has been found in amniotes (Peale, Jr. et al., 1998; Pearce and Evans, 1999; Robb et al., 2000; Stein et al., 1998). Furthermore, when considered within the context of the profound differences in early morphogenesis of *Xenopus* and axolotl (Johnson et al., 2003a; Shook and Keller, 2008b), it is not surprising that the GRNs governing early development diverged, nor is it surprising that the mGRN of axolotl embryos is conserved in mammals (Bachvarova et al., 2009a; Bachvarova et al., 2009b). Indeed the absence of amplified copies of *Nodal* and *Mix* in species at the base of deuterostomes (Sodergren et al., 2006) as well as mammals, strongly suggests that the simplified network we uncovered is conserved in vertebrates at large.

To explore the concept of a simplified network for mesoderm specification, we chose to study the urodele amphibian *Ambystoma mexicanum*, the axolotl. We cloned and characterized the axolotl *Mix* (G.Swiers) and *Nodal* genes. Southern blot experiments suggest that, in the axolotl, *Mix* is present in single copy and only two *Nodal* orthologs are identified. To further investigate the role of AxMix, AxNodal-1 and AxNodal-2 in mesoderm specification, we used antisense morpholinos targeted to the splice junctions

of *AxNodal-1/2* and *AxMix* to unambiguously disrupt their expression. Knockdown of AxNodal-1 but not AxNodal-2 blocks the induction of mesoderm, phenocopying the effects of chemical inhibition of Nodal signalling at both a morphological and molecular level. Unexpectedly, knockdown of AxMix also blocks the induction of mesoderm (work carried out by G.Swiers), demonstrating that these two factors act together in a pathway for mesoderm specification. Furthermore, AxMix positively regulates *AxBra* expression inferred from the loss of *AxBra* in AxMix morpholino embryos. Supporting this, overexpression of AxMix mRNA can rescue mesoderm, and *Brachyury* expression, in axolotl Mix morpholino caps. Similar results are not possible with *Xenopus* embryos due to the gene amplifications that evolved in the *Nodal* and *Mix* gene families. Indeed, morphants of several of the *Xenopus Mix* genes gastrulate with no failure in mesoderm specification, although FGF signalling is upregulated (Colas et al., 2008; Kofron et al., 2004; Trindade et al., 2003).

Similarly, although mesoderm specification in *Xenopus* can be prevented by chemical inhibition of Nodal signalling, there is no evidence that expression of any one of the *Nodal* gene family members is crucial to the production of mesoderm (Ho et al., 2006; Jones et al., 1995; Osada and Wright, 1999). The expansion of *Mix* and *Nodal* genes in *Xenopus* with varying expression patterns suggests that the purifying selection acting on these proteins has been relaxed to allow functional divergence and a rapid mechanism to establish cell fates during embryogenesis. Indeed, recent studies have demonstrated sub-functionalisation in the *Xenopus Nodal* gene family, each *Nodal* being sequentially involved in mesendoderm induction and

gastrulation movements (Luxardi et al., 2010). Perhaps the amplification of the *Nodal* and *Mix* genes renders the mesodermal GRN of *Xenopus* resistant to perturbations that would be lethal in axolotls.

Gene expansion within a GRN is likely to lead to sub-functionalisation of genetic interactions within the network which could include apparently novel connections. We have identified a critical difference in the role for Mix in axolotl previously obscured in *Xenopus*. In *Xenopus* embryos Nodal signalling induces co-expression of the *Mix* genes and *Brachyury* in the mesendoderm (Lemaire et al., 1998; Wardle and Smith, 2006). The subsequent negative regulatory loop between these factors causes Brachyury to segregate with the mesoderm and the Mixes to segregate with endoderm. However, in axolotl embryos, we detected only limited co-expression of *AxBra* and *AxMix*, restricted to the ventral mesoderm. Overexpression analysis in *Xenopus* reveals that activation of mesoderm and endoderm is only in response to AxNodal-1 signalling, not AxNodal-2. Similarly, in axolotl embryos we find that *AxFGF-8*, *AxSox17*, *AxMix* and *AxBra* are downstream of AxNodal-1 signalling, with the activation of *AxBra* being dependent on AxMix activity. Based on the available evidence from *Xenopus*, we expected a Mix morphant to promote mesoderm and suppress endoderm (Lemaire et al., 1998). However, we observed the opposite effect with increased *AxSox17* expression and a loss of mesoderm in AxMix morphants. This observation reveals a position role for AxMix in mesoderm induction prior to any role in its suppression, and this is not apparently conserved in *Xenopus*. We demonstrated this interaction directly by ectopic overexpression of *AxMix* in axolotl embryos, expanding the AxBra domain in response to forced AxMix

expression.

Studies with mouse embryos have lead to conflicting results with some studies implicating Mix in mesoderm production, and others in its repression. Based on our observations in the axolotl, we knocked down *Mixl1* in EBs and showed a clear inhibition of *Brachyury* expression. This is consistent with the absence of *Brachyury* expression in the primitive streak (the site of nascent mesoderm production) of *Mixl1*$^{-/-}$ mouse embryos, suggesting that the role for Mix at the top of a hierarchy or transcription factors leading to mesoderm specification is conserved in vertebrates. On the basis of these findings we have constructed a gene regulatory network for mesoderm specification in axolotl embryos containing a key change from the *Xenopus* network in which AxMix activates *AxBra*, and consequently, the mesoderm (Figure 6.1).

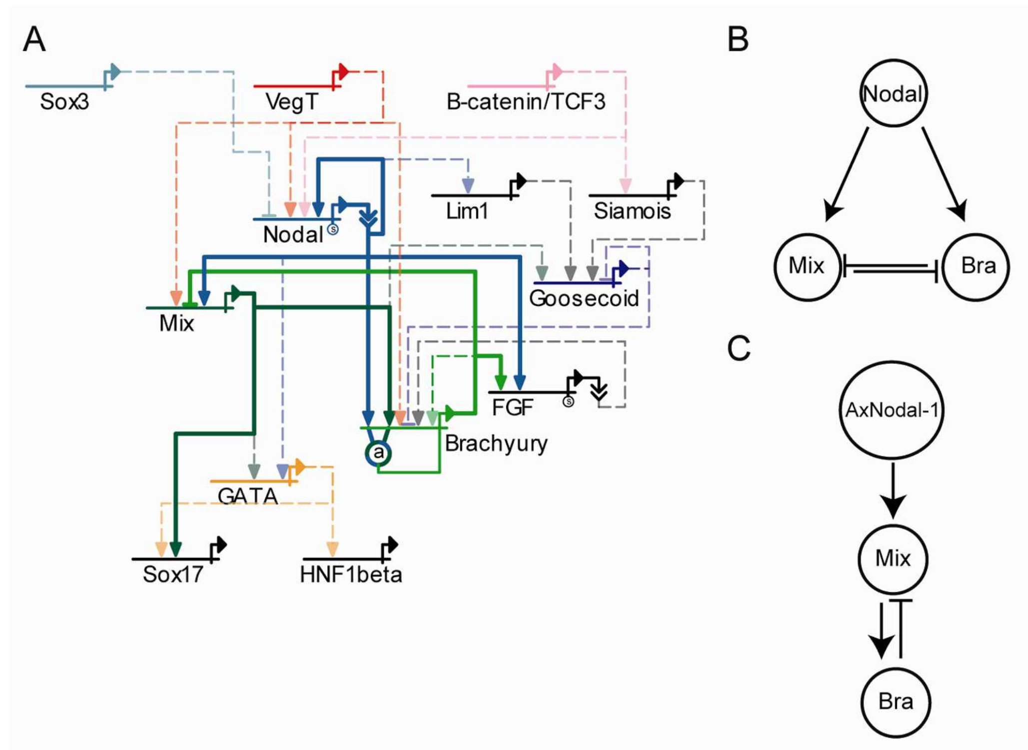


Figure 6.1 – The presumptive mesoderm sGRN for the axolotl
Solid lines indicate experimentally verified links; dashed lines indicate presumed links from *Xenopus*. Models comparing the relative roles of Nodal, *Mix* and *Brachyury* in *Xenopus* (B) and axolotl (C) development.

Germ plasm, maternal determinants and the mesoderm GRN

Previous genetic studies in *Drosophila* and *Xenopus* have shown that two essential contributions of maternal factors are their importance in the continuation of the germ line and to direct embryo pattern formation, especially germ layer specification (White and Heasman, 2008; Zhou and King, 2004). In most sexually reproducing organisms, the gametes are derived from a precursor stem cell population, called the primordial germ cells (PGCs). In fact, germ plasm has evolved repeatedly in metazoans (Extavour and Akam, 2003; Johnson et al., 2003b; Johnson et al., 2003a), providing an example of convergent evolution and implying a role in selection. However, the embryos of mammals, axolotl and amphioxus do not contain germ plasm and germ cells are induced by the intermediate mode at later stages of development (Bachvarova et al., 2009b; Bachvarova et al., 2009a; Johnson et al., 2003b; Johnson et al., 2003a). Therefore, the existence of germ plasm may have contributed the evolution of complexity in the *Xenopus* mesoderm GRN, whilst the axolotl mesoderm GRN retains the simpler, conserved network.

When development is robust to changes in genotype and environment, this robustness is termed canalization (Siegal and Bergman, 2002). As originally proposed by Waddington, canalization of a system evolves as a result of stabilizing selection and more complex GRNs will evolve to be more canalized. The robustness that results from canalization is generally considered a selective advantage, increasing the intensity of stabilizing selection by buffering genetic and environmental variation (Kitano, 2004). Therefore, the resistance of the mGRN to genetic perturbation offers a mechanistic

explanation for the accumulation of amplified *Nodal* and *Mix* genes in the *Xenopus* genome. However, unlike in *Xenopus,* the ancestral location of the PGCs in amphibians is the ventral mesoderm (Nieuwkoop, 1947). Expansion of the Mix and Nodal genes would likely disrupt the induction of PGCs in this position. Thus the evolution of a predetermined germ cell lineage in anurans enables the potential for change in the mesoderm network, in agreement with previous suggestions (Crother et al., 2007; Johnson et al., 2003a). Furthermore, the expansion of *Nodal* and *Mix* genes in teleosts (Fan and Dougan, 2007), which also contain germ plasm, suggests this may be a generalized mechanism leading to canalized development.

Wnt/β-catenin but not VegT or Vg1 regulates mesoderm formation in axolotls

The localised maternal mRNAs *VegT* and *Vg1* are involved in establishing the body plan and inducing both mesoderm and endoderm in *Xenopus* (Heasman, 2006). In contrast, we have demonstrated that in the axolotl, as with the germ plasm, *VegT* and *Vg1* are not localised. This demonstrates that the mechanism of mesoderm induction is not conserved between axolotl and *Xenopus*. Furthermore, homologous RNAs from lungfish and sturgeon, which also retain basal vertebrate traits, do not localise in oocytes. This strongly suggests that localised mesendodermal determinants are a derived trait and therefore the mechanism of mesoderm induction in *Xenopus* may also be derived.

To investigate the role of these maternal determinants, we compared mesoderm induction in *Xenopus* and axolotl in response to VegT, Vg1 and

β-catenin signalling. Our results in *Xenopus laevis* are consistent with all previous reports, demonstrating that *XlVegT* induces both endoderm and mesoderm (Clements et al., 1999; Horb and Thomsen, 1997; Kofron et al., 1999), and that the Wnt/β-catenin pathway acts alongside VegT signalling to form dorsal axial structures and dorsal mesoderm in *Xenopus* embryos (Katsumoto et al., 2004; Xanthos et al., 2002). Indeed, we see similar synergistic effects between AxVegT and β-catenin, with both together able to induce mesoderm in *Xenopus* animal caps. Based on data from *Xenopus*, we would expect AxVegT to play a key role in both mesoderm and endoderm formation in axolotls.

Using dominant-negative mutant constructs to disrupt the AxVegT and Wnt/β-catenin pathways, we reveal that AxVegT knockdown causes a failure in gastrulation movements in axolotl embryos similar to *VegT* depletion in *Xenopus* embryos. However, β-catenin knockdown axolotl embryos respond differently to *Xenopus*. Axolotl β-catenin knockdown embryos fail to gastrulate, whereas equivalently treated *Xenopus* embryos are able to gastrulate. Intriguingly in whole embryos the VegT and β-catenin dominant negative constructs affect the expression of the two *Nodal* genes in different ways. *AxNodal-1* expression is much lower in β-catenin knockdown embryos, whereas *AxNodal-2* expression is more sensitive to VegT than β-catenin inhibition.

The differential regulation of AxNodal-1 and AxNodal-2 is supported by overexpression assays. A*xNodal-1* and *AxNodal-2* are both induced by AxVegT, while β-catenin preferably modulates the level of *AxNodal-1*

expression but not *AxNodal-2*. Cycloheximide experiments reveal that *AxNodal-1* and *AxBra* are direct targets of β-catenin whilst *AxNodal-2* is a direct target of AxVegT, although our findings suggest other unknown factors may also be involved in the activation of both *Nodal* genes. Analyses of the promoters suggest that TCF/LEF and T-box binding sites may be required for the activation of *AxNodal-1/2*, *AxBra* and *AxMix* in response to AxVegT and Wnt/β-catenin signalling in axolotl embryos. Thus, our results suggest that *AxNodal-1* and *AxBra* , not AxNodal-2, are direct targets of Wnt/β-catenin signaling. In addition, our results suggest that AxVegT acts on the promoter of *AxNodal-2* through T-box binding sites, whereas both *AxBra* and *AxMix* can only be weakly induced by AxVegT. Although *AxVegT* overexpression can weakly induce *AxNodal-1* expression in animal caps, our results suggest the activation involves indirect effects downstream of the AxVegT pathway. Together, these data explain our most surprising observation in axolotl animal cap explants; β-catenin alone is sufficient to induce mesoderm even in the presence of a dominant negative VegT construct.

The frog *Xenopus laevis* has been used as a model animal to study the molecular mechanisms of vertebrate development, and has provided extensive knowledge on the roles of VegT pathway and Wnt/β-catenin signalling in body plan formation. Our findings highlight key differences in mesoderm induction between the two species, *Xenopus* and axolotl. In *Xenopus*, germ plasm and mesendodermal determinants are localised in the vegetal cytoplasm. Specifically, the maternal transcription factors VegT and Vg1 specify the endoderm and then produce secreted molecules (Nodal-related TGF-β ligands) to induce mesoderm specification in the

overlying ectoderm (Agius et al., 2000; Zhang et al., 1998). VegT therefore plays dual roles in early *Xenopus* development, firstly as an upstream inducer of endodermal and mesodermal genes and, secondly, as a co-factor to activate dorsalizing signalling and stabilized β-catenin (Katsumoto et al., 2004; Xanthos et al., 2002). Therefore, in *Xenopus*, both dorsal and general mesoderm induction are dependent on VegT (Agius et al., 2000; Kofron et al., 1999) and are the results of the interplay of VegT and β-catenin.

In the mouse, Wnt/β-catenin signalling in the primitive streak is not induced by localised maternal factors but by extra-embryonic signals from the juxtaposed extra-embryonic ectoderm (Rodriguez et al., 2005). In the mouse embryo, the activation of Wnt/β-catenin signalling is important for axis formation and also essential for the production of mesoderm and definitive endoderm (Haegel et al., 1995; Liu et al., 1999). In contrast, genome sequence fails to reveal *VegT* orthologs in mouse and human. Potentially, the localisation and function of the VegT orthologs important for germ layer specification are a synapomorphy amongst anuran amphibians (Nath et al., 2005). Although vertebrate T–box genes have been divided into at least eight different groups, T-box gene orthologs related to *VegT* in *Ciona intestinalis* (Takatori et al., 2004), zebrafish (Griffin et al., 1998), chicken (Knezevic et al., 1997) and mouse (Chapman et al., 1996; Chapman and Papaioannou, 1998) appear to be involved in later steps of mesoderm development and not in germ layer or germ cell determination. Therefore, the available evidence indicates that Wnt/β-catenin signalling along with the maintenance of Nodal activity is the major mesoderm inducer in the mouse embryo (Ben-Haim et al., 2006; Kemler et al., 2004).

However, in the axolotl mesendoderm determinants such as *VegT* and *Vg1* are not localised and VegT is dispensable for the induction of mesoderm in animal cap explants. Moreover, β-catenin knockdown embryos fail to gastrulate and have low expression of mesodermal genes as seen in β-catenin depleted mouse embryos (Marikawa, 2006; Morkel et al., 2003).

Activation of the Wnt/β-catenin signalling pathway results in the activation of *AxNodal-1* independent of VegT accompanied by the up-regulation of mesodermal markers, demonstrating that Wnt/β-catenin signalling alone is the mesoderm inducer in the axolotl. The apparent similarity with mouse may represent an evolutionary conserved mechanism of vertebrate body axis formation between axolotl and mouse. Indeed, our results indicate that although AxVegT is a weaker inducer for mesodermal and endodermal gene expression, AxVegT knockdown embryos have no effect on the expression of *AxNodal-1*, which we have demonstrated is necessary and sufficient for mesoderm specification in the axolotl.

Taken together, we propose that *AxNodal-1* represents the ancestor of the sub-functionalized *Xnrs* in *Xenopus,* and is responsible for promoting mesoderm and endoderm specification in response to Wnt/β-catenin signalling. This implies no role for VegT in the specification of the mesoderm, yet VegT dominant negative axolotl embryos do not gastrulate with an apparent failure of mesoderm specification. How then can these data be reconciled? Perhaps the explanation lies in understanding the role of AxNodal-2, the axolotl nodal gene most similar to mammalian nodal genes. *AxNodal-2* is activated by AxVegT, not Wnt/β-catenin signalling, having two

putative T-box binding elements and only one TCF/LEF responsive element in its promoter, elements that are also found in the mouse Nodal promoter. A role for nodal has been reported in the maintenance of pluripotency (Vallier et al., 2009) and recently a *Nanog* ortholog, essential for pluripotency, has been identified in the axolotl (J.D. Dixon, A.D Johnson pers. Comm.). Unexpectedly the overexpression of VegT-ENR in whole axolotl embryos leads to the downregulation of both *AxOct4* and *AxNanog* expression, suggesting that *AxVegT* may contribute to the regulation of pluripotency in axolotls (data not shown). Notably, Nanog mutants fail to gastrulate and resemble the VegT-ENR embryos we report here (C Jackson, A.D. Johnson. Pers Comm). Further studies will be required to characterize these factors which act as downstream of AxVegT pathway, including AxNodal-2, AxNanog and AxOct4.

Work presented in this thesis describes the cloning and characterization of the axolotl *Nodal* genes, orthologs of Nodal signal molecules involved in the specification of mesoderm and endoderm during early development. We have provided evidence to show that only a single *AxMix* and two *Nodal* genes are present in the axolotl genome. Furthermore, our experimental data demonstrate that a simplified gene regulatory network for mesendoderm induction does exist in the axolotl and reveals a novel role for Mix in the regulation of Brachyury. In addition, the role of VegT and β-catenin have been characterized and compared to *Xenopus*. Our preliminary data indicates that β-catenin is the key factor mesoderm determinant in axolotls, as is the case in mouse embryos. These findings are summarized in an updated axolotl mesendoderm GRN (Figure 6.2). Further analysis of the

regulatory circuits driving *AxNodal-2* and the pluripotency network in response to AxVegT will help to uncover the linkages between pluripotency, mesoderm induction and the localisation of maternal molecules during embryogenesis.

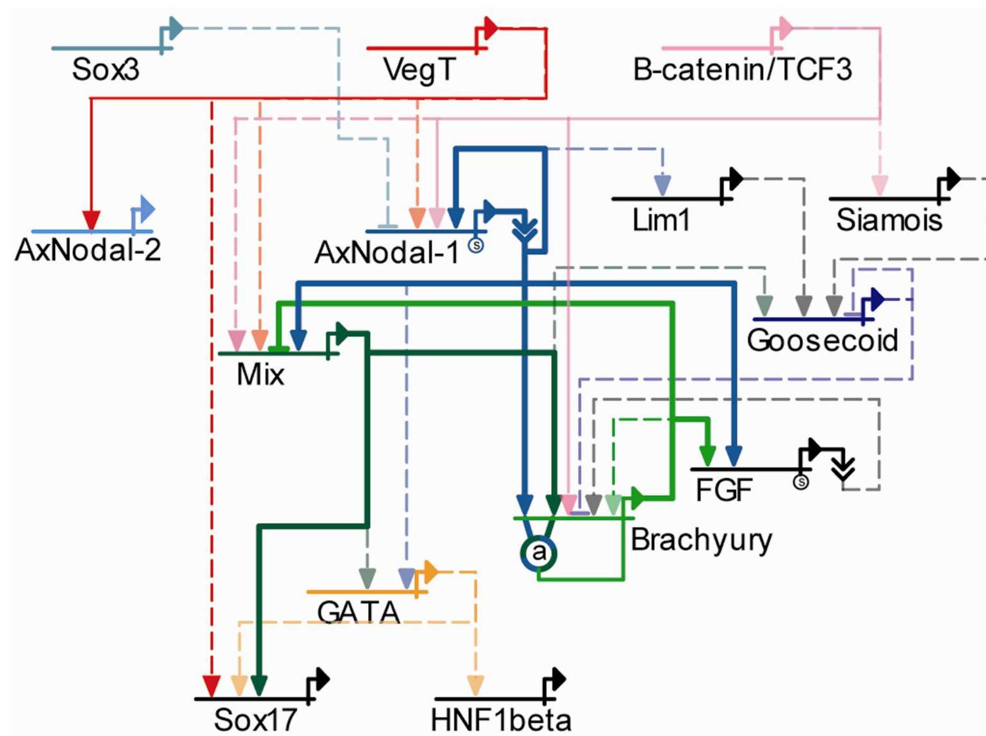


Figure 6.2 – An updated axolotl mesendoderm sGRN

Solid lines indicate experimentally verified links; dashed lines indicate presumed links from *Xenopus*.

<u>Axolotl as a model to study vertebrate development</u>

In addition to the evidence we have presented above, the comparison of axolotl and *Xenopus* during gastrulation, mesoderm induction and primordial germ cell development suggest that urodele amphibians have much to offer as a model system for experimental analysis of early vertebrate development.

1. Mesoderm origin and gastrulation

Many chordates internalize mesoderm by bending an epithelial sheet of cells inward (invagination) and/or by rolling a sheet of cells over an inflection point (involution) (Shook and Keller, 2008b). Basal chordate embryos predominately use invagination and a small amount of involution to accomplish the primary internalization of their presumptive mesoderm and endoderm through an open blastopore (Rhee et al., 2005; Swalla, 1993). The mechanisms of gastrulation in basal chordates differ from vertebrates; studies on amphibians provided different models of mesoderm internalization used by most anamniotes. Due to the initial differences in surface cell layers between urodeles and anurans, these two amphibian groups have different mechanisms for invagination and involution during gastrulation (Beetschen, 2001). The models of primary internalization can be divided into two main systems, the open blastopore model and dorsally restricted blastopore model (Figure 6.3).

In all anurans, the presumptive mesoderm involutes around the blastopore lip during gastrulation, in association with supra-endoderm. The presumptive mesoderm in the superficial epithelial layer is then restricted to portions of

the notochord and somitic mesoderm. Marginal zone tissues involute to form the lining of the gastocoel, resulting in a continuous epithelial connection between the outer epithelial surface of the embryo and that of the gastrocoel, and then ring the entire circumference of the blastopore (Minsuk and Keller, 1996; Shook et al., 2004). Therefore, the anuran presents an open blastopore model around its circumference. In contrast, in all urodele amphibians, the open portion of the blastopore is only restricted to the dorsal side. The presumptive notochord follows the same pattern of internalization as found in anurans, involuting dorsally during gastrulation to form part of the gastrocoel lining. However, the presumptive somitic and lateral-ventral mesoderm involutes around the lateral and ventral blastopore lips and than immediately ingresses adjacent to the endoderm (Imoh, 1988; Lundmark, 1986; Shook et al., 2002). Thus, the lateral and ventral blastoporal lips are the bilateral equivalents of the primitive streak of amniotes, a similarity between urodele amphibians and amniotes. Moreover, the mechanism of mesodermal internalization (ingression) is surprisingly similar in urodeles and amniotes (Shook et al., 2002).

Alongside the differences in gastrulation movements and mesoderm patterning, anamniote vertebrates have two epithelial types; multi-layered and pseudostratified epithelium (Figure 6.3). In embryos like those of anurans, the mesoderm epiblast has a discrete superficial epithelial layer and shows no interdigitation with the underlying deep cells. However, in urodele embryos, the mesoderm epiblast is a single-layered, pesudostratified epithelium and the deep cells continuously interdigitate with the superficial cells during gastrulation. Further studies also suggest that the ancestral

vertebrate probably has similar type of mesoderm epiblast which resolves to a pesudostratified epithelium during gastrulation.

Figure 6.3 – A comparison of Anurans (*Xenopus*) and Urodeles (Axolotl) during early development (A-D) *Xenopus* (E-H) Axolotl

(A) *Xenopus* have multi-layered epiblast with many layers of less organized deep cells, **(E)** whereas axolotls have single-layered (pseudostratified) epiblast. **(B)** The superficial view of cell fates, *Xenopus* start gastrulation with a smaller proportion of presumptive mesoderm than axolotl **(F)** whose surface mesoderm contains precursors for notochord, somite and lateral-ventral mesoderm (L-V mesoderm). **(C)** *Xenopus* internalize (arrows) their presumptive mesoderm by involution around the blastopore and aiding the blastopore closure. **(D)** a sectional view as indicated by the dashed line in **(C)** Mesoderm originating in the superficial layer remains in the gastrocoel roof and form an open blastopore mode with continuous epithelial connecting the outer epithelial surface. **(G)** In the axolotl, the gastrulation involution is restricted dorsally (arrow). **(H)** a sectional view as indicated by the dashed line in **(G)** Following the dorsally involution, most presumptive mesoderm ingresses laterally and ventrally adjacent to the endoderm, indicated by ingressing bottle-shaped cells, and the open portion of the blastopore is restricted to the dorsal side. In all figures the arrowhead marks dorsal. Figures are adapted from (Shook and Keller, 2008a; Shook and Keller, 2008b).

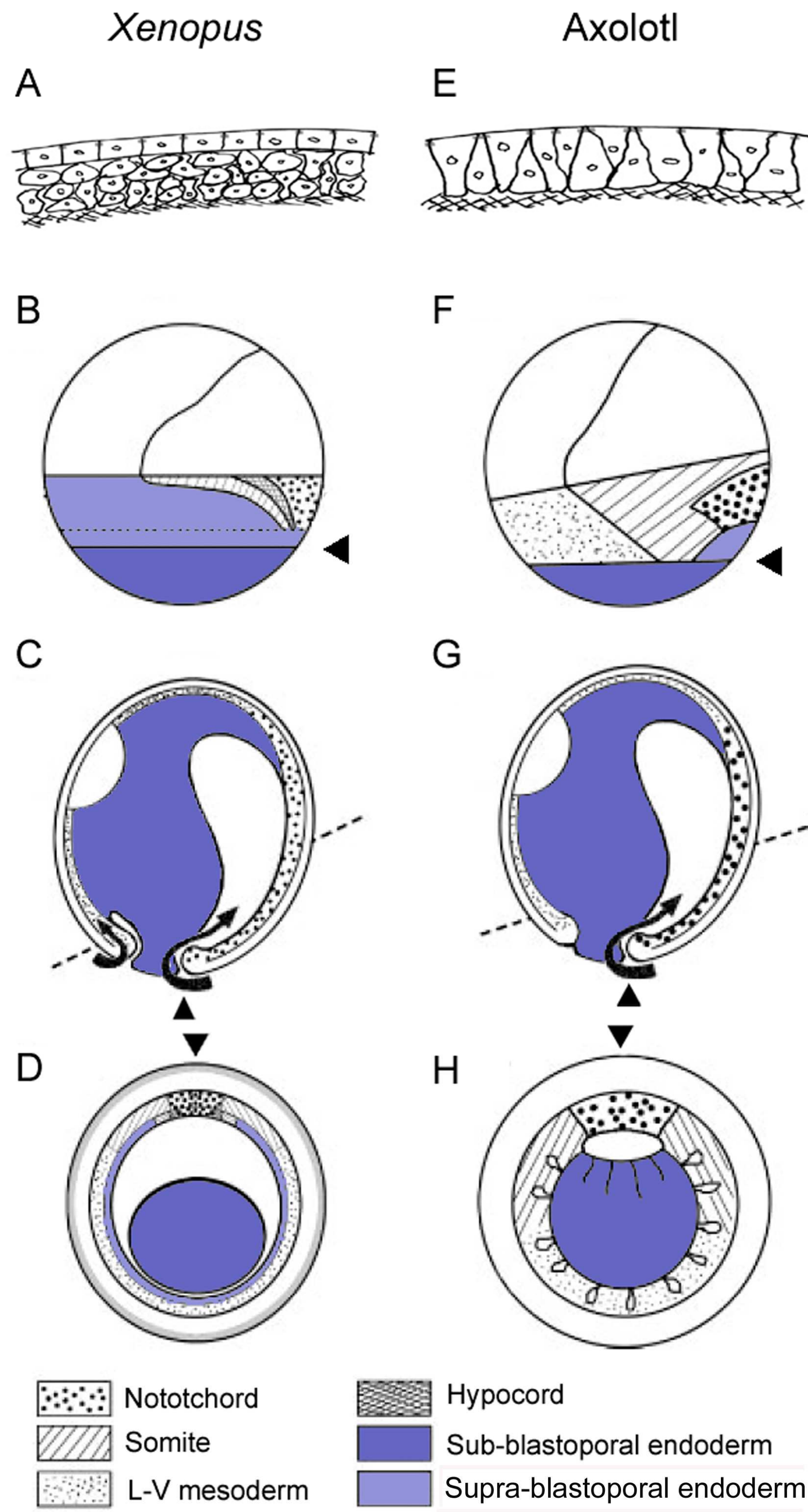


Figure 6.3 – A comparison of Anurans (*Xenopus*) and Urodeles (Axolotl) during early development

2. Primordial Germ Cell (PGC) development

Most cells which constitute the body of multi-cellular organisms eventually die after a certain number of cell divisions. However, germ cells, which differentiate to gametes and are responsible for reproduction, are potentially immortal. In vertebrate embryos germ cell formation can be classified into two main types; preformistic (in anurans and so on), and intermediate (in mammals, urodeles and so on) (Wakahara, 1996). PGCs in anurans are formed by the preformistic mode, germ cells are formed and segregate from somatic cells at a very early stage of embryonic development. They are often predetermined by the presence of a germ cell-specific germ plasm, which originally localises in the vegetal region of the embryos. In contrast, no germ cell-specific germ plasm can be detected during early development of urodele embryos. PGCs in urodeles are induced by the intermediate mode; germ cells are formed at a late stage of development from pluripotent embryonic cells (PEGs) (Wakahara, 1996). Humphrey's and Nieuwkoop's studies discovered the localisation of PGCs in the presumptive lateral plate mesoderm on the basis of characteristics such as large spherical nuclei and finely dispersed chromatin (Humphrey, 1929; Humphrey, 1928; Humphrey, 1927; Nieuwkoop, 1947). With no detectable germ plasm in mouse, PGCs are found in the presumptive extraembryonic mesoderm in both pregastrulation and early-streak stage embryos, demonstrating mouse PGCs are of extra-embryonic mesodermal origin (Lawson and Hage, 1994). Recombinations of the ventral vegetal mass with different regions of the animal ectodermal hemisphere in urodeles demonstrate that PGCs can be induced from ectoderm cells under the influence of mesoderm inducing factors from the vegetal endoderm (Michael, 1984; Sutasuaya and

Nieuwkoop, 1974). Thus, it seems reasonable to conclude that the mesodermal origin of urodele PGCs is basically identical to the mammalian pattern, but not to the predetermined endodermal origin of anuran PGCs. Further experimental observations show that overexpressing mRNA encoding the *Xenopus* mesoderm inducing factors eFGF and BMP-4 in axolotl animal caps can result in the formation of PGCs and the PGC specific marker *AxDazl* (Johnson et al., 2003a). In addition, germ plasm has not been identified in early echinoderm embryos, PGCs in sea urchins are not predetermined; rather, they most likely arise in response to regulative influences during development so supposedly regulative germ cell specification is probably a primitive mode, whereas the predetermined mode is derived (Ransick et al., 1996).

In addition, the ancestral amniote shared two significant features with those hypothesized for the ancestral vertebrate. They both have a pseudostratified mesoderm epiblast and a dorsally restricted blastopore, with involution of the notochord to form the roof of the gastrocoel and has ingression (subduction) of lateral-ventral and somitic mesoderm after involution around the lateral blastopore lip or through the blastopore plate (Shook et al., 2002; Shook and Keller, 2008b). As described above, these are also features shared by urodele, but not anuran amphibians (Shook et al., 2004; Shook and Keller, 2008a). Therefore, it suggests that the amniotes may arise from a urodele-like anamniote ancestor. Furthermore, the PGCs induction in axolotl embryos seems to retain a primitive trait conserved in the mammalian lineage (Johnson et al., 2003a). We believe that there are many differences between anamniote and ancestral amniote. However, if we consider the evolutionary

changes between the urodele amphibians and the amniotes, the conserved regulatory mechanisms might shed some light on their functional basis. Therefore, these features highlight the axolotl as an advantageous model system for the study of development.

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Appendix

Genome sequence of AxMix, AxNodal-1 and AxNodal-2

Gray shadow: exon

***Italic*: intron**

AxMix genome fragment (5'-3') – Intron1 and 2

1 ACGGGAACCTCCAGAACGCCTTTTCGGCAG*GTAAGAGCCGTCAATAATGC*

51 *TTCCTTTCCCTCTACTGCATGTCAGCTTACCCATGCCCACCCCCCTTCCC*

101 *CCAGAGCCCCTCATCCATCCCTCCGGTGTCCTCACAGTGCTCCTCAACTC*

151 *CCCGGCCCCTCGCCTCCACAATCCCTCATCTCCCAGTATCTTTCATCCCG*

201 *GCCTTCCTCCACCCTTCATTCCAGAGCCCGTTACCTCTCGAAATGTTCAC*

251 *CTCACCACTGTCTCCAACCCTTGCATAACTGAGCATCCCTTCGGTCCCCC*

301 *GCCCTCAACCCCAGCAGTGCCCCCTTCTCCATGGCAGGTCACCAGCCGCG*

351 *GGCCAGGGCTCTCCCTGGGTAACCTCCCCCTCGCCATGCCAGTCCCCCAG*

401 *CCGTGGGACTCTCGCCATACCAGGGCACTATTTGGGGGCCTCCCCCTCAC*

451 *TATGCCAGCTAATTATCTGGTTGCCTCACCCTCGCCATGCCAGGCCACTA*

501 *GTTGGGGGGCCTCCCCCTCACCATGCCAGGCCTCTGCCGCATGATGGGAA*

551 *CTTTTATCGTAGCATTTCTCTCCAATCAGAGTCAGAGAACGAAGCGCGTT*

601 *GCTGTTAATTAACCTTGTAACTGAGCTCTGATGTGCCTATATGAAGAAAC*

651 *GCTATCGGGATTGCAAATCCCAGCGAAGCTTGACATTCAGATTATAAATA*

701 *TTGCCAGGTGAAATCATAACTGGGCTGGTGGGATATTGGGTGCACACAGC*

751 *TGCTGCCCCCCATAACAGACCGCCCACGGTCTCGCTGACAGGGACCCTGC*

801 *GATGTATGCGACGCTTCTGGTCGCCTCTTAAACTATCGACGGTTTTTAAT*

851 *TCGCACGGAGGGTGTTGTGGGGGGTGCAGGTGGGCGGTTTAGCAAAGTAC*

901 *CTGTGACTTGTGTGACGCCCCTGCATTAGCCAGGATATAACGCCTAGACT*

951 *GGTGTCACTTCCTTTAGGCAAAATACCGCAGTGGGCGGGGGAGGGAGCGT*

1001 *CGGTAAGTATATGTTTATGTGGGCGGGGGAAAAGTAGGTTCACATCCGTT*

1051 *AGACGAAGTTTCAGATAAAACCGGTGCTTCCAAGTTAATGTTCGCCTGCA*

1101 *ATATTTTATATGATATGATCGTTTATTTATATAGCGCCTATACGCAATGT*

1151 *TTCAAAGCGCTACATTTCAGACACCTGCGCACGATTTCACGAGTATCTGC*

1201 *CACCACCCGAGGACTTTCTCCTTGGGAAGATATACTGTGAGAAATGACTC*

1251 *TTTATTACAGAAATCTGCCACAGGGGGCCTGACAAGAAATGTATTAGGAT*

1301 *AAACTATTCCCCAAATCCTGCAGACTGTCGCGTTTCCGGTGGTTGGCGTT*

1351 *TGGAAGTATTGGCTAGTTAGATTGCTCAGTGTGTTAACAGCATGCTGCAT*

1401 *CTGCTGGTCAATAGTAGTATCCGATGTTTGATGTGCAAGACTGAGGTTAA*

1451 *GCGCTATATAAACACTATCTTTATTTGGAAAAAATACACCGCAAGGATAT*

1501 *CATGGCAAGCGAGGAAACTGTGAGCAGAAACTCACGATAGCCAGTAAAAA*

1551 *GTGCATCTCCTAGGCCATGTTAACTCCTTTCTTTGCCAACAACGTCGCAA*

1601 *TAAAGGATTTCGCCCAAAATGCAGTTTCGTAGCTGGCCCACTCGTGACAC*

1651 *AGGTGTCGGTGCTTTTGAAACAACTTTCAAAGTAACATCGACTTTTTAAG*

1701 *TAGTGCTCATCGAAAACGGAACTGCGTGGGAGCTTTAATCACAGGAGCCG*

1751 *TTACCACGAAATGAAAGATACATATTATATTGCTCTCGGAAGGGTAAAAT*

1801 *GCGGAGTCAGGGCGGTTATGATTTGAACCCACGACCCGTATGTTACATAC*

1851 *GCACCCATCTCTTAAACTTTAGAATTATTGTGCAAGCCAATACATGTGTT*

1901 *CTATTAAGTTTTCCAGTGTGTGAAAGTAAGTGTATGCATTTCGCCTGTTT*

1951 *CTATGTCAGGGTCATTATTAGCATGCATTATTTCTTTCTACTTCTAGCGT*

2001 *TTATCTGACTCCGAGGCCTTTGCAGAACGAGTCCGAATACTATTAATATT*

2051 *GCGTCTCTTCTTTTGCAG*TAGGAGGTTGTGAAGAGTCCAGCGGCGCCAGC

. . . .

2351 ACCTGCGGGAGGAGCTGGCCGCCCTCACCTTGCTGCCGGAGTCCAGGATC

2401 CAG*GTGAGACCCGTGGATAGGCCGGCTGGGCGCATGGTACTGTTCACACA*

2451 *TGTCACCTCGGGGCTTGGTCTGCAGCCGTCCGCCTGAGTGGCTCTCGAAT*

2501 *ACTCGGGGCTGCTGTTTGGGCCCTCACTAGTAAGGGAAGCGGTTTCGCCC*

2551 *AGATCTCATTGCACAGATTTTTTAAATATATATATATATTCACACACACA*

2601 *CACACAACATGGCTTGCAAACACTAGGAGTAGCTGGGAAATGAACCTCAA*

2651 *AACAACTTGTACATAATGGTGACCATTCTTTTTTGACAATCCGATTGTAA*

2701 *AAATAAAAAAGCAACAATGATAAATAGATGGTGTACAGAGGAGGGATACC*

2751 *AATTGTACGTTGCAGTAAAGCATGGTTAGGTTTTAAAGAGGAGCCTGGGA*

2801 *AGTAGATCCTGGTAGGTACGGGCATATGCACGTAAGGGGAGTGTATACGG*

2851 *GGTAGCCAGGTTTTCTGGTCAAGTTATCCAAATAGTACATTTTTTGGGAG*

2901 *GACAGACTTTAGTGGGGTCGTTTTTAGAGTTCTCTGAACCTTTCGGGATT*

2951 *GCATTTTTCTTAACTGCATTTGGAGCTGAAGATCTGTGCTGGGGAAAAAC*

3001 *CTCAGCGGCCATACCATGGAAAAGTGTAGCGGGTCTAAGCAAGTGGGGAA*

3051 *AAAAGGCCCCAGTAGACCTTCAAACCAAGATGTTGGCAGGATACAAATAA*

3101 *GTCTGTAATTCTATTGTGAGCGAGGCCCGTAGTAGATTAGACGCCTACTT*

3151 *AACGCGTTATCGCACACATATTGAACAGAGACTGCCAGAAAAAGATAATA*

3201 *TGTAATTAGTAAAAAATTCCACTGCATGGGTCCAAAGATATAACTGGGTT*

3251 *GTTAATATGGGTTTCTCCAAATGAGTGCATTGATCTCATCCCGCTGAAGA*

3301 *GTATGTAAAGGTCATGTGTGTTAAGACACGTGGACCCTTTAGGAGCCCGA*

3351 *GTCCACCCGCCTCTGGGCCGTTCTGCAACGTTCCCCCTTCAGCATTCCTC*

3401 *CGTTAGCTTTTATTTGCACATGTCTTGCTTCTTTTTCTGACACCTTTGCT*

3451 *TGTGTCCAACCCAGGTCTATTTTATGCAATGAGTTTGGTGTTAGTTTAGG*

3501 *TTTCCGCCTCTGATAATGAACCACGTGGCCGCCATATGACAAGATGGCTC*

3551 *CTTGCTGATATGAGATTCAGCCCTGAGCCAATGAACCACGTGGCCTCTAT*

3601 *ATGACAAGATGGCGCCTTGCTGATATGAGATTCAGCCCTGAGCCAATGAA*

3651 *CCACGTGGCCTCTATATGACAAGATGGCGCCTTGCTGATATGAGATTCAG*

3701 *CCCTGAGCCAATGAACCACGTGGCCTCTATATGACAAGATGGCTCCTTGC*

3751 *TGATATGAGATTCAGCCCTGAGCCAATGGGCCTTACTTACTGTGCATATA*

3801 *TTCGGCAGCCCCAATGTGGGTAAATCCATGTGTTTTGGTATTTTACTCTG*

3851 *CTCACTCTATTTTTTCTTCTTTTTTTTCTTAAG*GTCTGGTTCCAGAATAG

3901 GCGTG

***AxNodal-1* genome fragment (5’-3’) – Intron1 and 2**

1 GGAGGCCGACACCGTGCTCAGCCTGGTGGCCAAAA*GTAAGCGGCTTCCTC*

51 *ATTATTCAATAGAATTAGGCCCTCAAATAGAACGAAGCAAGTTTGAACTA*

101 *GCAGAGCGATTGTGGTAGCGTGCCTTAATGGCATCCTATTTCTTGCTTTT*

151 *TTTCTTGTCCATTCCTTCCTTCCTTTTTTAACCTGTCAATCCCAAATACT*

201 *GTTTTTTTTCCTTAAAGTTTGTGTCACCGTGCTGTCGTCATATTCCCATG*

251 *TTGTCTATTTTCACTGGAGTGGTTTATTGAAGTACCTTGCAGCCTTGTGT*

301 *TTATTGTGGAATGTCTCTGATTTTGCGAGGACATGTGATTTGAGTTGGAA*

351 *GGGGCCATGCGTTGATGCAATTTGCTTTACGCAGCGGTAGCAAGGTGAAT*

401 *GTGTGCTGCAAAGAAGGAGGGCGCTTTTTGGTCTCTTTTTTTGACAGTCC*

451 *CTTCTTGCAGTCGCTCTAAAACTCGGATTCACCATCCCGTGCTCCCCAGT*

501 *GTAGTTGCAGTCGGTAAATATGTGGATTTGGGCCTGTGGGTTGTTGGTGT*

551 *GCAGGTCGTCTAGGCATCGCTTTCACTAAGTCACACCTCCAACAGGAATC*

601 *ATTCCTAGCTGTATCCTATATGCTCTGCGGCGTGTGCACCGTGTGCCTTA*

651 *ATGTGGACGCATGGCTATAGGCCAAATGCACCTTCTTCTCAATGTCTCTC*

701 *TGTGGGTACTTCTCTTGAGAGTAGCCATAGCATTCCCAATGCCCCTGCAT*

751 *GCCCTCTTCTTCCTGCCCCCTCTTCACTTCTGGATACATGCCCATTCCTT*

801 *TCATGGCCCCTTCTGCCTCCTCTGTGAGCTCACATCACTTCCTCTGCCCC*

851 *AGCCACATTGCCTTTCTGGCGGCCGTGTTCCTTGCGCAGTCATTCTCACA*

901 *GCTGACCTGCTGGCAGGACACATCTCTTCGCCTTCCCATCACCCTGTGAG*

951 *TATATGTCCCTGCCTCCTCTCGGCCGTGCACCCCCCTGTGTGTACCCGTT*

1001 *TCATTTCGTAGGTGTCCTCTTTGGATATTTCAAGTTACCACCCTGTAGGT*

1051 *ACAGGCCACTTCCTCAGCCTGTTTCTGCCCACCCTCTTCCATCACAGCGC*

1101 *CCCAACCAGTGAATATGAGAAATATGTCTACTATCTATGAATGAGCCAGC*

1151 *TTTGGCCATACTTGCATATTGTTTTGCATATTGTTTTGCATTGCATTGTA*

1201 *ATGGGTACGCTCCCTCGCCCTGTCAGTTTGACCCCGGGCTGTGTGTTGCG*

1251 *CGGGACAGGGGGCGTGGGGCTGTCTGCTGCCTCCTGCCTCGGTTGATGAA*

1301 *AGCTCGTGCTGGGCCTGGGCTGCTAAAATGCTTATTCATGTCTCGGTTTG*

1351 *TGTATTGCGCGTCAGACGTCTGTGAAGAGAAGCCGCTCCCCAATACACAT*

1401 *CGGCGCTGTTTTGACATCTGCCCAAAGCAGACTTGTCCCGTGCAAGGGGG*

1451 *TACATGTTTTTTTAACCTCGCTGTCTTCCCGGGTCGGTTCCTGCGACTTT*

1501 *CCTGGCGCGATGTTTTGTGCCGACCTTTCACACTTGGCGCATCTCTGTCA*

1551 *CTATTTACGTTACTAGACATGGTTTGTGTTTTGTTATCCTGCTTTGGTGT*

1601 *TTCATTTATAACTAAAAGGGTTTTCAAGTCACTGAGCAAAACGTACAGTG*

1651 *TTGTAATTTTCAGTTAGTTTTACGGTTAGCAGATGGCTCCATGCCAACAA*

1701 *CGATGCCATATTCCAGCAGTGTGCTTAAATGTAACCGCCCAGTGTGTTAT*

1751 *GGGGGGCTATGGGAGTAATAGTCTTGGTTCACTGCAACCTAACATAAAAA*

1801 *GCCACACGTTGAAATTTTGGTTTTTCGGGATGGCTGTCGAGGAGCTTAGC*

1851 *ATTCTTAGTCTGTTTTCATTATCATTTTAGTGCCTAATGAATGCACAATC*

1901 *TAACGGACACGTCTTCTCTTCCCACAG*CCTGTCTACAACAGAAGGACCGA

. . . .

2701 GTGGATGAAACGTTCAGCCCGACGAATCATGCCTACATGCAG*GTAAGATG*

2751 *CTTTCATCAAAAGGACATGTCCAGTTGTTAGATTATGCCTTATGCTAGCA*

2801 *TGGTACACATACCACCTGAGTTAGAAAATGGAGCCAGTCTAGAGTCCATA*

2851 *GACCTAATTTCATGGGCAATGGGTTTATAGGTTGAGAATCCCTGAGTTCA*

2901 *GAGTAAGTGAAGATGAGGGGTACCGGGTTTTGGAAGTTGCTGATTGTACC*

2951 *TATTGTTGAGGGCCCTCCCTTGAACCAAGGTTTAATGAGTACAGAGGGAG*

3001 *ATAGATTGTTATTAGTTAACTGAGTGTTGATAGTCATCACGTTCTTGCCA*

3051 *AGATGGCACCCGTCATTCCCTAAGCAAAGGACTCCCCACTGTGTTCCCAT*

3101 *CCATTATTAGCAATAGAAAGAGGGCTTCAACTTGTTTGTAGGCTTGGGAA*

3151 *TGCGGTCTTGTGTTTTTACTTTGTAAATATGACTGAGATTTACAACCACA*

3201 *ATTCACTCATCATGTCTTCTCTCTATTGCAG*AGCTTGCTGAAGCTGTACC

3251 ACCCGACCCGAGTG

***AxNodal-2* genome fragment (5'-3') – Intron2**

1 CCACCAACCATGCCTACATGCAG*GTAAGAAATATGGAATCTATTTGTGGA*

51 *AATATGAATAGTACACTTACACTTTCGTGTTTATAGTACTGCCTCTTTAA*

101 *AACCTTTAAAGGGAAAGTATCTGGGAAGCAGCAATTCAAGGTCTAGGGTT*

151 *ATCAATTAATTAGGAAGCTATCCTACATGGATATGGGCAGCTGTATAGCA*

201 *GTGAACATAGCATTATTATATATGGGCTACACATGCATTCTGCAAAGATA*

251 *GTGTATTTTTTAAAGAAATGAATAATCCAACAATATTATTCAAAAGGAGA*

301 *GGGGCATTTGCAAATACACATTTAGAAAGGAAGTGGCTAACACAAAAGCA*

351 *AGCATTTCAAACGCCCACCCACATATTTTTAGACACAATGAGCCAGCTTC*

401 *TCGAATCATTTTTTGGGGCTTTTTTTGGAGCAGGTCCGGAGATGCTCTAG*

451 *TACATCTCCCCACTTTTGCCAAAAGAAAGAGAAGCCAAATTAACAGCGCC*

501 *TTTTCCCCCTTTGTGCAAATTAAGGGGCGTTTCGGGGCATTTAAAAGCGC*

551 *ATCACGAATTCACAAAAGGGATGTGCTTTTAAATGCCCTGAAACGAGAGT*

601 *CATCTCCCTGTGCCTTTTGAAACATTCACAGGTGGAGGTGCAAATAATGG*

651 *CAGGGTCATTTCTGGCCTGTCTTGGTGAAATGTGCTCTGAGCAACCACGT*

701 *GGTTGCAGGGAGCGTGTCNAATGAAAGCATGCAAGGGGGCTGGACCTCTT*

751 *TGCTCTACAGTTCAGCCAATCCTGAAATGCTCAGGTCACCTTTGTGAATA*

801 *AGGTGACAGAGCATTTCTCCAAAGCAGCGAATATGAAATAAGCGATTTCT*

851 *CTTCGCTTATTTATTTGCGCTGCTTTTTGAGAATCTGGCCCAGTGGATCA*

901 *GCGCAAATATGGATACAATTACTAACTACTAAGCAAAAAACATCCAACTG*

951 *CTTCCCAAATTCTTTCGAAACCGTTCTTGATAATTGATGCCCCAGAGATT*

1001 *ACAAAGGTTTCTGTTTATGTACAGACAACACTGAAAGAAGATTTCAGGTA*

1051 *AAAGTGCATGTCGGCACATTCATAAAGTTATCAGGTAACTCTTGGTTCCA*

1101 *GGCTAGAAAAACAAGCCTAAACACACTCCTGCACAGCGGGAGGAGGCCGC*

1151 *TAATGTCTGTGCTGGAAATGCCCTGCTGCCACTGGGTGGGAGCTGCTGGG*

1201 *GCATATACTTTTATACTTTGCTAGCAGTGTTCTAAGAAAATCCCCTCTAG*

1251 *CTGTTTCTTTTTCTTTTGGGTATTGATTCGTTTTGTGAAATACAATGCTG*

1301 *ATTCCTAATTTGAATTTTCCTCTGTTAGGAAAGGCTAGTTAAATTAAATG*

1351 *TGCATATTTCACTTTAGGATACGTTCAGTCCCTATGCGTATCTTAATGAT*

1401 *CCTCTGCACTATTTTCTAGACTTAATGACATTTGAAGGCCTTGTTTTATA*

1451 *GGACTACCTCAGTACCTTTCCGGTAAATAGGCTTTGTTACGATTGCACTA*

1501 *TCCTACAGCAAAAGCACTTTGCCCTGTCTGGCCCAGGGCCAATCCCTGCC*

1551 *CTTACATCCTTGGAACGCGAAGTCGCATTAGGTATGTTTGGATTACTTAC*

1601 *CAGTGATCTTCATTCATCTTTGTCCGGCTCTTCCTCGTCACAGTACTTTA*

1651 *TTTATTTTGAGGTACCCATCTCTACAAATGATTTAGTTCACTTGGTTGGC*

1701 *TACTCCAAAATAAGCTTCACTCCTGGTATTGCTACAGCCATAATATTAAC*

1751 *AGTTACAAATCAGATATTGAATGTAAGTTTATTTGATTTACGATATTAAC*

1801 *GACTATTTTTGTGACATCATTTTCTGCTATGAAAATTAAGGGGCCCCAGA*

1851 *CATGTTTTGCCACCCGGGGCCTTGCAAATGCTAGGGCTGGTCCTCTTCAT*

1901 *TGGCAGAATTCAAGATACTATGTTGACAACTAGACGGGACTTGTGATGGT*

1951 *GGAAAATCCCACAGTGAGATTAGGCAGTATGTGTTGCCTGAGAAGAGCAT*

2001 *CTGCCCACCTTGGAAATCCGTCAGAATGTTAACCTCTGTTGCTGCCATTC*

2051 *TTTGAGGTTATAAACTCCTACTACACATTGTGTATATATTTCGCAGCCTT*

2101 *CCTTACAGACGCATAATATTTTGACAGTACTTTACAGAAGAAAACCCAGA*

2151 *AGTCCCACAGCTAATGTAACAGGGAGCATATGGGTGACAATTTTCAGAGT*

2201 *AAGTAGTACTCCCATCAGTTCCATCAGATAAATGCTGAAATCCTGAATAA*

2251 *AGGTTCTTCTTTATGACAGCAAAGAAGAAATGCAGGCATGAACCCACTGT*

2301 *GTTTTCCGGGAATCTTACCATAGTGTTGCCACGATTGTGGCAACATCACT*

2351 *AAAACTGCGCCTGAGCACGGTACGAAAATCCCAACCACCCGTTACTGCAC*

2401 *CCAAGCAGAGCNGGGGTTGCGGGGGTGTCCCCCGCTCTCCTTGGGCGCAG*

2451 *TAAAAGAGGTCGGGGTTGTGTGGGGGTGTCCCTCGCTTACCATTACAGTA*

2501 *AGCGGGGGATNAGATNACAAACAAAAAANGACTCTGCAGTGTCAGCACTG*

2551 *CCCCACGTAA*· · · · · · · · · · · · · · · · · · ·

2601 · · · · · · · *GCATGCATGGCAGTGCGCAAGCAGANCCCAGATGN*

2649 *ANTACCTGTNTCAGGTGTGGTCTATGGGGGCCCAGTGGGTTACTGCTATG*

2699 *CAAGGCTGTGTGGCCTTATAACACTCATCACATTTCTACTCCTTTTTGGA*

2749 *AATGTNCTAAATCGCACGGAAGGGGACCTTTTCACGTAAATTCTAACAGG*

2799 *CATGGTACCCATCAGTACAATTCAGGGTCTACTAGAATTACCCCCCATGG*

2849 *TATTAAAGCGCATCAATTAATACATCAGTCGAATCAGCACGTGCCTGTAC*

2899 *CCGAATGCATGGTACTGCAATAGTCCGTCTGATTTGCCATACCGAAGGGT*

2949 *TCTGTAATGTCTGCTGTGAACCATTCCTTATACAAAGTGAAAGACTGAAA*

2999 *TGCAGGCTGGTCTTGCCATAGTTCCCTGCAGAACTTTCAAAGTCTAATGA*

3049 *AATGGGAAAAGGGGCTTTATAATGCTTTGCTAGTCTACATTTCCCATCGG*

3099 *TAAATATAAGTGCTCTGTATATTTTAAGACACCTAAGCAAACATATTGCA*

3149 *AACTTTGCATAGGCAAACTGGCTGCATCATTGTGTTTCAGGCTTGATAAT*

3199 *GGTTCCCTGTCCATTTCGATTAAAAAGACAAGAATTCTATTCTCATACAT*

3249 *CCAAGTAGGGAGTCAGTGTACATTGGTTATGTAGATGCAGATAAAAGGGG*

3299 *GTCACAAGAGTTCGTGTCCCACTGAAAATGGGTTGTAGTACTTGATGGTC*

3349 *ACCACTATCCATGACGTAGGTTGGAATTGCTTATGTCTTGCGACCTTTGT*

3399 *CATAAAAGAGGCGGATTCCAACCAATGTTGGGGGATGGCATTGGAGTGTA*

3449 *AAAGCCTGGATTTGTAAAATCCCAAAACCGGATTAGCAAAGTATGACGCA*

3499 *TAGATTCTAGATATTAATTTTGATTTTCAAGCAATTACAGAAGATACTCC*

3549 *AGCTGCATTTTAGTACGCAAACCACTCAACAATATATTATTCAAAATTGT*

3599 *TAAAANCANCCATCACCTCAGTTAACCATGACTCAGCGGGCCCACACTTC*

3649 *AAAAGGGGCCCTCAATGCAGCGCACTTCCAAGAGCTTGTAAAACAAGATC*

3699 *AGAGTCTAGGTCAATCGACTCACACACGGCCTTCCGCAAGGCCTTAAAAA*

3749 *CCAAGCTCTTCATCTAAAGAGCCTGTCATTCTAGGTATGCTTTATTAATG*

3799 *TATTCACCATGTATAGGAGACTATATACCTAGACAAGTTTAGTAACCAAC*

3849 *TAGCATGCATGGCAGTGCGCAAGCAGANCCCAGATGNANTACCTGTNTCA*

3899 *GGTGTGGTCTATGGGGGCCCAGTGGGTTACTGCTATGCAAGGCTGTGTGG*

3949 *CCTTATAACACTCATCACATTTCTACTCCTTTTTGGAAATGTNCTAAATC*

3999 *GCACGGAAGGGGACCTTTTCACGTAAATTCTAACAGGCATGGTACCCATC*

4049 *AGTACAATTCAGGGTCTACTAGAATTACCCCCCATGGTATTAAAGCGCAT*

4099 *CAATTAATACATCAGTCGAATCAGCACGTGCCTGTACCCGAATGCATGGT*

4149 *ACTGCAATAGTCCGTCTGATTTGCCATACCGAAGGGTTCTGTAATGTCTG*

4199 *CTGTGAACCATTCCTTATACAAAGTGAAAGACTGAAATGCAGGCTGGTCT*

4249 *TGCCATAGTTCCCTGCAGAACTTTCAAAGTCTAATGAAATGGGAAAAGGG*

4299 *GCTTTATAATGCTTTGCTAGTCTACATTTCCCATCGGTAAATATAAGTGC*

4349 *TCTGTATATTTTAAGACACCTAAGCAAACATATTGCAAACTTTGCATAGG*

4399 *CAAACTGGCTGCATCATTGTGTTTCAGGCTTGATAATGGTTCCCTGTCCA*

4449 *TTTCGATTAAAAAGACAAGAATTCTATTCTCATACATCCAAGTAGGGAGT*

4499 *CAGTGTACATTGGTTATGTAGATGCAGATAAAAGGGGGTCACAAGAGTTC*

4549 *GTGTCCCACTGAAAATGGGTTGTAGTACTTGATGGTCACCACTATCCATG*

4599 *ACGTAGGTTGGAATTGCTTATGTCTTGCGACCTTTGTCATAAAAGAGGCG*

4649 *GATTCCAACCAATGTTGGGGGATGGCATTGGAGTGTAAAAGCCTGGATTT*

4699 *GTAAAATCCCAAAACCGGATTAGCAAAGTATGACGCATATAACAATCAAT*

4749 *CTGTATCATATGTAACCATGCAAATGTGCATAAGCGAATGTATCTAAACT*

4799 *GACCTTTGGGGCCGCTTACTGTAACCCTCAAGCAAGCCTTCTTAAAAACT*

4849 *CCTTATAGCGCCTCGTTGCCTGTGGGCTGTAGTGCGCTCTGCAAATGCTA*

4899 *AGATAAAAATAAATAAAATAAAAATAGGTGACAGCTTGATCAGAGCCAGT*

4949 *ATAATCATGTCGCACCCATGCATTCTTGCTGCTTTTTTGTCTCGGGAGGA*

4999 *TAGCTCAGCGGCAACGTGCCCGCCTTGGAAGCAGGATGACATGGAGCAAC*

5049 *ACAGGTTCGATCCCTGGGTCTGCGCTTAGAAACCTCTTGGTATTAAGCGT*

5099 *GCTATAAATAACCTATCATATCATATATGCTTTAAGCAATAGAAATATGA*

5149 *CATTTTTAATGAATGACAGTTCCAGTAATATTCTTCCTTCTCCTTTTCTT*

5199 *CTTCAG*AGTCTTCTGAAATTGTACATTCCAG

Degenerate fragments of VegT and Vg1

Sturgeon VegT (5'-3')

1 GAGATGATCATCACTAAGTCTGGNAGGNGGATGTTTCCAGCATGTAAAAT

51 CAGCGTGACTGGCCTAAATCCCAAAGTGAAATACCTGATGATGATGGACA

101 TGGTGCCCTATGATGACCATAAGCACAAGTGGAGCCGGAATAAATGGGAA

151 GTGAATGGCGAGGCTGAGCCACACCTTCCCAACCGACTGTTCATCCACCC

201 GGAGTCCCCAGCGCTAGGGGAGAAGTGGATGCAGTACCCCGTCTCCTTCA

251 ACAAGCTGAAACTCACCAACAACACACTCAACCAGAACGGCCTGGTTATT

301 TTGCACTCCATGCACAAATACCAGCCTCGTCTCCACATTGTCCAGGCGAC

351 CGATCTGTACAGCCAGCAGTGGGGCCCTTACCTCAGGTTCACTTTCCCAG

401 AAGCTGCATTCATTGGAGTCACGTCCTACCAGAACAATGAGATAACAAAA

451 CTGAAAATTGATAACAACCCTTTTGCCAGGGG

Sturgeon Vg1 (5'-3')

1 CCTTCAGTCCTGTGGAAGATATTTAATAAAAAGACAACCGCCAAGGGATC

51 CAATTCTGGTACTGAAAACGATTCTTGTAGAGTGTCAGAATTCAACGTTC

101 GTGGGAATATTGTTCGGTTTATTCAAGATCAAGGGAACGCAATTCCTGGT

151 ACAAGTCGCCAGTGTCCAATGTGTACGGAGAGGCATCTCAATTTTAATAT

201 TTCTGTTTTGGAGGAAGTTGAACAGCTGACTCTTGCTCAGCTGGAAGTAA

251 CATTCAATCGAAATTCTTACCACCGAACCAGGAACGCCAGGACCTTCAGC

301 TTGTCCCTTTATAAAGTTTTAAAGACGGCGTTAAAAGGGGTGTCTCCCGA

351 AAGCAGTCGCAAACTGCTACTGTCGCAATCCTTTGAGTCAGTTCACAAAT

401 CCATCAACTTCAACCTTACAGACATTGCTGCGACTTGGAGAGATCATAGA

451 AGGAACTTTGGGATGGTTCTCGTGATACATCCCGATCTGACTAGCGACCA

501 AGATGACCCAGTTAAAGTAATTTCTTCTGAAAACGAATTGGGTCATCCTC

551 CTCACTTTGGAGCTCAAGCTCTACTTTACACATCATTGGTGGCCGTTTCT

601 TTGAACCCACTTCAGTGCANGTCTCGAAGAAAAANGAGCGCGTATTACCT

651 CCCAGTTACACCAAGCAATGTGTGCAAACCAAGGCGACTCTACATTGACT

701 TTAAAGATGTTGGCTGGCAAGATTGGATCATTGCTCCCCAGGGATACATG

751 GCAAATNACTGTCAAGGANNATGCCCCTTTCCATTAAGTGAAAGCCTCAA

Lungfish VegT (5'-3')

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1    AAGTGGAATAAAGATAAGTGGGATGTGGCTGGGAAAGCAGAGCCGCAGCC
51   CCCTTGTCGGACATATTTCCATCCGGACTCACCAGCTCCCAGCAGCCATT
101  GGATGAAGCAGCCAGTTTCCTTCCAGAAACTCAAACTCACCAATAACACC
151  CTGGACCAACTCGGACATATCATTCTACATTCTATGCACCGCTATCAGCC
201  ACGGTTCCACGTAGTACAAGCAGATGACTTGTTCAGCGTCCGCTGGAGCG
251  TCTTTCAAACCTTCACCTTTCCCGAGACAGCTTTCACTGCAGTCACTGCC
301  TACCAGAATGACAAGATTAAAAAGCTGAAGATTGACAACAACCCTTTTGC
351  TAAAGGCTTCAGAGAACATGGATCACACATAAACCGAAACAGGTGTGGTT
401  CATCCGAGACCTGCTCAACAAAGTCTCAGAAGAGGAAAAACATAAATGAA
451  AACAGTCCAGAGCAAGAACGAGCAGATTTAAGGAGGTCCAAGTTTCTGGA
501  CGAGGAGTGCCCTGTAGAAACATCTTGCAAAGAGGAGAGGAGGAGCCCCA
551  TCGCAGTGGGCAGGTATTCACCATGGGCAACAGAACAAGATGGATCCCAT
601  GGACTGCACGTGGAGTCCCCACTCTCAGTGGAGCAGAAGGAGGCGTACGG
651  TGGGGAACAGCAAGTCCCTACACCCTCCTCCTCCTATCAGTCCTACAGGT
701  TCCAAGGTTTAAGCAAAGCCTCTAACAGTGAGTCCGTTCCTGGTGACTAC
751  AGAGGGAGAATTCCAGACATTGCCACAGTGCCTGAACAGGATGTCAAGCC
801  AACCTTGGAGAATGGTACTAACCCCTCTGCTTGCCCTCAGGCCTCTCAGG
851  ACTACTTGGGAGCAATCAACATGGCCACGGGAAAACATGGAGTCATAGGT
901  CATGTGTACAACCCATACAACACAGAGCAAGGGCTGGGCCAGTGGACTAC
951  TGCACCCCATGGCCAGTATGGGTCAGTGGGCTACACTCACCTTCCAACAG
1001 ACTACAGTGCCCAGAACGTACCCGCGTATCCCCACAGCAACATGGCGGAC
1051 TGGAGCCA
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Lungfish Vg1 (5'-3')

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1    GAGGAATTCAATGTTCCTGGAAACATTATTCGTGTTTTCCCAGATCAAGG
51   TTATTTTGTTCACAACAAGAAGCAAGAGGGTTTGAGCTGTATTGAAAAAC
101  ACATCTACTTTAACTTTTCTGTGCTGGAGAAAGATGAGCTCCTAACTATG
151  GCTCAACTAGAAATAAGACTCCGACACAATTCCTACCATCTTCCTGTACT
201  TGACCAGATCTACAACTTGAACATTTATAAGGTGTCGAAGATAACTTTAA
251  AAGGGGTCCCTACTCTCGAGTCCAATAAAAAACCTTTCATTTCACAGTCT
301  TTCAAGCTACTCCACAAATCTCTCTTCTTTAATCTCACAGACACTGCAGA
351  GACTTGGAGGAACCATAGCAATAATTATGGGCTAATCCTGGAGATTTCTT
401  TGAGCTCTGAGCAATATGTGGGAAATGCAATAACTTCATCAGATGATTTG
451  GATCATTGTGCTGTAATTCATCAGTTCCTTCAGACATCCATGCTTGTGGT
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501 ATCTTTAAATCCTCACCAGTGCAGATCATCAAGGAGAAAAAGAAATGCCT

551 TTTACCTTCCCATAACCCCTAGTAATGTTTGCAGAAGGAGAAGGCTCTAC

601 ATTGATTTTGGAGATGTTGGCTGGCAGGATTGGATCATTGCCACTCAGGG

651 ATATATGGCTAATTTTTGCCAAGGAGAATGTCCATTTCCTCTTAGTGAAA

701 GTCTAAATGGAACAAATCATGCTATCTTACAAACCTTAGTTCATTCTTTC

751 GATCCAGCAGGGGCACCACAACCTTGCTGTGTCCCCATTAAATTATCCCC

801 AATCTCAATGCTGTATTATGACAACAATGACAATGTGGTGTTGAGGCATT

851 ATGAAGATATGGTGG

Promoter sequences of AxNodal-1, AxNodal-2, AxBrachyury and AxMix

Orange: putative TCF/LEF binding site

Blue: putative T-box (VegT) binding site

AxNodal-1 promoter (5' - 3')

1 CGACGGCCCGGGGTAATACGACTCACTATAGGGCACGCGTGGTCGACGGC

51 CCGGGCTGGTCTGCATCATAAACCCATCACTGTATAATTCCTAGTGAGAG

101 GAAAAGTGGGAAGTGGGAGTTTCACTGGAGGAAATGGCAGATTACTGAAA

151 TCACTATCTCGGGTGTCTATTTTTAGGGCATGGTTACAGACGTATCCCAT

201 TGAAGGGAGGCAGACTGGACTAATGGTGTATTTTCCTGCTGGATCGATGT

251 TCACCCCCTCCCAATGCCCCCTGGTGGGGGCTGCATGAGAATGGGCTAAT

301 TGCCGGACAATGACCTTGGCACCGCTGACCCTCTTGATGGAGGAGGCGCA

351 CATCAAAGGGATGCTGGCGAGAGGGCTCTTCAAAGGCGGGGGCACGACTG

401 GTATATAGGGGCCGGCCCGGGCTCCAAGCAGCATTGAGCAGAGAGTCTCA

451 CTGGAACAAGGTTCTCCTGAGCTGTCAGGGCAAGGAGAGCCGAAGGCAGC

501 CGGCCTCCCAGCGTTCACAGCCCGACAAATACTGAGGGTCGCCCCCGCCA

551 GGAGAAGGCACACGTCTGAGAGCAGCAGCCAAGACAGTGAGCGGAGGGTG

601 CGTCCAGCCGGGAGACTTTACACCTGAGACATTTGGCTACTGAGGGTGCA

651 GCAAGCAGGCGGATTTTCGCTTTGAGGGTGCAGTCACCCTATCTCTGAGG

701 GTGCGGACAGGCAGTAAGACCTCCCAACCACTTCCGAGGACTAGTACCGG

751 CACTTGGAACGCAGAG

AxNodal-2 promoter (5' - 3')

1 GACGGCTCGGGCTGGTGTTGATTAATGTTTATTAAATGCCAATGCTCTTT

51 TGTCTGGTGCACTGTCTTCTAAGGGGAAAGGTCAATTGAATCAAACTTGT

101 GTATTTCACTTTCAGATATATACACTTGCTGTGCGTCTATTCATAGGTGC

151 CAGCTTTTGATTTTTCCGATGGGGCCAGATGTTCCCACACCTAGTGATGC

201 GAGGGAGCTGTACCTATCCCATAGTCGAAACACCACATTTTACAGCACAT

251 TTTGCAGCAGCATTGAGTAAAAAGCCATATTATTTACAGGAAGATATAAA

301 TATAACATCTCAGCAACAGTCACCTAACCTGCTCAGTTGCCGTTGCAACA

351 TTTGGTTATGGGATTCCCTGCTTATCCTTTCAATTCCTGCACCCCGCCAA

401 ACAGTTGTGGCCTGCGCACCCCCTCCAATGAAGGCTCAGTGGGTAATTGA

451 TGAGGCTCCCTTCTCTTTTTGTCTGCTACCACAAAATAAAGCCTCACTTC

501 GGATATTGCCACATAGGTAAACGTTAACAGTGACCATTCTTATACTGGAC

551 GTCACTGTGTATGTGTCTCCTGCCTCACTAATGGCAACGTGTTTTTATGT

601 TCTGTAATATCATGGGTCAGCATTTATGCAAATAAAGGGGCCCCGCACAT

651 CTTAAGCCTCACAGGGCCCCTCAAATCCTANGCCGGCCCTGACTGGGANG

701 GGAGGCGGGGGGGTTGAATGACCTTGTATCCCCTGACCTAGGGACACAAG

751 TCTCATTGCAGGCCCCTGTGTGTATTCTACAGCCAAAGCGAAACCTCGTT

801 GGAATACGCATGCCTNTTCGGGTGTCATGTTTTGTGTGTGTTTGTGTTTT

851 ACGCCCGGTAATATAAATCTCTATATCACATCTCCTGCATACGCGTTTTC

901 CCCCTTCTTTGATTTCTCTTCGCGTTGCCNTGACGCCGCCGACCTTCCAT

951 ACACGAGGGCCGACCGACTGCAACTCATTCCCGTGGCCATTTTCGAAACT

1001 GCAGCCCCAGTCTACTTAAAGCGACACCTGCGGAACTGGCTTCGCTTCTG

1051 CGGGTCACTGTTGCGAGGGGACACAATATATCCATCGCACGACGCTGCAG

1101 CTCCTAGAACGCGAGGACCAGGAACGACTGCATTTCGCCTGCGTGCTCTC

1151 CATCAGAAGTCCGTGCTGGGTGACCGATGATTCTGCAGCACTTTTCAGCG

1201 AAAATATAAAATAAATGTTTTTAAAAAACCATCACAAAGACGCTCGCTTC

1251 ATTAAACAGCGCAGTGCGTGCCATAAATGCCTCCGTCTGAACACTTTTTT

1301 GGGGTAGCCTAGGTCGGTCATATAACAGTCAATGCACTGCTGTCCTTACA

1351 GACATGAGGACTTGGTGGGTTCTGGCACCTAACTTCTGCGCTTCTTGTGC

1401 ATACACGCACAATCCTCGCCTAGTTTTGAACGCCCCTCCCACCCCATCAA

1451 AGCGCGATTTCTTAAATAGGTGTCTCCTCGCCCCAAGGGGACTGTGGCAC

1501 ACCGGGGGTCTGCCTCTCCGATTGGCCAGAGACCCTGAAAGCCACATAAC

1551 GCGGTCTCTGACACATGCCTGATGGAAGGCGGATTATAAAGCCCCAAGGG

1601 GCGCCAAGTAAGTCTCTGCCCACACCCACACTGGAGCCCCACAGGGCCAC

1651 A

***AxBrachyury* promoter (5' – 3')**

1 GGTCCAACACCATTGGTGTGCTAGAGTATGAGTAGACCAGCATTTATATG

51 TTTTGGTGTACAGCTTTGCTCCCAAAGTGGAAATAGATGTGTACTTGTCA

101 TTGATTTGGACGAGTTTGTATGTGTTCCATACCGTTGGTGAAGTAGCGTT

151 GGAAAAGCCAATAACTGATATGTTTTGGTATATAGCATTTGCTCGAAGAG

201 TGGAAATTGACCTGTGCAGGTTATTGGATGAACAAAGTTTATCTATATCG

251 TTGCACGCCAGGATTGATATGCTTTGGTCTACAGCATTCGCTCATAGTAT

301 TGAAATAGACACATGCGTATGACTAGTTTGGAAGGGTTTGTATGTGTTCT

351 AAACCGTAGGTGGTGTAGAGTTGGGGTATACCAGTAGACTGGAACTATGT

401 ATTGCTCTAAATTGTGGAGTTGGGACAGATTAGTGTATAGCATTTGATCA

451 AACTAATAGGTTTTGGTCCATACCATTAATTCTGTTTCAGTTGAAATAGA

501 AACGTGCACATCATTAGTTTGGAAGGGTGTGTAGGTGTCGTAGAGTTGGG

551 ATGGACCAGGTTTAGGGGGTTGATATTTGTTGGTCTGTAGTGTTGGCATT

601 ATAGGGTCAGAATAGACCACAGTTGATAAGTTTTGGTTTACAGCATTGAC

651 TCATCGAGTGGAAATAGACACCTGTCGATCATTGGATGGATAGGGGGTTG

701 ATATGTGTTGTGCTGGACTGTTGGCATTATAGAGTCAGAATAGACAATGT

751 TTGATATGTTTTGGTCTACAGCATTGACTCATAGAGNGGAAATAGACAAC

801 TGCCGATCATTGGATGGATAGGCGGTTGATATGTGTTGGTCTGTACTGTT

851 GGCATTATAGAGTCAGAATANGCCACGGTTGATATGTTTTGGTCTACAGC

901 ATTGACTCAGAGCGCAGAAATAGACCTCTGCAAATCTTTAATTTGGAAGG

951 GTTCATATGGGTTGGTCTACATCATAGGTGTGGTAGAGTTGGCAGAGGGC

1001 TCTGATGTTTGGGTCTATAGTATTTTCTCATAGAGTGGAAATAGAGATGT

1051 GCATACCATTGGTTGTGAAGGGTTTGCATTTCTCGGTCCATACCGTTGGC

1101 GTTGTAAAGCTGGAACAGACCAGTAGGCTGGAACTATTCATTGGTGTCAA

1151 TTGTTGGGACCATAGAGTTGGGACAGACCAGTGTATTTAGCATTTGATCA

1201 AAGGGTTATGTGTTGCTCCACAGCGTTGGTTCATATAGTGGAAATAGAAA

1251 CGTGCAAGTCATTGATTTGGAGGGGTCCATATGTGTTTTTCTTTTTTTTA

1301 TTTTTTTTTATTAGCCATTGATCAAAAATGTGATCTTATACACAAATGAC

1351 AAAAACGTCATAATAACTTATAACACGAATACATAGATAGACTTATACAC

1401 ACATGTATATACACTGGGTGTGTTAGAGCTGGGGTAGACCAGGGTTCTGA

1451 TGTTGCGGCCTATAGCATATGCTCTTAAGAGTGGAAACATACATGTGCAT

1501 ATCATTGGATTATCANGGTAATCTGTGTTGGTCTATGCCGCTGCCATTGT

1551 AAAATCAGAATATACCAATGTATAGCATTTAACAAGAGTTTTGTTTTGGT

1601 CTAATGCATTGGTTCGTAGAGTGGAAATAGACACCTGGTTCCCCCTTTCA

1651 CTCTCCAGATGTTTCGGACTAAAATTCCCACCAGCTCTAGTCAACATAGC

1701 CAATGGTTTAGGATCATGGGAGTCATAGTCCTAAACATCTGGCGAGCCGC

1751 GGCTCTCAGAGCTATGGAGAACCACTGCTTCGCGAGTGATGTGCAGACGA

1801 GTGTGGTGTCTGCCTGGCGTCTGATTATTGGTCATAGAAATAGACCCGTC

1851 TGTGAGCAGCCAATCTCTACAGATGTGTTGGGCCGATCAGCCTCTGATTC

1901 GGAGTTCTGTAAACTGGACTAGCCCCCGACTTCAGTGCCGACTACGGGTG

1951 CGCTTTGACCATGGTGAGAAGCGCGGCGGCGGGTGTGGGCTTTAAAGACG

2001 GTCCGCAGCTGCAGGGCTGCAGAAGAAGCAAGTGTCGGAGAGAGGATACC

2051 TGCTGGGGGCGGTTAGGGCAAGAGCGGAGAGGAGGGGAGGGGGCGGGGCA

2101 GGGCAGAACCAGCGTGTGAAGTGGAGCAGAGGGGCGGGGAGGGAGGCTCC

2151 CGGGGAGGGGAGATAGTAAGGTGAGGAGTGGACACACACGAAGCGGATAG

2201 AAGAGCAGGGACGAGCGCCAGGCAAGGGTGGAGCGGAGGTGCACGGGCGG

2251 CAGGGCTGTAGAGGTGCAGTTGAGCCGTGCGTGGGATTGGTGTAGAAGTG

2301 CGCAGAGTTGGAGCAGAGTTGCGGGAAAGGCGGAGAAGTGCGCAGAGGGG

2351 CTGCAGAGTGCAGGGGATCCACCGGCACCGAGGAGGATTCTGCGGAGCCC

2401 AGCCGGAAGA

AxMix promoter (5' – 3')

1 TCCGAAGGTTTCTTGACAAGAGGGTGCACAANAGAGCGCTTTAAGAGGAA

51 GGGGAAGCNTACAGTGGTGAAGAAGTTGGCCAGAGCCNGAGCGAGGGTGT

101 CAATATGGACCTTGTTGGTTTTGTACAAACAGAGAAGAACCTGTTTTGAC

151 GAGACTTTTTCNTGGTTCAGACTTGTCTATAGATCTCATCAGGGGAGAGC

201 AGAGGAAAGGAAGAGAAAGAGGGAGGAGAGGCGGATGCAGGGGGGCCTGA

251 CGTAGCNGGGGGAGAAGTGGGATTAGGTAGAGAGGGCAAGAGGGTGGACA

301 GGATGTCCTGGGTTTTAGAGATGAAGTTAGAAGCCAGGGCTGTGCAGGGG

351 GCCTTGGAGGGGGCAGGGGAAGTAGAGACTGCGGAAGGATTGGCCAGCTC

401 CTTGCCGATTTTATAGAGTTCGGACGAGTTGTTAGATGCTGNAGATACAC

451 GGGCTTGGATGTGGGCTCTCTTCTTAGTGCGGGCAGAGAGCCGGTATCGC

501 CTTTGGAGCAGGCGACGTGCCAGTTTGTCAGAGGATGCACAAGAAGCCTA

551 CCATTTCCTCTCACCCACCCTACACTCGTGTTTAATGGTGACGAGGTCAG

601 AGTTGTACCAAGAGTTGCATTTGGCTTTGGGCTTCAAACGGATCCTCATG

651 ACAGGGGCAAGAACATGAAGAGTATCAGAGATAGCAGAGTGGAGCATGGA

701 AGGGACTGTGTCAGGATGAGAGTCAGCCAGAGGTGGCATAGGGGGAGAGA

751 AAGTGGAAGCGAAAGCGGCAGCGAAAGCCTCAACCGACACGCTTCATGGG

801 CCAGATGACCGAGAAGGTAGGTGGCAGTGCTGGAGCTGGCTTGGCCTGCG

851 GGGTAGAGAGGGTGAGGTGGGAGGAAAGGGGAAAGTAATTGCTCCAGGAG
901 AGAGGAGTGATGAGAGGGTTAGAGAGGGGGAGGTCAGGGGAGATGAGAAC
951 ATCAAGATTGTGCCATGCAGTTGAGTCGGGCCAGAGGGAAGAATAGAAAG
1001 AGAGAGAGAGTCACAGAGGTCGCGAAAAAGTCCAGAGGAGGGACAGGAAG
1051 CATCCAGGTGGATATTGAGATCTTCCAGGAGGAGGAGTTGAGTGGTCGAG
1101 TCCAGGAGGGAGGAGGAGAGGTCAGCCCACTCGGAGCGGAAAGAGGTAGT
1151 CTGACCAGGTGGCCGATAGTAGCAATAGTGAAAGAGTGTGCCCGGGAGAG
1201 GGAGAAAGCCTGCAGACAAGGCATTCGAAGGATCTCTGCATGAGAGCGGG
1251 AAGATCCACACTCCTGCAACGCACTCCGAGATACAAAGATGTTGGCTTAA
1301 GTAGCGAGCTCACTCAGTTCTGGCAAGATAGCCCAAAGAGATTTTGCGCT
1351 AACAGAGAGATTAAATGTCTCTGANAATTAGGGAGAGGTAGTCTGGCTCG
1401 CCAGTGGTACAATTCAAGCTGCTACCACTGCTGTCCAGGGCTCAAAGCTA
1451 AGTCTGCAATATTGATATGGACAGCGTCCCTCACAGTCCTCGTCTAGCCG
1501 TAGCCTTGCCGTAGACTGCCAAGACAGCAGACACCTCCGCGAACGCCCTG
1551 CGTTAGAGCTCCTCACGCTGCAGGTGTAGACACAGTAGATAGACTCCCAG
1601 GAACAAAGTAGCAGTCTCCACTGCCAAGACTACAGACACCTCGCACACAC
1651 CTCCACCATAACATAGTGCACAAAAGAGTCTGATTTTTTTTACCATTGA
1701 GAAAATGTGGTTCCTTACATGATATTGTGATAAAAACCTATTTGCTCCAT
1751 TCTGTAATATCTAAGCATTTGCTCAATGTGTAAATTTGTGGAAATGTTAA
1801 TCTGTCTCCAGATCGAATACCTGTCAGATCCGGGTGTCAGGTGCATCCAT
1851 AACAGAGGACTTTGTTGCGTTCTGTTAATTTAATTTTGCGCGCTCTACTA
1901 TTTTGGAATATTTACACATTGGACCAGGCTGGGAGGACAGGAAGGGGAGA
1951 ATTTTCAGCAGAAGCAGCCTTGCTGTATAAATGATTGATATATTATTTTG
2001 TAATCTCGTGCGCAGGTCACCGTGCTCTGTGTTTCGTGCAGTTTGATGTG
2051 CGTGCCAACTTGCCGACTAGAGTAACCCGTGCTTCCAGTCCATAGAGTAA
2101 CCCGTGCTTCCAGTCCATAAATGCTGTAGGTCCATTTTCAAAATAGTGCC
2151 TCCCCTGCATCGACTTCTTTCTGGGTTTTTTTCTGGAGCGAACGAGGACT
2201 TCGTGGATCTGTTCCCAGGTCTTTAAAGGAACTGTAAACTCTGTGACGCG
2251 TGAAGATTCGGCTCAGTGGTAACATTTCTCCCCCTGACACAAGGTTGGAC
2301 GCAAACTTCAAGGGCTCTGGAGGAGCCCCAGCCACGCCAACTGAGATGGG
2351 GAATTATTTTTTATGAACAGCTAGTCGCTTGATACATTATGATTTCCCCC
2401 CTGTTATAAATTAAGAGTTTCTATTCGGCGCAGTCACCTTATAAAAAGGG
2451 GCCGTATGTCTCAGTTTCCTTATCTAAAATAAGTGGGCATTGATTAAAGC
2501 TGCCCCCCGCCTATATTAGACCTCTGTAATCCGGGGTGGGAAGTGTTTGC

2551 CAGTGGATGGCCCCTTGAATCATGTGTCTGGCGGACAGGTCGTGTCTTTG

2601 ATAGTTTGAAGTGGCCATTGGTGTTTTGGTTGGTGGGTGGGGGAGCAGGC

2651 CTTGTTTGAGCGTGCACCTTCAGGGGATGTGTATTGACACGGGCCCAGCC

2701 ACCCCATCCGCGCCTCACACAAAGACAATGTTATTGGAGGGGGATGTGTA

2751 TTTGTCTGCGGCCCCTGGGTGCTCTCGCACCCCCAGCATCCCTCCAGAGG

2801 AAGCCCTGATAATGGTGTCTGGGCGGGTTTGGCCGTCACCTCCCCGGCCC

2851 CTTGCAGTCTGCGCCCCGCAGGGAGGGCTTCGCACCTGCCGCCTTTGATC

2901 ACCTGGCGGTCTATCCGCCCCGCCTACCGCCATAAAGGGTCCCAGGAGGG

2951 CAGGCGGCCTCAAAACAAAGCTGCACCTCCAGGGAGTGGGACTTGGACAG

3001 TGTAGAACAGCAGCGGGGACAGGAAGGAAACGAGTCCGTCCGTGCCTGGA

3051 CCCTGGGGCCTGGG