Functional studies of novel genes involved in embryogenesis of the vertebrate *Xenopus laevis*

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1. Gutachter:

2. Gutachter:

3. Gutachter:

Vorsitzender des Prüfungsausschusses:

Tag der mündlichen Prüfung:

Abbreviations

amino acids

A; (Ala)	Alanine
C; (Cys)	Cysteine
D; (Asp)	Aspartic acid
E; (Glu)	Glutemate
F; (Phe)	Phenylalanine
G; (GIy)	Glycine
H; (His)	Histidine
I; (Ile)	Isoleucine
K; (Lys)	Lysine
L; (Leu)	Leucine
M; (Met)	Methionine
N; (Asn)	Asparagine
P; (Pro)	Proline
0; (GIn)	Glutamine
R; (Arg)	Arginine
S; (Ser)	Serine
T; (Thr)	Threonine
V; (VaI)	Valine
W; (Trp)	Tryptophan
Y; (Try)	Tyrosine

A	Adenosine
Amp	ampicilin
AP	Alkaline phosphatase
APC	Adenomatous Polyposis Coli
ATP	Adenosine 5’ triphosphate
ATRA	All-Trans-Retinoic Acid
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
BMB	Boehringer Mannheim Blocking Reagent
BMP	bone morphogenetic protein
bp	base pair
BSA	Bovine Serum Albumin
C	Cytidine
ca	circa
cDNA	complementary DNA
CHAPS	3-[(-Cholamidopropyl)dimethylammonio]-1-propansulphate
chordin	chd
cm	centimeter
CTP	Cytidine 5’triphosphate

Da	Dalton
DAI	dorsoanterior index
dATP	Deoxyadenosine 5'triphosphate
dCTP	Deoxycytidine 5'triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine 5'triphosphate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxynucleotide 5'triphosphate
dpc	days post conception
ds	double-strained
DTT	Dithiothreitol
dTTP	Deoxythymidine 5'triphosphate
ED	effector domain
EDTA	Ethylene-diamine tetra-acetate
EGTA	Ethylene-glycol-bis(2-aminoethylether)-N,N'-tetra-acetate
FGF	fibroblast growth factor
g	gram
G	Guanosine
gsc	goosecoid
Gsk3	glycogen synthase kinase 3
GTP	Guanosine 5' triphosphate
h	hour
H4	histone 4
HCG	human chorionic gonadotropin
HEPES	N- (2-Hydroxyethyl)piperazine-N'-2-ethane sulfonic acid
HMG	high mobility group
IFABP	intestinal fatty acid binding protein
k	kilo
L	liter
LB	Luria-Bertani
LFABP	liver fatty acid binding protein
MAB	Maleic Acid Buffer
MARCKS	myristoylated alanine-rich C kinase substrate;
MBT	midblastula transition
METRO	message transport organizer
min	minute
MLP	MARCKS like protein
MO	morpholino
MOPS	3-(N-Morpholino) propanesulfonic acid
mRNA	messenger RNA
NBT	Nitro-Blue-Tetrazolium
nr	Nodal related

O.D.	Optical Density
PBS	Phosphate buffered saline-solution
PCR	Polymerase Chain Reaction
pg	picogram
PVP	Polyvinylpyrrolidone
RE	Restriction Endonuclease
RNA	Ribonucleic acid
RNAse	Ribonuclease
rRNA	ribosomal RNA
ODC	ornithine decarboxylase
PKC	protein kinase C
PSD	phosphorylation site domain
RA	retinoic acid
RT	room temperature, reverse transcription
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SDS	Natriumdodecylsulphate
ss	single-strained
T	thymine
tBMPRI	truncated BMP receptor I
tBMPRII	truncated BMP receptor II
Tet	Tetracycline
TGF-β	transforming growth factor β
Tris	Trishydroxymethylaminomethane
U	unit of enzyme activity
UV	ultraviolet light
XCYP26	***Xenopus*** cytochrome P450 26
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranosid
XMLP	***Xenopus*** MARCKS like protein
XXBP-1	X-box binding protein 1
β-TrCP	β-transducin repeat-containing protein
M	Molar
µm	micro meter
ºC	grade Celsius
% (v/v)	Volume percent
% (w/v)	weight percent

1. Introduction

1.1 Imperative roles of Developmental Biology

The understanding of mechanisms of the embryonic development, in particular, controls of differential gene expression, has grown dramatically within last two decades. Recently two exciting topics have brought attention to the whole scientific community and, via the media, to a wider world audience: the isolation and culture of human stem cells (Shamblott ***et al.*** 1998; Thomson ***et al.*** 1998) for potential use in transplantation medicine (for review, see Tiedemann ***et al.***, 2001) and the cloning of Dolly, a sheep generated with a nucleus from an adult cell (Wilmut ***et al.***, 1997). This increase in the understanding of embryonic development and cell differentiation will have a tremendous impact on developmental and biomedical research and on the way the medicine is practiced.

In the last century, the Nobel Prize for physiology and Medicine has been issued to developmental biologists twice. Hans Spemann and his assistant Hilde Mangold were awarded this honor in 1935 for their famous organizer transplantation experiment (for review, see Trendelenburg and Grunz, 1996), and Edward Lewis, Christiane Nüsslein-Volhard and Eric Wieschaus in 1995 for their discoveries in ***Drosophila*** that led to a fundamental understanding of how genes control development in fly embryos (for review, see Lewis, 1998). Almost 80 years after the discovery of Spemann organizer, insights into developmental biology have been expanded from the cellular to the molecular level. A large number of genes involved in the early embryogenesis have been identified and characterized. And the developmental processes can be simply explained in terms of “switching on or off” gene expression. Moreover, with the completion of genome sequences of humans and of several other species, one of research attentions is now focusing on the functions of genes which are involved in developmental pathways.

The goal of this study is to isolate genes involved in early embryonic development in ***Xenopus laevis***, and furthermore to explore how these genes direct the complex developmental processes and the corresponding genetic controls.

1.2 *Xenopus laevis* as a model system for embryogenesis

Several animal model systems are used for studying early embryogenesis including ***Xenopus laevis***, ***Caenorhabditis elegans***, ***Drosophila***, zebrafish, the chick and the mouse. ***Xenopus laevis***, the South African clawed toad with large quantities of embryos year-round, turned out be a very suitable laboratory animal which could easily be maintained and induced to spawn with a simple gonadothropic hormone injection. As a potential and excellent model organism for investigating the mechanisms of development, it has large embryos which are accessible at all developmental stages, develop rapidly in simple salt solutions at ambient conditions and heal well after surgery, making possible the grafting and tissue explant experiments. An added advantage is that gain-of-function experiments can be carried out by overexpression of the injected mRNA. Despite unavailable for genetic manipulations, loss-of-function studies are practicable in ***Xenopus laevis*** by using dominant negative mutants of growth factors and receptors and transcription factors fused with the known activation or repression domains. Alternatively knockout studies in ***Xenopus laevis*** carried out with antisense oligonucleotides (Baker ***et al.***, 1990), antisense RNA (Harland and Weintraub, 1985) and morpholino (Heasman ***et al.***, 2000; Zhao ***et al.***, 2001) have been proved available. Moreover, the introduction of transgenic techniques to ***Xenopus laevis*** has exploited the potential of this model system for studying zygotic effects of the transgene expression and for characterizing the regulatory sequences of promoters (Chan and Gurdon, 1996; Kroll and Amaya, 1996). A gene trap approach based on the transgenic techniques were also carried out successfully to produce mutagenesis in ***Xenopus laevis***, leading to a possibility to identify novel genes with respect to mutant phenotypes. However, there are also some limitations of ***Xenopus laevis*** as a model of embryogenesis which should be considered. For example, it is a tetraploid species that does not favor genetic analyses. While in studying early embryogenesis, it is possible to complement deficiencies of individual systems by using a combination of different developmental models. ***Xenopus tropicalis*** is a diploid and has a much shorter generation time, which likely makes compensation for the disadvantages. The integration of unlimited information from different developmental model systems will undoubtedly provide perspectives in the elucidation of gene functions, thus aids in the understanding of

early embryonic development.

In addition, ***Xenopus laevis*** has also been successfully employed in the study of tumorigenesis which on the other hand sheds light for studying the embryogenesis. For example, the viral oncogene that encodes polyoma middle T was found to induce the formation of mesoderm in otherwise naive ***Xenopus*** tissue (Whitman and Melton, 1989). This led to the identification of the cellular proto-oncogene ***P21^{ras}*** as a key player in the endogenous process of mesoderm formation (Whitman and Melton, 1992). The continued examination of developmental events in ***Xenopus*** has helped to elucidate signaling pathways that are involved in tumorigenesis (e.g. Wnt signaling in colonrectal cancers, Pennisi ***et al.***, 1998) and has also provided mechanistic insights into functions of previously identified proto-oncogenes (e.g. the role of FRAT1 in T-cell lymphoma, Yost ***et al.***, 1998). Clearly, ***Xenopus laevis*** will become a highly effective addition to the arsenal of tools for the study of human malignancies (for review, see Wallingford, 1999).

1.3 Patterning the body plan of *Xenopus laevis* in the early embryogenesis

The establishment of body axis is by far best studied in amphibians compared to other animal models. The knowledge about the axis establishment has dramatically increased in the last two decades.

Maternal determination of the animal–vegetal axis

The ***Xenopus*** embryo exhibits prominent external animal-vegetal polarity even before fertilization. The animal pole sitting uppermost of ***Xenopus*** embryo has a heavily pigmented surface, while the vegetal pole located toward the opposite end is unpigmented and contains yolk-rich material. This polarity will influence the subsequent cleavage pattern. As many other organisms, the proper development of the ***Xenopus*** embryo depends on the asymmetrical distribution of maternal mRNAs and proteins which are preexisiting cytoplasmic factors responsible for the cell fate determination in the early embryonic development. The maternal mRNAs are localized either in the animal or vegetal pole although the ***Xenopus*** oocyte is radially symmetrical. The process of localization of these mRNAs in ***Xenopus*** oocyte occurs

during the long period of oocyte differentiation and growth, which is accompanied by the elaboration of oocyte polarity. Some of the vegetally localized mRNAs, such as ***Vg1***, ***VegT***, and ***Xwnt11***, are involved in the axial patterning and the germ layer specification. Others, such as ***Xdazl*** and ***Xcat2*** located in the germ plasma, are likely to play a role in the specification of germ cell fate (Kloc ***et al.***, 2001). The differential localization of maternal mRNAs in ***Xenopus*** oocyte follows one of two pathways: the message transport organizer (METRO or early) pathway and the late pathway (Forristall ***et al.***, 1995; Kloc and Etkin, 1995; Kloc ***et al.***, 2001). mRNAs that follow the METRO pathway can be first detected at the mitochondrial cloud in stage I oocytes. Subsequently the localized mRNAs are translocated to the cortex within the period of late stage I or early stage II, where they remain throughout the oogenesis. Several METRO-pathway mRNAs have been identified as possible candidates of cytoplamic determinants, including ***Xwnt-11*** required for the β-catenin pathway that specifies the dorsal identity of embryo (Ku and Melton, 1993) and ***Xcat2*** involved in germ cell development (Mosquera ***et al.***, 1993). mRNAs following the late pathway are excluded from the mitochondrial cloud and are found throughout the cytoplasm in stage I oocytes. Between late stage II and early stage III, late-pathway mRNAs localize to specific domains of the vegetal hemisphere including a crescent-shaped region in proximity to the nucleus (Chan ***et al.***, 1999), a wedge-shaped structure in the vegetal cytoplasm (Kloc and Etkin, 1995), and the vegetal cortical region (Melton, 1987). ***Vg1***, a member of the transforming growth factor-β (TGF-β) protein super-family which follows the late pathway, can induce dorsal mesoderm and secondary axis (Dale ***et al.***, 1993; Thomsen and Melton, 1993). ***VegT***, a T-box transcription factor whose localization belongs to the late pathway too, is involved in the specification of both endoderm and mesoderm (Zhang and King, 1996; Kofron ***et al.***, 1999). Similar to ***Vg1*** and ***VegT***, ***Xenopus Bicaudal-C*** (***xBic-C***) mRNA also localizes in vegetal cortex (Wessely and De Robertis, 2000).

Specification of the dorsoventral axis

The dorso-ventral axis of ***Xenopus*** embryo is trigged by sperm entry. An unfertilized ***Xenopus*** egg is radially symmetrical along the animal-vegetal axis. Upon fertilization, the plasma membrane and the cortex in the egg rotates about 30º relative to the rest of the cytoplasm that remains stationary. The dorsoventral axis is

defined by the site of sperm entry in the animal region which marks the future ventral side of the embryo and overlaps with the first cleavage plane that divides the egg bilaterally into right and left halves.

The crucial developmental consequence of cortical rotation is the formation of a signaling center in the dorsal vegetal region on the side opposite sperm entry. This signaling center is known as the Nieuwkoop center, which, lying on the dorsal vegetal region of embryo is active from the early cleavage stage to the late blastula stage. It exerts a special influence on surrounding tissues to induce dorsal axial structures. A proposed function of the Nieuwkoop center is to induce the cells immediately above the equatorial region to form the dorsal signaling center—the Spemann organizer.

Several dorsal determinants with the ability to induce a complete secondary axis are involved in the Wnt/β-catenin signaling pathway, such as siamois and β-catenin. During the embryonic development, in the absence of Wnt signaling, β-catenin is continuously phosphorylated by the glycogen synthase kinase-3 (GSK-3) complex. Afterwards the phosphorylated β-catenin binds to a protein called β-transducin repeat-containing protein (β-TrCP), and is then modified by the covalent addition of a small protein called ubiquitin. β-catenin tagged with ubiquitin finally is degraded by proteosome via a ubiquitination-dependent pathway (Figure 1-1). The resulting low level of β-catenin cannot induce target genes expression. Adenomatous Polyposis Coli (APC) and Axin protein family normally facilitate the addition of phosphate groups to β-catenin by GSK-3. The activity of GSK-3β can be inhibited by GSK-3β binding protein (GBP), which however, is not a linear component of this pathway. In contrast, when a secreted Wnt molecule is recognized by a transmembrane receptor of the frizzled family, a cytoplasmic component called Dsh is activated. Dsh in turn suppresses the phosphorylation of β-catenin imposed by GSK-3β and therefore leads to a blockage of subsequent ubiquitination. This results in an accumulation of β-catenin in the cytoplasm. Thereafter β-catenin translocates into the nuclei and interacts with members of the high mobility group (HMG)-box transcription factor family, such as Tcf/Lef1, to activate expression of target genes (Cadigan and Nusse, 1997). In ***Xenopus***, genes responsible to dorsal determination, such as ***siamois*** (Brannon ***et al.***, 1997), ***twin*** (Laurent ***et al.***, 1997), the TGF-β factor ***Xnr-3*** (McKendry ***et al.***, 1997) and ***cerberus*** (Nelson and Gumbiner, 1999) have been shown to be inducible by β-catenin.

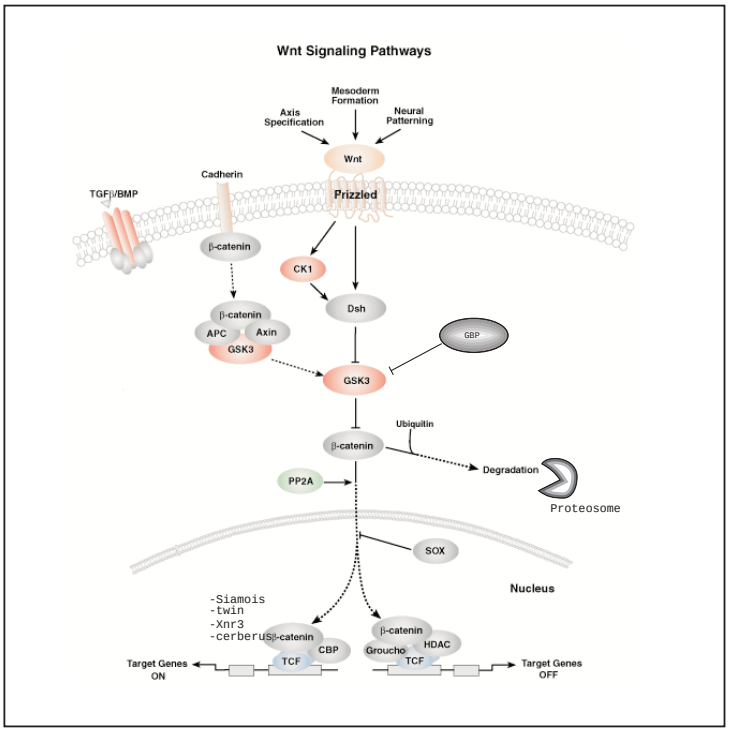


Figure 1-1. **The schematic diagram of Wnt/β-catenin signaling pathway in the dorsoventral axis patterning.** In the absence of Wnt signaling, β-catenin is continuously phosphorylated by GSK-3 complex, and subsequently degraded by proteosome via a ubiquitination pathway. In the case that a Wnt ligand binds to frizzled receptor, the Wnt receptor transduces the signal through Dsh, resulting in the inhibition of GSK-3. The suppression of GSK-3 activity leads to the accumulation of β-catenin. β-catenin thereafter translocates to the nucleus, and activates the transcription of targets genes including *siamois*, *twin*, *Xnr3* and *cerberus*. In addition, the GBP can also inhibit GSK-3 activity but is not a linear component of the pathway (Modified from the protocol of Cell Signaling Technology).

The functions of Spemann organizer during embryogenesis

As stated before, the Spemann organizer, lying on the dorsal blastopore lip of gastrula, is induced by the Nieuwkoop center. Signals originating from the Spemann organizer are involved in further patterning along both the anterior-posterior and dorso-ventral axes and inducing the central nervous system of the embryo.

In *Xenopus*, the organizer is formed as a consequence of signaling by TGF-β and β-catenin (Harland and Gerhart, 1997; Heasman, 1997; Niehrs, 1999, 2000). Transplantation a dorsal blastopore lip to the ventral side of the blastocoel of another embryo at the same stage led to an embryo containing a secondary body axis (Spemann and Mangold, 1924). Meanwhile several genes specifically expressed in the organizer have been identified, which can be primitively classified into four groups based on the expression regions (for review, see Chan and Etkin, 2001). The

first group of organizer genes is transcriptional targets of the Wnt/β-catenin pathway, including ***siamois*** and ***twin***. They are expressed in the dorsal vegetal region before the appearance of dorsal lip during the blastula stage. Overexpression of either ***siamois*** or ***twin*** induces ectopic secondary axis with a complete head (Lemaire ***et al.***, 1995; Laurent ***et al.***, 1997). The second group of organizer genes is expressed in the prechordal mesoderm region of head organizer at the gastrula stage and most of them can generate ectopically incomplete secondary axes lacking anterior structures. The members of this group include genes encoding transcription factors (***gsc***, ***Xlim-1***, ***Xanf-1*** and ***Xotx2***), growth factor antagonists (***noggin***, ***chordin***, ***follistatin***, ***frzb***, ***dickkopf-1*** and ***Xolloid***), as well as growth factors ***Xnr1-4*** and anti-dorsalizing morphogenetic protein (***ADMP***). The third group of organizer genes is expressed in the anterior endomesoderm, such as ***Xnr-1***, ***-2***, ***-4***, ***Xblimp*** and ***Xhex***, which can regulate specific genes expression in the anterior endomesoderm. Some members of this group such as ***cer*** and ***dkk-1*** (Glinka ***et al.***, 1998) can produce ectopic head without a trunk by overexpression. The last group of organizer genes is expressed in the chordamesoderm (trunk organizer), which includs ***chd***, ***noggin***, ***dkk-1***, ***Xnot2*** and so on. Because of the overlap of expression domains, a gene may be classified into two groups simultaneously. It should be emphasized that the organizer tissue is not a constant population of cells but rather a dynamic structure in which considerable cell movements and rearrangement take place during the gastrulation (Chan and Etkin, 2001).

Specifically, the organizer is a source of secreted antagonists that bind to growth factors in the extracellular space and prevent them from binding to their cognate receptors. Briefly the antagonists can be classified into the following groups (De Robertis ***et al.***, 2000): (1) BMP antagonists such as chordin, noggin and follisitatin. Chordin and noggin bind to BMPs directly in the extracellular space, preventing BMPs from binding to and signaling through its cognate BMPs receptors. The follisitatin binds to BMPs via different mechanisms and the resulting complex can then bind to the BMP receptor but no further available signals arise; (2) Wnt inhibitors encompassing the Frzbs and the Dkks two types. Frzb-1, containing a domain similar to the Wnt-binding region of Frizzled Wnt receptors, can bind to Wnt ligands and thereafter antagonize their activities. And DKKs, a new class of Wnt antagonists, cooperate with Frzb-1 in the formation of head structure by combining inhibitions of BMPs signaling. There are evidences that the Wnt antagonists expressed by the

Spemann organizer act as different biological activities. For example, overexpression of ***crescent***, a frzb type protein, causes cyclopia, whereas overexpression of ***frzb-1*** leads to enlarged eyes, indicating that different Wnt antagonists bind to overlapping but distinct sets of Wnt signals; (3) Cerberus protein, a multivalent antagonist that can bind to Xnrs, Xwnt-8 and BMP-4 in the extracellular space. These three signaling pathways are required for trunk development. And secretion of cerberus by anterior endoderm serves to maintain a trunk-free zone so that the head territory can develop (De Robertis ***et al***., 2000); (4) TGF-β/Nodal receptor antagonists including Antivin/Lefty that has been isolated from frog, fish and mouse. Mouse mutants lacking Lefty-2 form excess mesoderm, a phenotype that is partially suppressed by heterozygosity for nodal, suggesting that the main function of Lefty-2 is to down-regulate Nodal signaling. In ***Xenopus***, Xnr-3 (a divergent Xnr) lacks mesoderm-inducing activity but can induce neurulation instead. Yet it is not determined so far that if Xnr-3 can also function as a competitive inhibitor of TGF-β receptors.

Taken together, the organizer is crucial for the proper early embryonic development and functions of three important aspects: self–differentiation, morphogenesis and induction. Cells of the organizer eventually differentiate a variety of mesodermal and endodermal tissues. Mesodermal derivatives include the notochord and prechordal plate head mesoderm (head mesenchyme and cartilaginous rods of the skull floor), whereas endodermal derivatives include pharyngeal endoderm (containing gill slits) and anterior gut tissues such as liver. Organizer-dependent morphogenesis consists of not only the movements of organizer cells but also the movements they induce in neighbors, especially in the somatic mesoderm. The organizer's inductions are often called primary induction to distinguish them from secondary and tertiary induction occurring after the gastrulation. Organizer releases inductive signals which primarily affect all three germ layers including dorsalization of the mesoderm, neural induction of the ectoderm and anteriorization of the endoderm (Harland and Gerhart, 1997), although not all organizer's factors play dorsalizing roles in early development such as ADMP (Moos ***et al.***, 1995).

The formation of the Spemann organizer and its functional activities are summarized as a model shown in Figure 1-2.

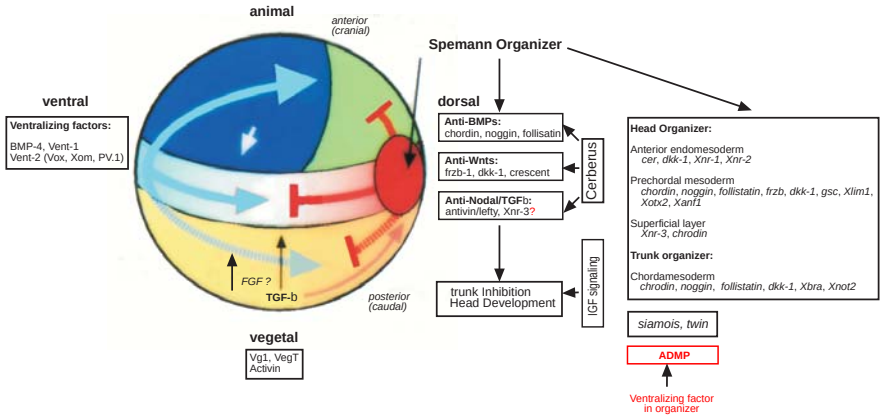


Figure 1-2. A model of the Spemann organizer formation and its functions. At the midblastula stage, higher β-catenin level on the dorsal side of embryo, together with the vegetally located transcription factor VegT and the maternal TGF-β family growth factor Vg1, generate a gradient of Nodal-related molecules expressed in the endoderm, in turn, this gradient induces the formation of overlying mesoderm; low dose of Nodal-related molecules leads to the formation of ventral mesoderm, whereas high dose leads to the establishment of the Spemann organizer. At the gastrula stage, the organizer secretes a cocktail of factors that refine the initial patterning. The organizer secrets proteins that bind to different growth factors in the extracellular space and block signaling through the cognate receptors. Crescent, frzb-1 and dkk-1 are Wnt antagonists. Cerberus is a multivalent inhibitor that antagonizes Xwnt-8, Xnrs and BMPs. Chordin, noggin and follistatin bind to and inhibit BMPs. Lefty/antixin blocks nodal signaling through a different mechanism, i.e. by binding to the TGF-β/Nodal receptor and acting as a competitive inhibitor. The organizer consists of two of structural regions, head organizer (prechordal mesoderm, and anterior endomesoderm), and trunk mesoderm (chordamesoderm). The organizer specific genes can be classified into four groups according to their expression territories (indicated in the figure). Head development is considered a default state, resulting from double inhibition of Wnt, BMP (dkk-1, Glinka *et al.*, 1998) or triple inhibition of Wnt, BMP and Nodal signal (cerberus, Piccolo *et al*., 1999), New evidence indicated that the IGF signaling is required and sufficient for head formation (Pera *et al*., 2001). (Modified from Grunz, 1997).

The patterning of the three germ layers

The basic body plan of *Xenopus* is derived from three germ layers, i.e. the endoderm, ectoderm and mesoderm. The endoderm arises from most of yolky vegetal region of the embryo. The ectoderm is originated from the animal hemisphere and then differentiates into epidermis and the future nervous tissue depending on the signal gradient. The mesoderm is formed from the marginal zone. During the gastrulation, the marginal zone moves into the interior through the dorsal blastopore and becomes subdivided along the dorso-ventral axis of blastula. The most dorsal

mesoderm gives rise to the notochord, followed ventrally, by somites (which gives rise to muscle tissue), lateral plate and blood island. The dramatic change depends on the molecular events that underlie both the specification and patterning of the germ layers.

Fate-mapping analysis in ***Xenopus laevis*** in the 32-cell stage has indicated that individual blastomeres can contribute to several organs (Wetts and Fraser, 1989; Moody, 1987; Dale and Slack, 1987). The endoderm arises from the yolky cells of tier D in the vegetal region of 32-cell embryo. These vegetally derived blastomeres later form the lining of the gastrointestinal and respiratory tracts, liver and pancreas (Chalmers and Slack, 1998; Wells and Melton, 1999). Many factors have been proved to be involved in endoderm differentiation. For example, the maternal determinant VegT is essential to endoderm differentiation (Lustig ***et al.***, 1996 Stennard ***et al.***, 1996; Zhang and King, 1996; Horb and Thomsen, 1997). Antisense oligonucleotides complementary to ***VegT*** mRNA inhibit endoderm differentiation in embryos (Zhang ***et al.***, 1998). Overexpression of ***VegT*** in the prospective ectoderm activates endodermal genes including ***Xnr-2***, ***Xsox17*** and ***Mix-1***. VegT and perhaps also other maternal determinants can activate cell-autonomous expression of the endoderm-specific genes, such as ***Xsox 17***, ***Mix-1*** and TGF-β related growth factors (***derrière***, ***activin B***, ***Xnr-1***, ***-2***, ***-4***). The TGF-βs activate further expression of endoderm-specific genes and reinforce the expression of TGF-β members in the vegetal region. The treatment of animal cap with activin or processed Vg1, a member of TGF-β, induces expression of the endoderm marker, ***XlHbox8*** and ***IFABP*** (Wright ***et al.***, 1989; Gamer and Wright, 1995; Henry ***et al***., 1996). Maternal Vg1 induces expression of ***Mixer*** (Henry and Melton, 1998), which can in turn activate the endodermal markers ***XlHbox8***, ***IFABP***, ***LFABP*** and ***edd*** in the prospective ectodermal tissues in the absence of mesoderm. Both ***Xsox17***α and ***Xsox17***β (Hudson ***et al***., 1997) have been shown to be able trigger endodermeral differentiation in the prospective ectoderm.

The blastomeres from tier B and tier C of 32-cell embryo contribute to the prospective mesoderm lineage, which is further patterned into dorsal, intermediate, and ventral mesoderm derivatives (Dale and Slack, 1987). Cells from the dorsal marginal zone contribute to dorsal mesoderm including head mesoderm, notochord, and somites, whereas cells from the ventral marginal zone form somites and blood

cells. Specification studies show that the lateral marginal zone gives rise to intermediate mesoderm, such as somites and pronephros, only as a result of interaction between the dorsal and ventral mesoderm, a process known as dorsalization.

The “two-step” model has been employed to elucidate the mesoderm induction in ***Xenopus laevis***. According to this model, at the mid blastula stage higher level of β-catenin on the dorsal side of embryo together with the vegetally located transcription factor VegT and the material TGF-β family growth factor Vg1 generate a gradient of Nodal related molecules expressed in the endoderm, in turn this gradient induces the formation of ventral mesoderm, whereas high doses lead to the establishment of the Spemann organizer. The Nieuwkoop center is the very region of dorsal endoderm that induces organizer tissue. And at the gastrula stage, the organizer secretes a cocktail of factors that refine the initial patterning (De Robertis ***et al.***, 2000). This model in principle is identical to the ‘three-signal’ model described elsewhere (Munoz-Sanjuán and Hemmati-Brivanlou ***et al.***, 2001)

Some signaling pathways and molecules are involved in the mesoderm pattering. Activins can induce the formation of different mesodermal derivatives in the prospective ectodermal tissue including both dorsal and ventral mesoderm in a dose-dependent fashion (Grunz 1983; Smith ***et al***., 1995). In contrast to activin which is required for the activation of mesodermal gene expression, FGF is required only for maintaining the expression of these genes but not for the initial mesoderm introduction (Isaacs ***et al.***, 1994; Schulte-Merker and Smith, 1995). Vg1 is also a participant for dorsal mesoderm formation (Joseph and Melton, 1998). Overexpression of ***Xnr-1*** and ***Xnr-2*** induces both dorsal and ventral mesoderm in the prospective ectodermal tissues. Organizer genes such as ***noggin***, ***chordin***, and ***siamois*** induce the dorsalization of ventral marginal zone tissue explants.

Xbra can induce ***VegT*** and ***Eomesodermin*** (***Eomes***) (Smith ***et al.***, 1991; Ryan ***et al.***, 1996). Overexpression of ***Xbra*** lead to the formation of ventral mesodermal cell types in the prospective ectoderm. While overexpression of zygotic ***VegT*** or ***eomes*** induces both dorsal and ventral mesoderm in the animal cap assay in a dose-dependent manner. Another TGF-β member, ***derrière***, can activate both dorsal and ventral mesodermal marker in the animal cap assay (Sun ***et al.***, 1999). In addition to TGF-βs and FGF, ***BMPs*** overexpression induces ventral mesoderm formation in

prospective ectodermal tissues including blood and mesendchyme (Dale and Wardle, 1999). A high level of BMPs in the ventral marginal zone induces blood which is the most ventral mesoderm indicated by the expression of both ***Xvent-1*** (Gawantka ***et al.***, 1995) and ***Xvent-2*** (Onichtchouk ***et al.***, 1996). A lower level of BMPs in the lateral marginal zone leads to muscle formation and only induces the expression of ***Xvent-2***.

Wnt signaling is involved in patterning the mesodermal layer. The activin/Vg1 and the Wnt pathways have been shown to positively and cooperatively regulated/induce organizer genes such as ***goosecoid*** and ***chordin*** (Crease ***et al.***, 1998; Harland and Gerhart, 1997). However, the function can be divergent in the different cell-stages. In the dorsal side, the ***Wnt*** is inhibited by ***cer*** and ***dkk-1***, and in the ventral marginal zone, the ***sizzled*** activated by ***BMP-4*** may restrict Wnt signaling to the lateral region (Salic ***et al.***, 1997; Marom ***et al.***, 1999). Therefore, in the marginal zone Wnt signaling is eliminated in the dorsal and ventral mesoderm by different Wnt antagonists. As a consequence, Wnt signaling is active only in the lateral marginal region to specify the formation of lateral mesodermal derivatives.

Blastomeres from tier A of a 32-cell embryo give rise to the prospective ectoderm, which differentiates into neural in the dorsal side and epidermal lineages in the dorsal side (Chang and Hemmati-Brivanlou, 1998). At the gastrula stage, the dorsal ectoderm in proximity to the organizer develops into the neural plate and the ventral ectoderm gives rise to the epidermis. The cement gland, various placode and neural crest are formed at the boundary between the neural plate and the epidermal region.

It has been shown that BMPs signaling mediates the decision between neural and epidermal cell fates (Grunz and Tacke 1989, 1990; Wilson and Hemmati-Brivanlou, 1995). Currently, the view for neural specification in vertebrates is that in the absence of BMPs signals, ectodermal cells will differentiate into neural tissue according to the ‘default model' (for review, see Weinstein and Hemmati-Brivanlou, 1999). It has been proposed that different levels of BMPs generate a morphogen gradient across the dorsal-ventral axis to specify different cells in the ectoderm. A low level of BMP-4 induces neural markers expression, while intermediate and high doses activate cement gland and epidermal keratin expression, respectively. A number of organizer genes including ***chordin***, ***noggin***, ***follistatin***, ***Xnr-3***, ***cer*** and ***dkk-1*** can induce prospective ectoderm to adopt a neural fate by exerting a dorsalizing activity. In addition, in spite of the controversial evidence that the FGF signaling is not required to the neural induction but involved in the posterior mesoderm formation (Kroll and

Amaya, 1996; Holowacz and Sokol, 1999), FGF signaling has been suggested to be involved in the neural induction and the anterior neural patterning (Hongo ***et al.***, 1999).

1.4 Gene identification from a cDNA library

Isolation of new genes and the understanding of these genes function and the corresponding genetic controls in the processes of embryonic development are key themes in the field of developmental biology.

Under this direction, cDNA libraries are constructed for different purposes. For identification of genes expressed temporally, the mRNAs from embryo at a certain stage are employed. Accordingly oocyte library (I. Dawid), gastrula cDNA library (C. Niehrs), neurula library (C. Niehrs), tailbud stage 24-26 cDNA library (J. B. Gurdon) and tailbud stage19-23 library (R. M. Harland) have been constructed for ***Xenopus laevis***. For isolation of tissue specific genes, the tissue specific mRNAs are employed. cDNA libraries of adult retina (J. Besharse) and adult heart (R. M. Harland) have been constructed for ***Xenopus laevis*** (Group leaders are indicated in the parentheses). Furthermore, for identification of genes which are only expressed temporally on a certain region of embryo, cDNA libraries are constructed rather specifically. For example, to isolate head organizer genes, an anterior endomesoderm (AEM) cDNA library was constructed for which the mRNAs preparation were from the AEM of late gastrulare/early neurulae (Shibata ***et al.***, 2001). Theoretically the rarest mRNA sequence from any tissue is likely to be represented in a cDNA library containing 10^7 recombinants; however, practically its identification is very difficult. Thus, for a variety of purpose, it is advantageous to prepare the normalized cDNA library. Based on this consideration, the normalized cDNA libraries for ***Xenopus*** early gastrula, neurula and tailbud have been constructed, which bring the frequency of each clone within a narrow range (Kitayama ***et al.***, 2001 GeneBank cDNA library list).

Screening cDNA library has been proved to be an efficient approach to identify genes involved in ***Xenopus*** development (Gawantka ***et al***., 1998). Successful screening requires a straightforward assay that can be scaled up for a high throughput. To date, mainly there are two methods used to screen ***Xenopus*** cDNA

libraries. One is the functional screen based on the phenotype shift after microinjections to embryos with mRNA from a single clone or clones pool. The potential promising clones are justified by morphologic changes of the injected embryos. ***Mixer*** (Henry ***et al.***, 1998) was identified in this way. The other approach to the study of embryonic patterning at large-scale is to use a randomly isolated cDNA and analyze expression pattern of the mRNA by ***in situ*** hybridization which allows direct access to the DNA sequence and reflects endogenous genes expression. The expression pattern often implies important clues for assessing the gene function and makes readily testable predications. Moreover, analyses of genes expression have led to spectacular discoveries regarding embryonic patterning which had not been revealed by morphology or experimental embryology (Levin ***et al.***, 1998). Several genes involved in the mesoderm pattern such as ***Xvent-1***, ***Xvent-2*** and ***Xblimp-1*** have been identified by this method. The combination of these two methods was also exploited to identify genes involved in organogenesis (Grammer ***et al.***, 2000). In this approach, to assay maximally the activities of injection, the injected embryos were monitored throughout early embryo and tadpole stages, which was followed by the analyses of internal development of multiple tissues and organs by ***in situ*** hybridization with markers—such as ***nrp-1*** (general neural marker), ***krox-20*** (hindbrain marker), ***Hoxb9*** (spinal cord marker), ***MyoD*** (somite marker for stage 21 embryo), ***Pax-8*** (kidney marker), ***Nkx-2.5*** (heart marker) and ***α-globin*** (blood island marker for stage 28). Clones which profoundly affect cell fate and embryonic were further characterized.

Some other new approaches have also been developed to identify secreted signaling molecules that play very important roles during the embryonic development. For example, ***Xenopus Kielin*** (Matsui ***et al.***, 2000) has been isolated from a signal trap cDNA library with yeast selection system. Alternatively, ***crescent*** was isolated as follows. The colonies pool from cDNA libraries constructed in cytomegalovirus (CMV) promoter-based expression plasmide was expressed in 293T cells grown in serum free medium containing ^{35}S methionine and cysteine, and positive clones were individualized in a second transfection step by sib-selection after the antoradiography detection of proteins secreted into the culture medium (Pera and De Robertis, 2000).

1.5 In this thesis

Activin plays important roles in mesoderm and endoderm patterning during the embryonic development. The cells from disaggregated animal caps which have been treated with low concentration of activin will differentiate into mesoderm, while with higher concentrations of activin, the cells will even differentiate into endoderm cell (Grunz 1983, Ariizumi ***et al.***, 1991). A cDNA library prepared with activin treated ectoderm under this direction would be more efficient to isolate genes involved in the mesoderm and endoderm patterning.

In this study, I have screened such an activin-treated ectoderm cDNA library of ***Xenopus laevis*** using the approach of large-scale whole-mount ***in situ*** hybridization introduced above. 51 new genes were isolated, and subsequently were assigned accession numbers by GeneBank. The expression patterns of ***Xenopus X-box binding protein 1*** (***XXBP-1***), ***Xenopus MARCKS-like protein*** (***XMLP)*** and ***Xenopus L-Arginine:glycine amidinotransferase*** (***XAT***) were potentially interesting. Therefore their functions have been further characterized.

XXBP-1 and the BMP-4 signaling pathway

As stated before, in ***Xenopus*** the patterning of the embryonic ectoderm has been suggested to be under the control of bone morphogenetic proteins (BMPs) (Weinstein and Hemmati-Brivanlou, 1997). Disruption of BMPs feedback loop in ectoderm initiates the process of neural induction, resulting in the suppression of epidermal-specific genes and the activation of regulatory and structural genes specific to the neural tissues (Hemmati-Brivanlou and Melton, 1997). During gastrulation, dorsalizing factors such as ***noggin*** (Zimmerman ***et al.***, 1996), ***Xnr3*** (Smith ***et al.***, 1995), ***chordin*** (Sasai ***et al.***, 1994), Cerberus (Bouwmeester ***et al***., 1996) and ***follistatin*** (Fainsod ***et al.***, 1997) antagonize BMPs signaling by binding to extracellular BMPs and preventing their interaction with cognate BMPs receptors. Without organizer signals, the ectoderm becomes epidermis, in part due to the activities of BMPs.

In addition to acting as a negative regulator of neural induction, BMPs are involved in the mesodermal patterning. The mesoderm patterning starts prior to the overt

gastrulation and can be defined into at least four mesodermal territories in the early gastrula stages: (1) the organizer expressing genes such as ***gsc***, ***Xnot***, ***chd*** and ***noggin***; (2) a dorsolateral territory expressing genes such as ***Xmyf5 and Xvent2***; (3) a ventrolateral territory marked by the expression of ***Xvent-1*** and ***Xvent-2*** and (4) a ventral territory expressing ***Xvent-1***, ***Xvent-2*** and ***sizzled***. The expression of these genes is regulated by BMP-4 and overexpression of ***BMP-2***, ***-4*** or ***-7*** leads to the ventralization of early mesoderm (Dale and Jones, 1999).

BMPs also play roles in limb development, tooth development and the regulation of apoptosis. The effects diversity depends on its intracellular co-factors that participate BMP signaling transduction, as well as crosstalk between BMPs signaling and other signaling pathways. In general BMP-2, -4 or-7 dimers bind to the type II receptor primarily, which then leads to phosphorylation of the type I receptor. This initiates a signal transduction cascade that is mediated by a family of proteins collectively known as Smads (Massagué, 1996). The activated BMPs type I receptors in turn phosphorylates an appropriate R-Smad (Smad1, -5 or -8). This phosphorylation then enables the R-Smad to complex with the Co-Smad (Smad4), and the R-Smad/Smad4 complex enters the nucleus to activate or repress target genes depending on which nuclear cofactors are present. Thus an extracellular signal is converted to a cellular response (for review, see von Bubnoff and Cho, 2001). In ***Xenopus*** embryos, Smad1 and Smad5 can mimic many BMPs functions and activate BMPs-inducible genes (Graff ***et al.***, 1996; Suzuki ***et al.***, 1997), while overexpression of ***Smad8*** phenocopies the effect of blocking BMP-4 signaling, leading to the induction of a secondary axis on the ventral sides of intact embryos and the neural induction in ectodermal explants (Nakayama ***et al.***, 1998).

A large number of genes have been shown to be involved in BMPs signaling in different cell types and tissues. In this study, it is demonstrated that ***XXBP-1***, a basic leucine zipper transcription factor, is involved in BMP-4 signaling pathway. Overexpression of ***XXBP-1*** leads to the ventralization of early embryos. Moreover, the overexpression can rescue the neural induction in ectodermal explants as well as the dorsalization in intact embryos imposed by a dominant negative BMPs receptors (tBMPRs). These results suggest that ***XXBP-1*** acts the downstream of BMP-4 receptors by mediating BMP-4 signaling pathway in the epidermal induction and the inhibition of neural differentiation. Furthermore, it has been characterized as a transcription activator by exploiting repressor and activator fusions.

XMLP and the MARCKS family

Phosphorylation of intracellular substrates by protein kinase C (PKC), which composes a family of diacylaglycerol-activated and Ca^{2+}-dependent serine/threonine related protein kinases, is an impetus for a wide range of cellular processes including differentiation, mitogenesis, neurotransmission and hormone secretion. Up to now at least 10 subtypes of this family have been found and each subtype has shown different enzymological properties and distinct cell type distribution (for review, see Nishizuka, 1984, 1995). In ***Xenopus***, it has also been demonstrated that PKC is involved in the response to endogenous inducing signals during neural induction (David ***et al***., 1987; Otte ***et al.***, 1988; for review, see Grunz, 1999a). One of the most prominent intracellular substrates for PKC is the myristoylated alanine rich C kinase substrate (MARCKS), which can be phosphorylated in many cell types by PKC. The members of MARCKS share three conserved domains, a myristoylated consensus following the glycine residue at position 2 in the amino terminus, the site of intron splicing, and the phosphorylation site domain (PSD), also called effector domain (ED) which contains three of four serines that are the only residues known to be phosphorylated by PKC in this domain. MARCKS related proteins have also been identified in mouse, rabbit and human, sharing striking similarity with MARCKS. They were termed as MARCKS like protein (MLP), also known as F52 or MacMARCKS. MARCKS is a ubiquitous 32-kDa protein, whereas MLP is mainly expressed in brain and reproductive tissues (Aderem, 1992; Blackshear, 1993).

MARCKS behaves as a PKC substrate and binds to calmodulin in a Ca^{2+}-dependent manner. However, there is no direct evidence that the protein contains a Ca^{2+} binding domain. Additionally it was also demonstrated that dephosphorylated MARCKS could bind to and cross-link filamentous actin ***in vitro***. Both functions are correlated with the PSD domain (for review, see Blackshear, 1993).

It has been reported that the expression of MARCKS increased sharply when Swiss 3T3 cells escaped from cell cycle and entered G0 (Herget ***et al.,*** 1993). Reduced expression of MARCKS has been described in various cell lines after oncogenic or chemical transformation. On the other hand, overexpression of the MARCKS in human tumor-derived choroidal melanoma cells (OCM-1) can cause the down-regulation of cell proliferation with high percentage of cells arrested in G0-G1 phase (Manenti ***et al.***, 1998). However the effect of MARCKS on the cellular functions is not yet well understood and even less for MLP. It is assumed that MLP has a

similar function as MARCKS because both are sharing high sequence homology. Recent gene knockout studies have indicated that, at least in mice, MARCKS is essential for the normal development of the central nervous system and postnatal survival (Stumpo ***et al.***, 1995). MARCKS gene knockout mice exhibited lethal neural defects during the development such as defect of cerebral hemisphere fusion, disturbance of forebrain commissures formation as well as cortical and retinal lamination abnormalities of the cortex and retina. But by expression of the non-myristoylated human MARCKS in this null MARCKS population all the neuroanatomical abnormalities could be rescued (Swierczynski ***et al.***, 1996). Expression of a transgene containing non-myristoylation and pseudo-phosphorylation sites was able to compensate almost the entire cerebral anatomical abnormalities of the knockout mice, however these mice also exhibited profound retinal ectopia (Kim ***et al.***, 1998). Chen ***et al.*** (1996) found that ***MLP*** deletion in mice prevents the closure of cranial neural tube in the developing brain characterized by embryonic exencephaly and postnatal anencephaly. It was shown in another ***MLP*** knockout mice system (Wu ***et al.***, 1996) that the neural tube defects caused exencephaly and spina bifida.

In this study, a novel homologue of the MARCKS like protein (MLP) in ***Xenopus*** was identified and characterized. It is demonstrated that ***XMLP*** is differentially expressed during early ***Xenopus*** development. Ectopic expression of ***XMLP*** led to eye, axis defects and apoptosis and also resulted in the disruption of expression pattern of ***Krox20***, suggesting that it may be correlated with a disturbed morphogenetic movements rather than an inhibition of induction processes. While overexpression of ***XCYP26*** caused an alternation of the expression pattern of ***XMLP***, indicating that retinoic acid (RA) may play an important role for its regulation.

XAT and the AT family

The L-Arginine:glycine amidinotransferase (AT, EC2. 1. 4. 1) catalyzes the transfer of a guanido group from arginine to glycine. The resultant guanidinoacetic acid is the immediate precursor of creatine. Phosphorylated derivatives of creatine play important roles in the vertebrate energy metabolism (Walker, 1973). The phosphocreatine serves as a high-energy reservoir in mammalian tissues, especially when the source of ATP is limited. It has been shown that the expression of ***AT*** can be tightly regulated by growth hormones and thyroxine induction (McGuire ***et al.***,

1980) and is inhibited by creatine (Guthmiller ***et al.***, 1994). In Wilms tumor ***AT*** expression is down regulated as well (Austruy ***et al.***, 1993), which suggests that it may play an important role in the limb-girdle muscular dystrophy type 2A (LGMF2A) (Humm ***et al.***, 1997a) (for review, see Humm ***et al.***, 1997b).

In this study, it is demonstrated that ***XAT*** is expressed in the organizer in the gastrula stages, and the posterior and trunk in the tailbud stages. The mechanism of its funcion and regulation during the early embryogenesis still needs to be elucidated.

2. Materials and Methods

2.1 Materials

Enzymes

***Dpn*I**	Roche, Mannheim
Lysozyme	SIGMA, Taufkirchen
pfu DNA polymerase	Stratagene, Amsterdam
Proteinase K	SIGMA, Taufkirchen
RNase A	SIGMA, Taufkirchen
RNase T1	Roche, Mannheim
RNase inhibitor	Stratagene, Amsterdam
SP6 RNA polymerase	Stratagene, Amsterdam
Taq DNA polymerase	Genecraft, Münster
T4 DNA ligase	MBI Fermentas, St. Leon-Rot
T7 RNA polymerase	Stratagene, Amsterdam
T3 RNA polymerase	Stratagene, Amsterdam
All other restriction enzymes	MBI Fermentas, St. Leon-Rot

Chemicals and kits

Agrose	peqLAB, Erlangen
acetic anhydride	SIGMA, Taufkirchen
All-trans-RA	SIGMA, Taufkirchen
Ampicillin	SIGMA, Taufkirchen
BCIP	Roche, Mannheim
BM purple	Roche, Mannheim
BSA	SIGMA, Taufkirchen
Cap-Scribe	Roche, Mannheim
Digoxigenin RNA mix or florescein-mix	Roche, Mannheim
Ficoll 400	SIGMA, Taufkirchen
Fluorescein-12-UTP	Roche, Mannheim

Formamide	SIGMA, Taufkirchen
Gene Ruler ™ 1kb ladder	MBI Fermentas
Goat Serum (GS)	GibcoBRL, Karsruhe
HCG	Schering, Berlin
Hepariin	SIGMA, Taufkirchen
Kanamycin	SIGMA, Taufkirchen
Leibovitz L-15	GibcoBRL, Karsruhe
NBT	Roche, Mannheim
Penicillin	SIGMA, Taufkirchen
Polyvinylpyrrolidone (PVP-40)	SIGMA, Taufkirchen
Qiagen Midi-preparation Kit	Qiagen, Hilden
Qiagen QIAquick purification kit	Qiagen, Hilden
Qiagen RNeasy kit	Qiagen, Hilden
RNA-Clean™	HYBAID, Egelsbach
Sephadex G50	SIGMA, Taufkirchen
streptomycine sulfate	SIGMA, Taufkirchen
Torula RNA	SIGMA, Taufkirchen
X-gal	SIGMA, Taufkirchen

All other chemicals used in this study were from Fluka, Merck.

General stock solutions and reagents

LB broth (1L):	20 g	Luria Bertani medium, SIGMA
LB agar (1L):	35 g	SIGMA, Taufkirchen
NZY broth (1L):	21 g	GibcoBRL, Karsruhe
NZY agar (1L):	21 g	GibcoBRL, Karsruhe
NZCYM broth (1L):	22 g	GibcoBRL, Karsruhe
5× TBE (1L):	54 g	Tris
	27.5 g	boric acid
	20 mL	EDTA (0.5 M, pH 8.0)

TE:	10 mM	Tris pH7.4
	1 mM	EDTA

Bacteria and animals

E.coli XL1-Blue MRF'	Stratagene, Amsterdam
E.coli XLOLR	Stratagene, Amsterdam
Xenopus laevis	Snake farm, South Africa

Vectors

PBK-CMV	Stratagene, Amsterdam
pCS2+	Turner, 1994
pCSMTEnR	Turner, 1994

Hardwares

Petriperm™Petri dish	Heraeus
Microloader	Eppendorf
Spectrum photometer	Pharmarcia
UNOII thermo cycler	Biometra
Micromanipulator	Singer Comp., UK
PV830 Pneumatic PicoPump	World Precision Comp., USA
Stereomicroscope	Zeiss
Axioplanmicrocope	Zeiss
Progressive 3 digital camera	Sony
Axiocam HRC digital camera	Zeiss
MC100 optical camera	Zeiss
Orthomat-camera	Leitz
LS-100 slide scanner	Nikon
Thermal cycler	Biometra

Primers (All following primers employed for RT-PCR were synthesized by Roth).

Genes	Primer sequences	Authors
XAT	Fw: 5'-GCAATGGAATCCACCTCCTA-3' Re: 5'-GGTTACTCCCCCAACTCCAT-3'	Zhao ***et al***., 2001a
XMLP	Fw: 5'-GTCTAATGGCTCCGCTGAAG-3' Re: 5'-GCTTCTGGAGATGCTTCCAC-3'	Zhao ***et al.***, 2001b
XXBP-1	Re: 5'-TCACTTGCTGTTCCAGTTCA-3' Fw: 5'-GCCCCCAAAGTGATCTTTAT-3'	Zhao ***et al.*** unpublished
goosecoid	Fw: 5'-AGGCACAGGACCATCTTCACCG-3' Re: 5'-CACTTTTAACCTCTTCGTCCGC -3'	Blumberg ***et al.***, 1991
Xnot	Fw: 5'-GAATCCGCACAGTGTTCACCCC-3' Re: 5'-TCCTCGTCCTCCTCAGTCTCCC-3'	von Dassow ***et al.***, 1993
Xwnt-8	Fw: 5'-GAGAGAAGAAGCTGCAAGAGGC-3' Re: 5'-GGCAAACAAATCCACTGGCCCG-3'	Smith and Harland, 1991
Muscle actin	Fw: 5'-GCGGCTTTGGACTTTGAGAATG-3' Re: 5'-TCAGCAATACCAGGGTACATGG-3'	Mohun ***et al***., 1984
MyoD b	Fw: 5'-AACTGCTCCGATGGCATGATGGATTA-3' Re: 5'-AATGCTGGGAGAAGGGATGGTGATTA-3'	Hopwood ***et al.***, 1989
Xvent-1	Fw: 5'-GCATCTCCTTGGCATATTTGG-3' Re: 5'-TTCCCTTCAGCATGGTTCAAC-3'	Gawanka ***et al***., 1995
Otx2	Fw: 5'-GGATGGATTTGTTACATCCGTC-3' Re: 5'-CACTCTCCGAGCTCACTTCCC-3'	Blitz ***et al.***, 1995
BMP-4	Fw: 5'-GCATGTACGGATAAGTCGATC-3' Re: 5'-GATCTCAGACTCAACGGCAC-3'	Dale ***et al***., 1992
XAG1	Fw: 5'-CTGACTGTCCGATCAGAC-3' Re: 5'-GAGTTGCTTCTCTGGCAT-3'	Sive ***et al***., 1996
Xbra	Fw: 5'-GGATCGTTATCACCTCTG-3' Re: 5'-GTGTAGTCTGTAGCAGCA-3'	***Xenopus*** MMR
NCAM	Fw: 5'-CACAGTTCCACCAAATGC-3' Re: 5'-GGAATCAAGCGGTACAGA-3'	***Xenopus*** MMR
H4	Fw: 5'-CGGGATAACATTCAGGGTA-3' Re: 5'-TCCATGGCGGTAACTGTC-3'	Steinbeisser ***et al.***, 1995
ODC	Fw: 5'-GGAGCTGCAAGTTGGAGA-3' Re: 5'-ATCAGTTGCCAGTGTGGTC-3'	***Xenopus*** MMR
Xvent-2	Fw: 5'-TGAGACTTGGGCACTGTCTG-3' Re: 5'-CCTCTGTTGAATGGCTTGCT-3'	Onichtchouk ***et al***., 1996
Xhox3	Fw: 5'-ATATGATGAGCCACGCAGCAG-3' Re: 5'-CAGATGCTGCAGCTCTTTGGC-3'	Ruizi Altaba and Melton ***et al.***, 1989
Epidermal Keratin	Fw: 5'-CACCAGAACACAGAGTAC-3' Re: 5'-CAACCTTCCCATCAACCA-3'	***Xenopus*** MMR
chordin	Fw: 5'-CCTCCAATCCAAGACTCCAGCAG-3' Re: 5'-GGAGGAGGAGGAGCTTTGGGACAAG-3'	Piccolo ***et al***.,1996

2.2 Embryos and explants manipulation

2.2.1 Preparation of Xenopus laevis embryos

1. Aliquots of 3000-5000 IU of human chorionic gonadotropin (HCG) were kept in vials at 4ºC. Dissolve an aliquot in 0.4% NaCl solution immediately before the injection to ***Xenopus***.

2. In the evening before the experiment, inject two adult female frogs with 600-800 IU (depending on the individual size) of HCG into the dorsal lymph sac, i.e. a semicircle of small slits on the dorsal side of the frog just above the junction of the back leg and the body, below which is a membrane joins the outer skin to the body wall. Hold the syringe such that the needle is almost parallel to the skin and push it through just on the 'leg side' of the marks, ensuring that it stays between the two layers. Penetrate the membrane under the marks and inject.

3. Keep the injected frogs overnight at 19-21ºC. Generally 6-8 h later, the frogs begin to lay eggs.

4. Kill an adult male frog, and remove the testes. Trim any extraneous tissue from them, rinse off any blood, and place in medium, either in 1× MBS (or 1× Barth's) solution if they are to be used in the next 3 days or modified L-15 medium if they are to be kept longer (up to 2 weeks). Store at 4ºC.

5. Crush one piece of the testis in 0.2 mL of 0.1× MBS (or 0.1× Barth's) with forceps and then take the resulting suspension up and down in 1-mL pipette tip until the tissue is well suspended.

6. Squeeze the female frogs gently but firmly around the abdomen so that they lay eggs into a Petri dish; move the frog so that the eggs fall into small groups.

7. Using a pipette, squirt the crushed testis over each group of eggs and then shake the dish to form a single layer of the eggs.

8. Leave the eggs for 5 min, then rinse and incubate in 0.1× MBS (or 0.1× Barth's).

9. After the eggs have rotated and the animal pole is uppermost (about 20-40 min post fertilization), discard the MBS and dejelly eggs with 20 mL of 2% cysteine solution. Detach the eggs from the plate by swirling the Petri dish vigorously. Notice that when the jelly coats are removed, the eggs lie close to one another, whereas before that the thick jelly separates them. Once all are in contact, wash them with at least four changes of 20 mL of 0.1× MBS (or 0.1× Barth's) and keep in a fresh Petri dish.

10. Incubate the eggs at 14-25ºC until they develop into the desired stages for microinjection.

1× MBS:

88 mM	NaCl
2.4 mM	$NaHCO_3$
1 mM	KCl
10 mM	HEPES
0.82 mM	$MgSO_4$
0.41 mM	$CaCl_2$
0.33 mM	$Ca(NO_3)_2$
pH 7.4	

1× Barth' Solution:

88 mM	NaCl
2.4 mM	$NaHCO_3$
1 mM	KCl
5 mM	HEPES
0.82 mM	$MgSO_4$
0.41 mM	$CaCl_2$
0.33 mM	$Ca(NO_3)_2$
0.2 mM	Na_2HPO_4
pH 7.4	

2% Cysteine

Dissolve cysteine hydrochloride with sterile Millipore H_2O to a final concentration of 2% and adjust pH to 8.0 with 5 M NaOH.

penicillin/streptomycin stock solution (1000×):

Dissolve penicillin, 100,000 units/mL, and streptomycine sulfate, 100 mg/mL, in sterile Millipore water. Store frozen.

L-15 solution for testis storage:

5 mL	L-15
1 mL	calf serum
4 mL	sterile Millipore H_2O
100 µL	penicillin/streptomycin stock solution

2.2.2 UV, LiCl and ATRA treatments

For UV treatment, fertilized eggs were dejellied thoroughly within about 20 min of the insemination, then immediately transferred to Petriperm™ Petri dishes with UV-permeable bottom and treated with UV transilluminator for 45 sec–1 min depending on the illumination intensity. The eggs were allowed to be settled quietly for 2 h or at least until the first cleavage at room temperature.

LiCl treatments were carried out with fertilized eggs at the 32-128-cell stage to obtain the dorsoanterior enhancement. The embryos were treated with 0.3 M LiCl solved in 0.1× Barth's solution for 5-10 min, afterwards washed several times with 0.1× MBS and cultured until the desired stages.

All-trans-RA stock (10^{-2} M) solved in DMSO was diluted with 0.1× Barth's solution to a final concentration of 10^{-6} M. Embryos were incubated in this solution from the stage 8 to stage 11, afterwards were washed several times with 0.1× Barth's solution and cultured until the desired stages.

2.2.3 Explantation of animal caps and marginal zones

2.2.3.1 Animal cap

1. Transfer the stage 9 embryos to a Petri dish covered with 1% Agar and filled with Holtfreter's solution.
2. Grasp the membrane with the very tips of one pair of forceps in the marginal or vegetal region while bracing the embryo against the side of the other forceps. With the other forceps, grasp the membrane close to the place where the first one penetrates; hold the membrane and pull away to remove it.
3. After the removal of vitelline membrane, roll the embryo animal pole up and gently push it back into shape in order to maintain a good blastocoel.
4. Insert into the blastocoel with sharp glass needles and excise the cap. Care must be taken to take only the animal cap tissue and not the marginal zone material.
5. The harvested animal caps finally were transferred into 1× Barth's solution and kept until the desired stages.

Holtfreter's Solution:

60.00 mM	NaCl
0.60 mM	KCl
0.90 mM	$CaCl_2$
0.20 mM	$NaHCO_3$
5 mM	HEPES
pH 7.4	

2.2.3.2 Dorsal and ventral marginal zone

Dorsal and ventral marginal zones (DMZs and VMZs) were isolated from embryos at stage 10-10.5 with the blastopore lip marking the dorsal side. Vitelline membrane was removed from the animal halve and the explants were isolated with glass needles. DMZs corresponded to an ~60º arc of marginal zone tissue centered on the dorsal lip midline, and VMZs, analogously the region opposite the DMZs. Both then were cultured in 1× Barth's solution till expected stages.

2.2.4 Microinjection

1. Place a needle into the holder on the micromanipulator.
2. Cut off the end of the needle at the point where the needle tip becomes less flexible using watchmaker forceps under the microscope.
3. Spin mRNA (from 2.5.1) for 5 min in a microcentrifuge to remove any debris that might block the needle, and then pipette 2-5 µL of the mRNA into the needle. Take up the RNA sample in to the needle from the back of the needle using a long-tip microloader.
4. Set the injector to the required volumes. It is suitable to use a volume of approximately 4 nL for embryos at the 1-4-cell stages. In general, an injection pressure of 10 psi and an injection time period of 0.3-0.6 sec are required, which gives excellent survival rates.
5. Transfer embryos used for the injection into a Petri dish covered with 1% agar and filled with 4% ficoll (dissolved in 1× MBS or 1× Barth's solution).
6. Drive the needle tip through surface of the embryo, give pressure to the needle and inject. Wait for a moment, and then withdraw the needle gently.
7. Once a set of embryos has been injected, pipette the embryo up gently and place them in the same medium as in step 5 in a fresh Petri dish.

8. Incubate the embryos at 18-21ºC.

9. After 1-2 h, replace the medium with 0.1× MBS and remove any unhealthy embryos.

10. Culture as in step 8 for the appropriate cell stages.

Notes:

Dorsal blastomeres determination:

Because of the cortical rotation, the future dorsal side tends to be lighter in colour than the ventral side, but this difference is an imprecise and unreliable indicator of where the future dorsal side will form.

Injection volumes determination:

The inject volume must be calibrated carefully. The following protocol describes the calibration procedure for a pressure injector.

1. Backfill the needle using a microloader fitted with a long narrow tip (of the kind used for loading sequencing gels) and mount on the injector.
2. Break the needle tip to produce an orifice of approximately 10 µm.
3. Place a small drop of paraffin oil on a microscope slide and mount the slide on the stage of a dissecting microscope.
4. Calibrate the eyepiece micrometer for the appropriate magnification and perform a trial injection into the drop of oil. The injected liquid forms a sphere within the oil droplet.
5. Measure the diameter of the sphere using the eyepiece micrometer and calculate the injected volume ($V=4/3\pi r^3$, where V is the volume and r is the radium of the sphere).

The different methods are employed for calibrating the injection volume including deposition of the drop directly on the stage micrometer (note that micrometer must siliconized for the drop to be near spherical). The injected volume can then be calibrated as described in step 5 above. Alternatively, allow the drop to hang at the end of the needle, where its diameter can be measured using an eyepiece micrometer. The drop must be measured quickly because evaporation causes it to shrink rapidly. Anther method is to inject the drop into a dish of paraffin oil. Approximate the volume of the drop by using the equation $V=4/3\pi r^3$ (see above) or using the figures shown in Table 2-1 below. Once the appropriate pressure has been established, the duration of the pressure burst can be used to control the volume precisely.

Table 2-1. The injection volume and the corresponding drop diameter and radius.

Diameter of drop (μm)	Radius of drop (μm)	Volume (nL)
125	62.5	1.03
140	70	1.44
150	75	1.07
160	80	2.15
170	85	2.58
180	90	3.06
200	100	4.20
225	112.5	5.90
250	125	8.20

2.2.5 Morpholino preparation for microinjection

2.2.5.1 Principle

As a new loss-of-function approach, morpholino antisense oligos have been adopted to the study of gene functions in the recent 3 years. In contrast to the traditional posphorothioate oligos, the morpholino antisense oligos (Figure 2-1) are non-toxic and when injected into fertilized eggs they prevent translation of the proteins in both cytosolic and membrane-associated fractions through the neurula stage (Summerton *et al.*, 1997). Generally, an antisense morpholino oligo, typically 18 to 25 genetic letters in length, binds the sense mRNA and prevents synthesis of the protein coded by that mRNA. A normal gene function and the inhibition of this gene function by an antisense oligo are illustrated in Figure 2-2. This new approach has been successfully used to deplete the β-catenin in *Xenopus laevis* (Heasman *et al.*, 2000).

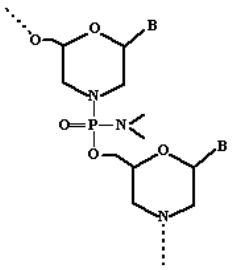


Figure 2-1. The chemical formula of morpholino (MO).

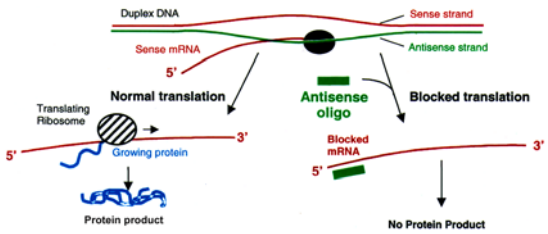


Figure 2-2. The blockage of protein translation imposed by morpholino. In the absence of morpholino, the protein will be synthesized by ribosome naturally according to codons in the mRNA. However in the presence of morpholino designed to bind to the complementary sequence in this mRNA, the protein initial site will be sequestered by the morpholino, hence no protein product will be synthesized finally (Cited from protocol of GeneTool.

2.2.5.2 Experimental procedures

Dissolve the morpholino in 1× Barth's with a concentration of 4 ng/nL as the stock solution, and store it at -20ºC. Dilute the stock solution to the working solution with 1× Barth's just before microinjection. The microinjection of morpholino was performed the same as that of mRNA.

2.2.6 Embryos fixation with MEMFA or HEMFA

Embryos were fixed in HEMFA or MEMFA at room temperature for 1 h with shaking, afterwards washed with 100% ethanol twice or three times and stored at –20ºC in 100% ethanol.

10× MEM:	MEMFA:	
1M MOPS pH 7.4	0.1M	MOPS pH 7.4
20 mM EGTA	2 mM	EGTA
10 mM $MgSO_4$	1 mM	$MgSO_4$
	3.7%	formaldehyde

10× HEM:	HEMFA:	
1M HEPES pH 7.4	0.1M	HEPES pH 7.4
20 mM EGTA	2 mM	EGTA
10 mM $MgSO_4$	1 mM	$MgSO_4$
	3.7%	formaldehyde

The 10× stock solutions without formaldehyde can be prepared and stored after filtration to remove bacteria. These solutions turn into yellow if autoclaved or aged.

2.2.7 LacZ staining

1. Fix embryos in MEMFA for 1 h.

2. After rinsing with 1× PBS twice for 10 min each, transfer the embryos to the X-gal staining solution and stain for 1.5-6 h at room temperature until the blue appears.

3. Wash the embryos with 1× PBS twice with shaking for 10 min each.

4. Refix the embryos with MEMFA for 40 min.

5. Rinse the embryos with 100% ethanol twice for 5min each, afterwards store them at –20ºC till whole-mount ***in situ*** hybridization.

X-gal staining solution (in 1× PBS, 10 mL):

1 mg/mL	X-gal	0.25 mL	40 mg/mL X-gal (in DMSO)
5 mM	$K_3Fe(CN)_6$	0.10 mL	500 mM $K_3Fe(CN)_6$
5 mM	$K_4Fe(CN)_6$	0.10 mL	500 mM $K_4Fe(CN)_6$
2 mM	$MgCl_2$	0.20 mL	100 mM $MgCl_2$
Add 1× PBS to 10 mL			

2.3 Bacteria manipulation

2.3.1 Preparation of competent cells

1. Grow a single bacterial colony in 100 mL of fresh LB broth at 37ºC for 16-20 h with vigorous shaking.

2. Dilute (1:100) the culture above to a certain volume of fresh LB broth and grow to early log phase ($O.D._{600}$ = 0.2-0.4) at 37ºC for approximately 2-2.5 h with vigorous shaking.

3. Aseptically transfer the cells to sterile, disposable and ice-cold 50ml-polypropylene tube (Falcon). Cool the culture to 0ºC by storing the tube on ice for 10 min (This procedure could prolong if the culture volume is very large.). All subsequent steps were carried out aseptically.

4. Collect cells by centrifugation at 3000 rpm at 4ºC for 10 min.

5. Decant the medium, and stand the tube in an inverted position for 1 min to allow the last traces of media to drain away.

6. Wash the pellet with 10 mL of ice-cold 0.1 M $CaCl_2$ and keep the cells ice cold in all further steps.

7. Collect cells as in step 4 and resuspend the cell pellets with 1/25 original culture volume of 0.1 M chilled $CaCl_2$.

8. Add 140 µL of DMSO per 4 mL of resuspended cells. Mix gently by swirling and hold on ice for 15 min. Then add another 140 µL of DMSO to each suspension, mix gently and keep on ice bath.

9. Dispense aliquots of the suspensions into chilled, sterile 1.5-mL Eppendorf tube quickly, afterwards fast freeze in liquid nitrogen and store at -70ºC.

2.3.2 Transfomation

1. Thaw 50 -200 uL of bacteria per transformation and keep them on ice.

2. Gently mix cells by hand.

3. Add 0.1ng-50 ng of DNA (or approximately 1/2 ligation) to cells, swirl gently, and put it on ice for 30 min.

4. Afterwards heat shock at 42ºC for 90 sec and then hold it on ice for 2 min.

5. Add 0.8 mL of LB and shake at 37ºC for 40 min.

6. Plate on LB-agar plates containing antibiotic, and keep the plates overnight at 37ºC.

2.4 DNA techniques

2.4.1 Extraction of plasmid DNA

2.4.1.1 Mini-preparation with TELT

1. Harvest the bacterial cells from a 1.5-mL culture by centrifugation at 6000 rpm for 5 min at room temperature.

2. Remove supernatant completely with vacuum system and resuspend the bacterial pellet in 150 µL of TELT solution.

3. Add 15 µL of freshly prepared solution of lysozyme (10 mg/mL in H_2O) to the cells, vortex and incubate at room temperature for 5 min.

4. Keep the cells in boiling water for 2 min and then cool them down on ice for 5 min.

5. Remove bacterial debris with a sterilized toothpick after full speed centrifugation at room temperature for 8 min.

6. Extract the supernatant with 100 µL of isopropanol with vortex.

7. Centrifuge with full speed for 15 min at room temperature. Discard the supernatant with pipette and wash the pellet with 200 µL of 70% ethanol.

8. Centrifuge as in step 7. Suck off the supernatant and dry the pellet in a vacuum desiccator for 5min.

9. Dissolve DNA in 30 µL of TE (pH 8.0) containing RNAse A (10 ng/µL). Incubate first for 2-5 min at room temperature, and then 5 min at 65ºC.

10. Spin down and collect the supernatant.

TELT:

50 mM	Tris/HCl pH 7.5
10 mM	EDTA
3.2 M	LiCl
0.5%	Triton X-100

RNase A stock:

1. Dissolve RNAase A in TE (pH7.8) to a final concentration of 10 mg/mL.
2. Boil for 10 minutes before use.
3. Store RNase A stock solution in aliquots of 50 µL at -20ºC.

2.4.1.2 Midi-preparation with Qiagen Kit

Plasmid DNAs midi-preparation was carried out using Qiagen Midi-preparation Kits and following the supplied protocol.

2.4.2 Recovery of DNAs from gel

The DNAs recovery from agrose gels were carried out using Qiagen QIAquick purification kits and following the supplied protocol.

2.5 RNA manipulation

2.5.1 Preparation of mRNA for microinjection

Capped mRNAs used for microinjection were ***in vitro*** transcripted with the Cap-Scribe as described below.

Mix the following component in order on ice:

To a total volume of 20 µL:

4 µL	the Cap-Scribe 5× buffer
0.5 ng	linearized template DNA
Add to 18 µL	DEPC-H_2O
2 µL	RNA polymerse

After incubation for 2 h at 37ºC, add 2 µL of a solution of DNase I (10 U/µL) to remove the DNA template. Purify the RNA product with Qiagen RNeasy kit and determine the concentration with spectrum photometer. Aliquot the RNA sample into convenient volumes (depending on the concentration measured) and place at -70ºC.

2.5.2 Preparation of RNA probe for whole–mount in situ hybridization.

1. Add the following components into a 0.2-mL Eppendorf tube on ice, mix and then incubate for 2 h at 37ºC.

To a total volume of 25 µL:

10 µL	template (1µg linear DNA)
5 µL	5× transcription buffer
1 µL	DTT (0.75 M)
0.5 µL	RNase black (40 U/µL)
4 µL	Digoxigenin RNA mix or florescein-mix
0.5 µL	RNA polymerase (20 U/µL)
4 µL	DEPC-H_2O

2. Add 2 µL of a solution of DNase I (10U/µL) to digest the template DNA for 15 min at 37ºC.

3. Purify the RNA product with Qiagen RNeasy kit according to its protocol.

2.5.3 Whole-mount in situ hybridization

In principle, whole-mount ***in situ*** hybridization was performed as described by Harland (Harland, 1991) and Oschwald (Oschwald ***et al.***, 1991).

2.5.3.1 Preparation of solutions

PTw:

1× PBS
0.1% Tween 20

20× SSC:

175.3 g	sodium chloride
88.2 g	sodium citrate

Dissolve in 800 mL of sterile Millipore water, adjust pH to 7.0 with NaOH, and adjust the volume to 1 liter.

100× Denhart's Solution:

2%	BSA
2%	polyvinylpyrrolidone (PVP-40)
2%	Ficoll 400

Hybridization Buffer:

50%	formamid
5×	SSC
1 mg/mL	Torula RNA (Type IX, Sigma)
100 µg/mL	heparin (Sigma)
1×	Denhart's solution
0.1%	Tween 20
0.1%	CHAPS
10mM	EDTA

5× MAB (Maleic Acid Buffer):

0.5 M	Maleic acid
0.75 M	NaCl
pH 7.5	

10% BMB:

Weigh Boehringer Mannheim Blocking Reagent in 1× MAB for a final concentration of 10% (m/v), and autoclave immediately. The BMB will be dissolved after the autoclave.

APB (Alkaline Phosphatase Buffer):

100 mM	Tris/HCl pH 9.5
50 mM	$MgCl_2$
100 mM	NaCl
0.1%(v/v)	Tween20

Freshly prepare the APB immediately before use.

NBT/BCIP:

nitro blue tetrazolium (NBT): 75 mg/mL in 70% dimethylformamide.

5-bromo-4-chloro-3-indolyl-phosphate (BCIP): 50 mg/mL in 100% dimethylformamide.

Goat Serum (GS):

Heat deactivates the complement for 30min at 55ºC. Store as aliquots of 10 mL at -20ºC.

2.5.3.2 Experimental procedures

Embryos were fixed in MEMFA (or HEMFA) for 1 h at room temperature. Replace the MEMFA (or HEMFA) with absolute ethanol twice or three times for 5 minutes each, and store in 100% ethanol at –20ºC.

Day 1:

Rehydration. The embryos were then rehydrated by washing with following solutions in order.

(1) 100%	ethanol	5 min	
(2) 75%	ethanol	5 min	
(3) 50%	ethanol	5 min	
(4) 25%	ethanol in PTw	5 min	
(5) 100%	PTw	5 min	4 times

Proteinase K treatments. The embryos in each vial were treated with 2 mL of PTw containing 10 µg/mL proteinase K for 10-30 min at room temperature depending on the cell stage they were in, the number of embryos and the temperature. It is approximately 20 min for gastrula-stage embryos, and a long period of 20-30 min for tailbud-stage embryos.

Acetification. After the proteinase K treatment, the embryos were immediately subject to the treatment of acetylate which neutralizes free amines and helps to prevent electrostatic interaction between the probe and basic proteins in the embryo***.***

(1) 4 mL	0.1 M triethanolamine pH 7.5	5 min twice
(2) 4 mL	0.1 M triethanolamine pH 7.5	
+ 12.5 µL	acetic anhydride	5 min
+ 12.5 µL	acetic anhydride	5 min
(3) PTw		5 min twice

Refixation. The embryos were refixed by incubating with following solutions in order.

(1)	PTw + 4% formaldehyde	20 min
(2)	100% PTw	5 min 5 times.

Hybridization.

(1) Remove approximately all 1 mL of PTw from the vial and add 250 µL of hybridization buffer (without mixing).
(2) Once embryos have settled through the dense layer of the buffer, remove the entire buffer and replace with 0.5 mL of fresh hybridization buffer per vial. Incubate for 10 min at 60ºC in a shaking water bath.
(3) Replace hybridization buffer and prehybridize for 6 h at 60ºC.
(4) Remove hybridization buffer and replace it with fresh hybridization buffer containing 0.5 µg-1µg/mL of probes. Hybridize overnight at 60ºC. The rest probe can be stored at -20ºC.

Day2:

Washing the embryos.

(1) Rinse the embryos for 10 min with 2 mL of fresh hybridization buffer and then wash with 4 mL of 2× SSC three times for 20 min each at 60ºC.
(2) Replace the washing solution with 4 mL 2× SSC containing 20 µg/mL of RNase A and 10 U RNase T1. Incubate vials for 1 h at 37ºC.
(3) Remove excess RNase by washing the embryos with 4 mL of 2× SSC once for 10 min at room temperature, and then with 4 mL of 0.2× SSC twice for 30 min each at 60ºC.
(4) Wash with 4 mL of 1× MAB twice for 15 min each at room temperature.

Antibody Incubation. All subsequent steps were carried out at room temperature except those indicated.

(1) Replace 1× MAB with 4 mL of 1× MAB + 2% BMB, and keep shaking for 15-60 min.
(2) Change the solution to 4 mL of fresh 1× MAB + 2% BMB + 20% goat serum, and keep it for 1 h.
(3) Replace the solution with 1.5 mL of fresh 1× MAB + 2% BMB + 20% goat serum containing 1/5000 dilution of the antibody. Rock the vials for 4 h.
(4) Remove the excess antibody by washing with 4 mL of 1× MAB twice for 30 min each.
(5) Change with 40 mL of fresh 1× MAB and keep shaking the vial overnight at 4ºC.

Day 3:

Color reaction.

(1) Wash the embryos with 4 mL of 1× MAB twice for 30 min each at room temperature.
(2) Replace the solution with 4 mL of APB twice for 5 min each at room temperature.
(3) Replace the last washing solution with 0.5-2 mL of BM purple or APB containing 2 µg/mL of NBT and 3.5 µg/mL of BCIP per vial and incubate at 4ºC until staining becomes apparent.

Bleaching.

(1) Wash the embryos with 2 mL of 100% methanol for 1-5 min.
(2) Rinse the sample with 2 mL of Millipore H_2O several times for 1-5 min each.
(3) Transfer the embryos to methanol containing 10% H_2O_2 and keep at 4ºC until pigment disappears.
(4) Rinse the embryos with 2 mL of Millipore H_2O, then keep them in 2 mL of MEMFA or HEMFA.

2.6 RT-PCR

2.6.1 Isolation of total RNA from embryos, animal cap, or marginal zone explants.

1. Homogenize 10 embryos with 1000 µL of RNA-Clean™, (for minimum RNA preparation, 2 embryos use 200 µL of RNA-Clean, 20 animal caps, 200 µL, and 10 marginal zone explants, 200 µL). Keep the samples on ice before preparation.

2. Add 100 µL of chloroform per 1000 mL of homogenate, cover the samples tightly, shake vigorously for 15 sec and hold on ice for 5 min.

3. Spin at 12000g for 15 min at 4 ºC.

4. Transfer the upper aqueous phase to a fresh 1.5-mL Eppendorf tube. Add the equal volume of isopropanol.

5. Let stand at -20ºC for at least 1 hour or overnight to precipitate the RNAs.

6. Spin the samples at 12000g for 15 min at 4ºC. Carefully remove the supernatant without disturbing the RNA pellet.

7. Wash the RNAs pellet once with 70% ethanol (0.8 mL of ethanol per 100 µg of RNA), i.e. vortex the RNAs pellet for several seconds and spin at 7500g for 8 min at 4ºC. Repeat the washing step with 500 µL of prechilled 70% ethanol.

8. Dry the RNAs pellet in air (about 10 min). It is important that the RNAs pellet does not dry out completely, as this will affect solubility and quality of the RNA.

9. Dissolve the RNAs pellet in 50 µL of DEPC-H_20.

10. Determine concentration of the RNAs by spectrum photometer. The OD_{260}/A_{280} ratio should be in the range from 1.6 to 1.9.

11. For the RNAs storage, add 3 volume of prechilled absolute ethanol at -20ºC and 1/10 volume of 3 M NaAc solutions (pH 5.2). Keep the RNA at -20ºC.

2.6.2 Isolation of total RNA from adult tissues

1. Various organs and tissues were taken from an adult sacrificed frog and then ground in a meat grinder with liquid nitrogen and stored at -80ºC.

2. Add 500 µL of lysis buffer containing 200 µg/mL of proteinase K for 200 µL of tissue powder.

3. Thoroughly homogenize the tissue power with the lysis buffer by pipetting with a fine needle, afterwards incubate for 45 min at 45ºC.

4. Extract the sample once or twice with 0.5 mL of phenol/chloroform and three times with 0.5 mL of chloroform.

5. Precipitate the RNAs with 3/4 volume of 8 M LiCl at -20ºC.

6. Dissolve the sample in 100 µL of DEPC-H_2O after washing with 80% ethanol.

7. Reprecipitate the RNAs with 3 volume of absolute ethanol and 1/10 volume of 3M NaAc (pH 5.2) and resuspende in 45 µL of DEPC-H_2O.

8. After centrifugation, treat 40 µL of the samples with 2 µL of DNase I (10 U/µL) for 30 min at 37ºC.

9. Store the sample at -20ºC.

2× Lysis Buffer:

1%	SDS
10 mM	EDTA
100 mM	Tris/HCl pH 7.5
100 mM	NaCl

Working Lysis Buffer:

2× lysis buffer	5mL
20 µg/µL Proteinase K	0.5mL (added immediately before use)
DEPC-H_2O	4.5mL

2.6.3 Reverse transcription

1. To a total volume of 12 µL, add the following components to a nuclease-free 0.2-mL microcentrifuge tube:

1 µg	total RNA
2 µL	0.1 mM Hexamer random primer
9 µL	DEPC-H_20

2. Mix well, incubate for 10 min at 70ºC, and then quickly chill on ice. Collect the contents in the tube by brief centrifugation, to which following components were then added and incubated for 10 min at 25ºC.

4 µL	5× First strand buffer
2 µL	0.1 M DTT
1 µL	10 mM dNTPs mix

3. Add 1 µL of RNase H^-Reverse Transcriptase (200 U/µL). Mix gently and incubate for 50 min at 42ºC, then 15 min at 70ºC to inactivate the reverse transcriptase. Afterwards store the sample at -20ºC.

2.6.4 PCR reaction

At first, the normalization of cDNA samples was carried out with housekeeping genes such as H4 and ODC using PCR. Keep the sample which gives the weakest band, and add H_2O to those sample that give stronger bands until all samples show the same amount of PCR products after a proper amplification. The normalized samples were then applied for PCR with specific primers. Always use 1 µL of reverse transcripts in the subsequent PCR system.

1. Set up the reaction by mixing the following reagents on ice:

18 µL	sterile Millipore H_2O
1 µL	2.5 µM Primers (forward, reverse primer together)
2.5 µL	2 mM dNTPs mix
2.5 µL	10× PCR buffer
1 µL	reverse transcripts
0.3 µL	Taq DNA polymerase

Add one drop of mineral oil or without oil in the case of using thin wall tube.

2. PCR was performed with following cycling parameters (for normal markers with less than 500 bp of products).

1	1×	denaturation	95ºC	3 min,
2	20-35× cycle:	denaturation	95ºC	1 min
		annealing	55ºC	1 min (depends on primers)
		extension	72ºC	1 min
3		extension	72ºC	10 min
4		end	4ºC	

3. PCR products determination

Add 5 µL of loading buffer to a 25 µL of reaction system after PCR, and load 20 µL of this in 2-2.5% agarose gel. Inspect bands on UV illuminator.

2.7 cDNA screening

A part of the phage cDNA library prepared from activin-treated ectoderm of ***Xenopus laevis*** was converted into a plasmid cDNA library by an ***in vivo*** excision of the pBK-CMV phagemid from the ZAP Express vector according the protocol provided by the manufacturer (Stratagene). A large-scale whole-mount ***in situ*** hybridization method was then used for screening this plasmid cDNA library.

After ***in vivo*** excision around 10,000 single colonies were picked up from the LB agar plates. Grow the single clones overnight in 2× LB containing 50 µg/mL kanamycine in the wells of a 96-well titration plate, respectively. The bacteria were stored at -80ºC.

2.7.1 Inserts amplification of a single clone to obtain templates for in vitro transcription by PCR

1. Setting up the reaction system for a large-scale screening cDNA.

To one reaction of 20 µL:

2 µL	10× PCR reaction buffer
0.16 µL	25 mM dNTPs mix
0.3 µL	10 µM CMV-F
0.3 µL	10 µM CMV-R
16.14 µL	H_20
0.1 µL	Taq DNA polymerase
1 µL	Bacteria culture solution

2. PCR was carried out with 96-well PCR reaction plates in UNOII thermal cycler with the following program using a pair of primers: CMV-F and CMV-R.

	95ºC	45 sec
40× cycles:	95ºC	45 sec
	56ºC	45 sec
	72ºC	3 min
	72ºC	10 min

CMV-F: CGC GCC TGC AGG TCG ACA CTA
CMV-R: GCA AGG CGA TTA AGT TGG GTA

3. Store 5 µL of PCR products for the subsequent transcription. The remaining 20 µL of product was loaded for electrophoresis to determine the efficiency of the amplification.

2.7.2 Preparation of the fluorescein labeled probes

1. Prepare reagents.

rNTPs mix:

50 µL	10mM	rATP
50 µL	10mM	rCTP
50 µL	10mM	rGTP
32.5 µL	10mM	rUTP
17.5 µL	10mM	Fluorescein-12-UTP (Roche)

2. Set up the transcription reaction.

Transcription reaction mix (total volume of 20 µL):

5 µL	PCR product
0.8 µL	0.75 M DTT
4 µL	5× transcription buffer
2 µL	rNTP mix
0.25 µL	RNase inhibitor (40 U/µL)
7.7 µL	DEPC-H_2O
0.25 µL	T7 RNA polymerase(50 U/µL)

The transcription reaction was carried out with 96-well PCR reaction plate in UNOII PCR thermo cycler for 4 hours at 37ºC.

2.7.3 Purification of RNA probe

1. Sephadex G50 preparations: Weigh 40 g of Sephadex G50 into 500 mL of Sephadex G50 medium solution and autoclave.

2. Pack such Sephadex G50 into a 96-well column plates and spin down the multi-column plate at 1200-1500 rpm for 30 sec to remove the medium solution.

3. Equilibrate Sephadex G50 column plate with 1× STE (50 µL for each well) twice.

4. Discard the flow through, and subsequently load the transcripts on the column plate and place it on a new 96-well microtitration plate (pay attention to the direction).

5. Spin down as in step 2 and the purified transcripts were collected in the microtitration plate. Usually 20 µL was obtained in each of the wells. Add the same volume of formamide into each well. Take 5 µL of this mixer from each well respectively and load on an agarose gel without ethidium bromide to determine the efficiency of ***in vitro*** transcription.

10× STE (50mL):

200 mM	Tris/HCl pH 7.5	10 mL	1 M Tris
20 mM	EDTA	2 mL	0.5 M EDTA
500 mM	NaCl	5 mL	5 M NaCl
Add DEPC-H_2O to 50 mL			

Sephadex G50 medium:

10 mM Tris/HCl (pH 8.0)	5 mL	1 M Tris/HCl pH 8.0
0.1% SDS	5 mL	10% SDS
0.3 M NaCl	30 mL	5 M NaCl
Add DEPC-H_20 to	500 mL	
Sephadex G50	40 g	

2.7.4 Large- scale whole-mount in situ hybridization

In principle, all steps are similar to the normal whole-mount ***in situ*** hybridization (see 2.5.3). Four sets of a special 24-well device were used for the hybridization instead of the vials.

To find a compromise between the large-scale throughput and the depth of analysis, a limit was imposed on embryonic stages to gastrula (stage11), neurula (stage13-15), and tailbud (stage28-30).

2.8 Sequences submission

Colonies indicating suggestive expression patterns were selected for sequencing. The sequence data were used to eliminate redundant clones and to compare to the DNA and protein sequences in GeneBank with BLAST (NCBI). Those which were not collected in the database were submitted to GeneBank, and got accession numbers shown in results.

2.9 Constructs used in this study

2.9.1 Primers design for mutagenesis

Names	Primer sequences	Notes
XXBP-1 subclone	Fw: 5'-GCGC**GAATTC**AATGGTGGTCGTGGGAGCC-3' Re: 5'-GCGC**CTCGAG**TTAAAAATGTACATCAAACTC-3'	***E.coR***I and ***Xho***I cutting site is in bold
VP16	Fw: 5'-GACTCCCAGCAGCC-3' Re: 5'-GCGC**TCTAGATTA**GTCAATTCCAAGGGCATCGGT-3'	***Xba***I site and the stop codon are in bold
Eve	Fw: 5'-AGCACGATCAAGGTGTGG-3' Re: 5'-GCGC**TCTAGATTA**CGCCTCAGTCTTGTA GGG-3'	***Xba***I site and the stop codon are in bold
ΔXXBP-1	Fw: 5'-GCGC**GAATTC**AATGGTGGTCGTGGGAGCC-3' Re: 5'-TAGGCCCAATCTTTGGCG-3'	***E.coR***I cutting site is in bold
Truncated XXBP1	Fw: 5'-GCGC**GAATTC**AATGGTGGTCGTGGGAGCC-3' Re: 5'-GCGC**CTCGAG**TTATAGGCCCAATCTTTGGCG-3'	***E.coR***I and ***Xho***I cutting site is in bold
XMLP G2A	Fw: 5'-GCGCGAATTCAATG**GCT**AGCGTAGAG-3' Re: 5'- GCGCCTCGAGCTATTCCTCCTGTTT -3'	alanine codon is in bold
XMLP S83A	Fw: 5'-CTTGAA**AGC**GAACCTCTT-3' Re: 5'-AACCTGAAGTTCGGTAAT-3'	alanine codon is in bold
XMLP ED	1: 5'-TGCCTGCTTTCCCGGTGG-3' 2: 5'-TTCAAGAACCTGAAGTTCGGTAAT-3'	
XMLP subcone	Fw: 5'-GCGC**GAATTC**AATGGGTAGCGT-3' Re: 5'-GCGC**CTCGAG**CTATTCCTCCTG-3'	***E.coR***I and ***Xho***I cutting site are in bold
CMV	Fw: 5'-CGCGCCTGCAGGTCGACACTA-3' Re: 5'-GCGC**CTCGAG**CTATTCCTCCTG-3'	***Xho***I cutting site are in bold
SD	2: 5'-TTGGTCTGCCTGCTTGTTGCT-3' 3: 5'-AAAACCAATGGCGACGCCCCC-3'	

2.9.2 The constructs for XXBP-1

The coding region of ***XXBP-1*** was subcloned into pCS2+ (Turner ***et al.***, 1994) by PCR with the primers of XXBP-1 subclone Rw and Re.

2.9.2.1 XXBP1-VP16

To construct XXBP1-VP16, the sequences encoding VP16 activation domain and the sequence encoding the first 126 amino acids of XXBP-1 containing the bZIP were fused together (Figure 3.2-14).

The sequence of VP16 activation domain (***VP16***, 261bp, Friedman ***et al.***, 1988) was PCR amplified from ***VPXvent-1*** (Onichtchouk, 1998) by pfu DNA polymerase with the primers, 5'-phosphorylated VP16 Fw and VP16 Re (see 2.9.1). The amplified product was ***Xba***I cut and gel purified. The sequence corresponding to the first 126 amino acids containing the bZIP of XXBP-1 was PCR amplified from pCS2-XXBP-1 by pfu DNA polymerase using primers: ΔXXBP-1 Fw and 5'-phosphorylated ΔXXBP-1 Re (see 2.9.1). The amplified products were ***EcoR*** I cut and gel purified. Afterwards the VP16- and bZIP-containing fragments were filled in ***EcoR***I –***Xba***I cut pCS2+ to get XXBP1-VP16.

2.9.2.2 XXBP1-Eve

To construct XXBP1-Eve, the sequence encoding the even-skipped repression domain (Han and Manley, 1993) was fused with the sequence from ***XXBP-1*** as that for constructing XXBP1-VP16. The even-skipped repression domain sequence (***Eve***, 777bp) was PCR amplified from ***EveXvent1*** (Onichtchouk ***et al***., 1998) with the primers: 5'-phosphorylated Eve Fw and Eve Re (see 2.8.1). The amplified product was phosphorylated, ***Xba***I cut and gel purified. Afterwards Eve- and the ***bZIP***-containing fragments were colligated with ***EcoR***I –***Xba***I cut pCS2+ to obtain XXBP-Eve.

2.9.2.3 XXBP1-EnR and truncated XXBP-1

The sequence encoding the first 126 amino acids of XXBP-1 containing the bZIP region with a stop codon at 3'-end was subcloned to pCS2+ to yield truncated XXBP-1. In addition, the bZIP-encoded region was also transferred to pCS2MTEnR to obtain XXBP1-EnR with ***Drosophila*** engrailed repressor domain at the carboxyl

terminal and 6 ***myc*** epitopes tag at the amino terminal.

2.9.3 The constructs for XMLP

The predicted ***XMLP*** open reading frame sequence was subcloned into pCS2+ with PCR using the primers of XMLP subcone Fw, and Re.

XMLP G2A mutant was generated from XMLP-pCS2+ with the primers of XMLP G2A Fw and Re (see 2.9.1) changing glycine at position 2 to alanine. XMLP S83A mutant was obtained by amplifying XMLP-pCS2+ with long distance PCR using a pair of 5'-phosphorylated primers: XMLP S83A Fw and Re. The PCR products were digested with ***Dpn***I to remove the parental DNA and afterwards the resultant was blunt-end ligated with T4 DNA ligase. The supposed phosphorylation site serine at position 83 was replaced by alanine. For the XMLP ED mutant, the fragment of 5'-ED was amplified form XMLP-pCS2+ with the primers of XMLP subclone Fw and 5'-phosphorylated ED primer 1, and the fragment of 3'-ED was amplified with 5-phosphorylated ED primer 2 and XMLP subclone Re. Afterwards 5'- and 3'-ED were cut by ***Eco***RI and ***Xho***I, respectively, and then coligated with ***Eco***RI- ***Xho***I- digested pCS2+. For XMLP SD mutant, the subclone was performed in two steps. First, 5'-SD was amplified from XMLP-pbk-CMV containing full sequence of ***XMLP*** with two primers: CMV-Fw and 5'-phosphorylated SD primer 2, in turn was cut by ***Eco***RI and gel purified. 3'-SD was amplified with 5'-phosphorylated SD primer 3 and XMLP subclone Re, cut by ***Xho***I, gel purified. The 5' and 3' terminal SD were coligated with ***Eco***RI- and ***Xho***I- cut pCS2+. Second, the resultant was amplified with XMLP subclone Fw and Re, and subcloned into pCS2+.

3. Results

3.1 Isolation of novel genes from an activin-treated ectoderm cDNA library of *Xenopus laevis*

I have screened a cDNA library prepared from activin-treated ***Xenopus laevis*** ectoderm, using large-scale whole-mount ***in situ*** hybridization. Based on the expression patterns, potentially interesting clones were subsequently sequenced. The obtained sequences were compared to the DNA and protein sequences in the GeneBank and 51 novel genes were identified. The novel sequences were then submitted to the GeneBank and assigned the Accession Numbers listed in Table 3.1-1. It should be noted that the deposit sequences in GeneBank are updating rapidly every day, therefore some of genes in the list may have also been isolated by other groups after my submission. The results shown in Table 3.1-1 were updated on March 10, 2002.

The cDNA clones encoding the ***Xenopus*** X-box binding protein 1 (XXBP-1), the ***Xenopus*** MARCKS like protein (XMLP) and the ***Xenopus*** arginine:glycine amidinotransferase (XAT) were further characterized because their suggestive expression pattern strongly indicate that they are deeply involved in the early embryogenesis.

Table 3.1-1. Novel genes isolated from an activin-treated ectoderm cDNA library of *Xenopus laevis.*

Accession Number	Main distribution of blast hit of DNA sequence database		
	score	E value	Homologue in ***Xenopus*** or other species
BF348693	178	$2e^{-42}$	***Gallus gallus*** PR264 mRNA, a potential splicing factor. ACCESSION X62446
BF348704	42	0.33	Myxoma virus, complete genome, also similar to SW: HCD2_BOVIN O02691 3-Hydroxyacyl-coa dehydrogenase type II, mRNA sequence. ACCESSION AF170726
BF348705	313	$6e^{-83}$	***Xenopus*** gene encoding a HMG1 homologue. ACCESSION D30765
BF348706	42	0.32	***Rattus norvegicus*** Aquaporin 1 (aquaporin channel forming integral Protein 28 (CHIP) (Aqp1), mRNA. ACCESSION NM_012778
BF348707	172	$1e^{-40}$	***Gallus gallus*** death associated protein 5-like (DAP5), mRNA sequence. ACCESSION AF093110
BF348708	42	0.34	***Homo sapiens*** chromosome 16 clone CTD-2270L9, complete sequence. ACCESSION AC008915
BF348709	40	1.3	***Homo sapiens*** genomic DNA, chromosome 6p21.3, HLA Class I region, section 13/20. ACCESSION AP000514 BA000025,
BF348710	613	e^{-173}	***Xenopus laevis*** xCIRP2 mRNA for cold-inducible RNA binding protein 2, complete cds. ACCESSION AB044535
BF348711	40	1.3	***Homo sapiens*** NF-E2-related factor 3 gene, complete cds. ACCESSION AF135116 5' similar to SW: CKR1_human P32246 C-C Chemokine receptor type 1, mRNA sequence.
BF348712	48	0.005	***Homo sapiens*** 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 2(PFKFB2), mRNA. ACCESSION NM_006212 5' similar to TR: Q9V9B6 Q9V9B6 CG9422 PROTEIN, RNA sequence.
BF348713	42	0.33	***Mus musculus*** GIG18 (Gig18) mRNA, complete cds. ACCESSION AF374476
BF348714	48	0.005	***Xenopus laevis*** rod arrestin gene, promoter, exons 1 and 2, and partial cds. ACCESSION AF053942
BF348715	127	$7e^{-27}$	***Homo sapiens*** similar to RIKEN cDNA 2700064H14 gene (LOC116225), mRNA. ACCESSION XM_057654
BF348694	42	0.3	Human DNA sequence from clone RP11-166O7 on chromosome 10, complete sequence. ACCESSION AL607082
BF348695	44	0.085	Human DNA sequence from PAC 76C18 on chromosome 6q16.1-21. Contains STS. ACCESSION Z98755
BF348696	44	0,085	***Homo sapiens*** 12q22 BAC RPCI11-513P18 (Roswell Park Cancer Institute Human BAC Library) complete sequence. ACCESSION AC007564
BF348697	44	0.084	***Homo sapiens*** chromosome 17, clone hRPK, 601_N_13, complete sequence. ACCESSION AC025775
BF348698	680	0.0	***Xenopus*** X-box binding protein-1 partial sequence. ACCESSION AF358133
BF348699	72	$4e^{-10}$	***Mus musculus*** 10 days embryo cDNA, RIKEN full-length enriched library, clone: 2610015J01, full insert sequence. ACCESSION AK01140
	64	$9e^{-08}$	Human chromosome 14 DNA sequence BAC R-109N23 of library RPCI-11 from chromosome 14 of Homo sapiens (Human), complete sequence. ACCESSION XM_027330
BF348700	632	$1e^{-179}$	***Xenopus laevis*** mRNA for retinoic acid receptor gamma B. ACCESSION X59396

BF348701	42	0.33	Human DNA sequence from clone RP3-401D24 on chromosome 6, complete sequence. ACCESSION AL137184 5' similar to SW: G731_HUMAN P09758 pancreatic carcinoma marker protein ga733-1 precursor.
BF348702	103	$1e^{-19}$	***Mus musculus*** 10 days embryo cDNA, RIKEN full-length enriched library, clone: 2610528A17, full insert sequence. ACCESSION AK012159 5' similar to TR: O46084 O46084 EG:63B12.4 PROTEIN mRNA sequence.
BF348703	668	0.0	***Xenopus laevis*** signal sequence receptor beta subunit mRNA, complete cds. ACCESSION AF346565
AI880935	56	$2e^{-05}$	Homo sapiens chromosome X clone RP11-54I20, RP11-66N11 map q28, complete sequence. ACCESSION AF274858
AI880936	103	$2e^{-19}$	***Geodia cydonium*** mRNA for XPB/ERCC-3-like protein. ACCESSION Y17148
	103	$2e^{-19}$	***Lycopersicon esculentum*** heat shock protein (HSP21) mRNA. ACCESSION U66300
AI880937	1043	0.0	***Xenopus laevis*** MARCKS-related-protein protein mRNA, complete cds. ACCESSION AF187864
AI880938	1423	0.0	***Xenopus laevis*** ATP synthase subunit β mRNA, complete cds. ACCESSION AF187862
AI880939	283	$1e^{-73}$	***X.laveis*** cytokeratin typeII mRNA (cytoskeletal protein). ACCESSION M13811
AI880940	111	$7e^{-22}$	***Urechis caupo*** mRNA for cytoplamic intermediate filament protein (structure protein). ACCESSION AJ004935
	111	$7e^{-22}$	***Homo Sapiens*** dolichy-phosphate beta-glucosyltranferase. ACCESSION AF102850
	111	$7e^{-22}$	***Lycopersicon esculentum*** heat shock protein (HSP21) mRNA. ACCESSION U66300
	111	$7e^{-22}$	***Mus musculus*** mRNA for receptor activity modifying protein 1 (Ramp1 gene). ACCESSION AJ314840
	111	$7e^{-22}$	Human mariner-like element-containing mRNA, clone pcHMT1. ACCESSION U48696
AI880941	1031	0.0	***Xenopus laevis*** glycine amidinotransferase mRNA, complete cds. ACCESSION AF187863
AI880942	147	$1e^{-32}$	***Rana esculenta*** mRNA for acidic ribosomal protein 1 (prp1 gene). ACCESSION AJ298875
AI880943	94	$2e^{-16}$	***Homo sapiens*** annexin A3 (ANXA3), mRNA. ACCESSION XM_034350
AI880944	547	$1e^{-153}$	***Xenopus laevis*** mRNA for high mobility group HMG-17 (hmg-17 gene). ACCESSION AJ272075
AI880945	58	$9e^{-06}$	***Homo sapiens*** similar to unnamed protein product (LOC160622), mRNA. ACCESSION XM_090399
	44	0.14	***Xenopus laevis*** desmoplakin-related protein mRNA, partial cds. ACCESSION AF169314
AI880946	58	$1e^{-05}$	***H.sapiens*** mRNA for sortilin. ACCESSION X98248
AI880947	182	$2e^{-43}$	***Rattus norvegicus*** protein arginine N-methyltransferase (PRMT1) mRNA, complete cds (metabolism). ACCESSION NM_02436
AI880948	1031	0.0	***Xenopus laevis*** liver RNA helicase II/Gu mRNA, complete cds. ACCESSION AF302423
AI880949	44	0.14	***Homo sapiens*** clone IMAGE: 205156, mRNA sequence. ACCESSION AF339786
	44	0.14	***Homo sapiens*** F-box protein FBX10 mRNA, partial cds. ACCESSION AF176705
AI940806	42	0.57	***S.cerevisiae*** DNA from chromosome XII right arm including ACE2, CKI1, PDC5, SLS1, PUT1 and tRNA-Asp genes. ACCESSION X91258
AI940807	113	$2e^{-22}$	***Mus musculus*** interleukin enhancer binding factor 2 (Ilf2), mRNA. ACCESSION NM_026374

AI940808	44	0.15	***Xenopus laevis*** Nkx2-5 (Nkx2-5) gene, promoter region and partial. cds. ACCESSION AF283102.
	44	0.15	***Xenopus laevis*** eomesodermin gene, promoter region. ACCESSION AF179418
AI940809	56	$4e^{-05}$	***Rattus norvegicus*** nuclear ubiquitous casein kinase 2 (Nucks), mRNA. ACCESSION NM_022799
AI940810	147	$6e^{-47}$	***G.gallus*** PR264 mRNA. ACCESSION X62446
AI940811	44	0.14	***Homo sapiens*** similar to putative (LOC115098), mRNA. ACCESSION XM_055230
	44	0.14	***Mus musculus*** cell division cycle 2 homolog (S. pombe)-like 2 (Cdc2l2), mRNA. ACCESSION NM_007661
AI940812	260	$1e^{-66}$	***Homo sapiens*** oxoglutarate dehydrogenase (lipoamide) (OGDH) mRNA (metabolism) ACCESSION XM_004889
AI940813	42	2.2	***Caenorhabditis elegans*** cosmid F20C5 complete sequence ACCESSION Z68161
Full sequence			
AF358133	***Xenopus laevis*** X-box binding protein (XXBP) mRNA, complete cds		
AF187862	***Xenopus laevis*** ATP synthase subunit β mRNA, complete cds		
AF187863	***Xenopus laevis*** glycine amidinotransferase (XAT) mRNA, complete cds		
AF187864	***Xenopus laevis*** MARCKS-related-protein protein (XMLP) mRNA, complete cds		
AF346565	***Xenopus laevis*** signal sequence receptor beta subunit mRNA, complete cds		

3.2 XXBP-1: a leucine zipper transcription activator involved in BMP-4 signaling pathway

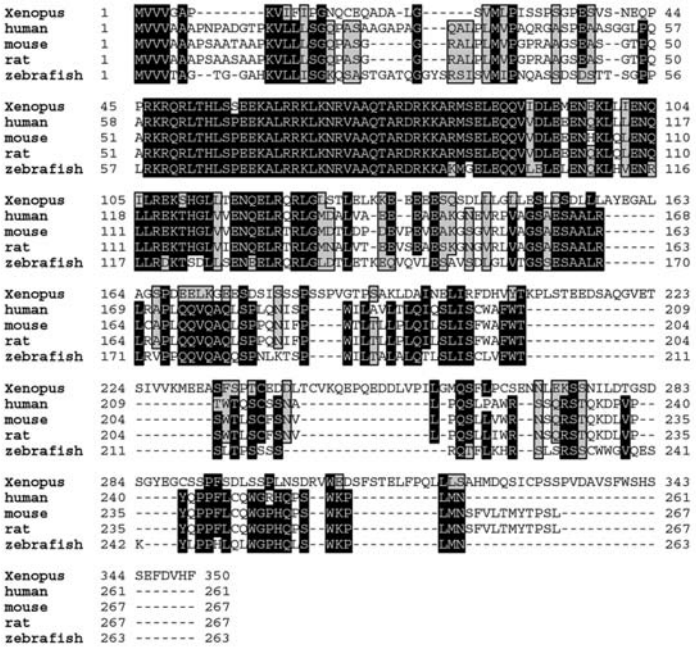
3.2.1 XXBP-1 is a new member of the basic leucine zipper transcription factors

The sequence of the ***XXBP-1*** cDNA (the GeneBank accession number: AF358133) revealed an open reading frame encoding a polypeptide of 350 amino acids. Between amino acids 85 and 121 of the protein, there is a hypothetical leucine zipper sequence characteristic of basic leucine zipper (bZIP) transcription factors, in which six leucines are spaced seven residues apart. NH_2-terminal flank to these heptad repeats of leucines, it is a basic domain abundant in arginine or lysine (Figure 3.2-1A). The predicated amino acid sequence is most similar to human X-Box binding protein 1 (hXBP-1, NM_005080, 66% amino acid identity; Liou ***et al.***, 1990), rat XBP-1 (JC4857 66% amino acid identity), mouse XBP-1 (NP_038870, 66% amino acid identity), and Zebrafish Treb5 (AY029577, 59% amino acid identity) as shown in Figure 3.2-1B. Although the sequences outside the bZIP domain are divergent compared to these proteins, it shares amino acid sequence of the inside leucine zipper domain and of the adjacent basic domain with them. Therefore it is likely to be

the *Xenopus* homologue and was named as *Xenopus X-box binding protein 1* (*XXBP-1*).

MVVVGAPKVIFIPGNQCEQADALGSVMLPISSPSGPESVSNEQPPRKRQR
LTHLSSEEKALRRKLKNRVAAQTARDRKKARMSELEQQVIDLEMENEKLL
IENQILREKSHGLLTENQELRQRLGLSTLELKKEEEEESQSDLLLGLLES
LDSDLLLAYEGALAGSPDEELKGEESDSISSSPSSPVGTPSAKLDAINEL
IRFDHVYTKPLSTEEDSAQGVETSIVVKMEEASFSPTCEDDLTCVKQEPQ
EDDLVPILGMQSFLPCSENNLEKSSNILDTGSDSGYEGCSSPFSDLSSPL
NSDRVWEDSFSTELFPQLLLSAHMDQSICPSSPVDAVSFWSHSSEFDVHF

(A)



(B)

Figure 3.2-1. XXBP-1 protein sequence and the alignment with its homologues. (A) Deduced protein sequence of XXBP-1. The bZIP domain is underlined. The DNA sequence of *XXBP-1* can be accessed in GeneBank using accession number: AF358133. **(B) The alignment of XXBP-1 and its homologue in human, mouse, rat, and zebrafish.** Black boxes indicate the conserved amino acids, and the gray boxes, the similar amino acids. Dashes represent gaps introduced into the sequence to obtain optimal alignment.

3.2.2 Spatial expression pattern of XXBP-1 suggests a role in BMP-4 signaling pathway

By whole-mount ***in situ*** hybridization (Figure 3.2-2), the expression of ***XXBP-1*** was first detectable faintly in the animal halve of embryo before the gastrulation. As the gastrulation proceeded, the staining was intensified, becoming more concentrated on the dorsal blastopore lip (stage 10, 11; Nieuwkoop and Faber, 1975). In addition, the signal was also detected on the whole ventral marginal zone and the animal halve. With the gastrulation ongoing, the staining was graded from the blastopore region anteriorly. The perspective neural plate appeared devoid of staining, while concurrently the signal was detected on the ventral side. At the middle neurula stages, ***XXBP-1*** were expressed ventrolaterlly at a lower level but the signals stretching from anterior to posterior along the neural crest were concentrated on the area adjacent to the most anterior of neural plate that corresponds to the cement gland primordium. This expression pattern is reminiscent of BMP-4, whose expression signals are also maximal in the cement gland primordium at this stage. Hence it inspired me to explore whether ***XXBP-1*** is involved in BMP-4 signaling pathway in further studies. In addition, a signal zone along the bottom of neural plate was detected on the notochord corresponding to the previous pattern on the dorsal blastopore lip. In the late neurula or early tailbud stages, the staining was found to be strong on the cement gland, stretching weakly to the hatching gland (Figure 3.2-2L, M). An additional signal territory also extended from the cement gland to the ventral side as shown in Figure 3.2-2M, and a more ventral view of this embryo was shown in Figure 3.2-2N. Though there is some evidence that human XBP-1 plays very important roles in the fetal liver development (Reimold ***et al.***, 2000), ***XXBP-1*** expression was not likely correlated with the liver primordium. The transversal section shown in Figure 3.2-3B indicated that staining zone lay closely under the ventral ectoderm, but not on the area of perspective liver. In the tailbud stages the signals lay on the cement gland as well as the pronephros (mainly on the glomus part), which was then confirmed by histological analyses shown in Figure 3.2-3C. By deliberately overstaining, the signals were also detected on the ear vesicle and the upper part of lens in the tailbud stages (3.2-2O—R).

In summary, the expression pattern of ***XXBP-1*** partially overlaps that of ***BMP-4*** and suggests that ***XXBP-1*** may play a role in dorsoventral patterning.

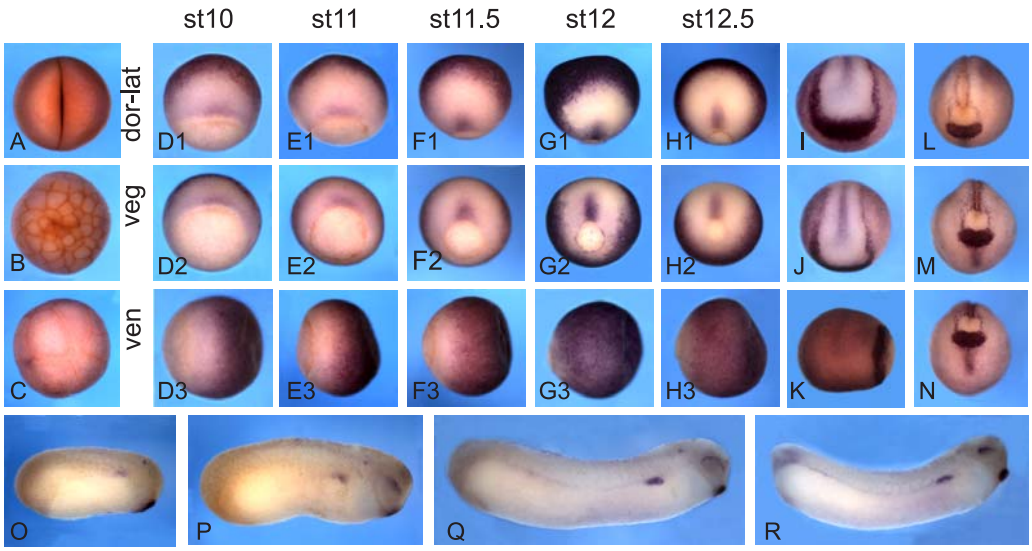


Figure 3.2-2. The spatial expression of *XXBP-1* revealed by whole-mount *in situ* hybridization. The expression of *XXBP-1* signal is detectable faintly in the animal halve of embryos at 2-cell stage (A) and stage 7 (B) before the gastrulation. (C) is the vegetal view of (B). As gastrulation proceeds, the staining is intensified, becoming more concentrated on the dorsal blastopore lip, and is also found on the whole ventral ectoderm (stage 10, D1-D3; stage 11, E1-E3). With the gastrulation going on (stage 11.5, F1-F3; stage 12, G1-G3 and stage 12.5, H1-H3), the staining is graded from the blastopore region anteriorly; the perspective neural plate appears devoid of staining, while concurrently ventral ectoderm is stained. (I), (J) and (K) show the anterior, dorsal and ventral views of the stage 15 embryo, respectively. Signals are concentrated on the area of the most anterior of neural plate (the prospective cement gland), stretching posteriorly along the neural crest in gradient. (L) and (M) display the anterior view of embryo at stage 19 and 20, respectively. The staining was detected strongly on the cement gland, stretching posteriorly to form weak signal strips on the hatching gland. An additional signal zone extends from the cement gland to the ventral side (N) (ventral view of the stage 20 embryo shown in M). (O)—(R) exhibit the expression pattern in a variety of tissues after the middle tailbud stages: the cement gland, the pronephros (mainly on the glomus part), the ear vesicle and the upper part of lens. (dor-lat: dorsal-lateral views of the gastrula embryos; veg: vegetal views of the gastrula embryos; ven: ventral views of the gastrula embryos)

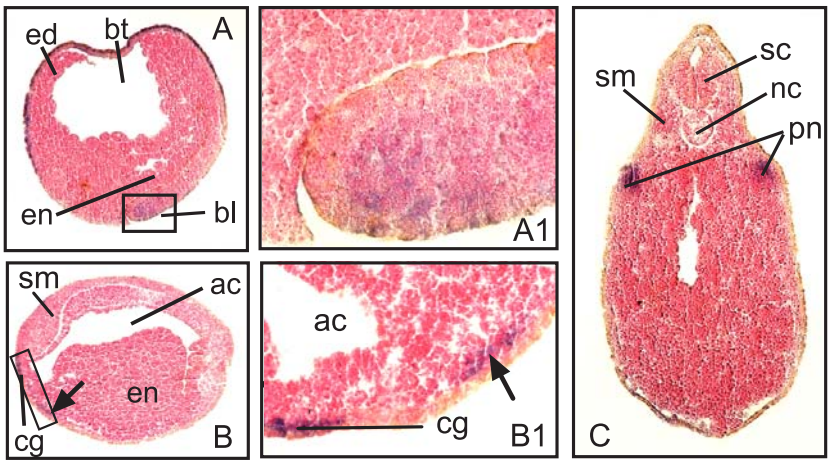


Figure 3.2-3. Histological analyses of *XXBP-1* expression. (A) The transversal section of stage 12 embryo. (A1) The magnification of the framed part shown in (A). Signals are detected on the ventral ectoderm and dorsal blastopore lip. (B) The view of a sagittal section of stage 18 embryo. Signals lie on the cement gland and some adjacent ventral mesoderm (arrow indicated). (B1) The magnification of the framed part shown in (B). (C) The transversal section of stage 28 embryo showing the *XXBP-1* expression signals on the pronephros (Abbreviation: ac, archenteron; bt, blastocoel; bl, blastopore lip; cg, cement gland; ed, ectoderm; en, endoderm; nc, notochord; pn, pronephros; sc, spinal cord; sm, somite).

3.2.3 The expression of XXBP-1 in the developing embryo and adult tissues

The temporal expression of *XXBP-1* and the expression level in different adult tissues were studied using RT-PCR analyses, which was performed with RNA extracted from embryos at different embryonic stages and that from different adult tissues, respectively, using the *XXBP-1* specific primers. The results revealed that *XXBP-1* was a maternal factor which was weakly expressed before the gastrula stages, up-regulated during the gastrulation and kept in a persistent level in the following stages (Figure 3.2-4). And *XXBP-1* was also shown as a ubiquitous factor which was expressed in all tested tissues with relatively higher expression level found in liver, lung and stomach (Figure 3.2-5).

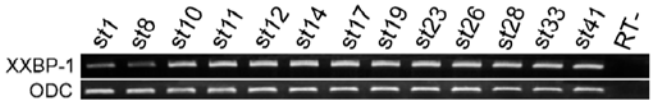


Figure 3.2-4. The temporal expression pattern of *XXBP-1* during the embryonic development revealed by RT-PCR analysis. 1.0 µg of total RNA extracted from embryos at different embryonic stages was used for the reverse transcription, and ODC was employed as internal standard for normalization. *XXBP-1* is a maternal factor which is weakly expressed before the gastrula stages, up-regulated during gastrulation and kept in a persistent level in the following stages. Top: *XXBP-1*; bottom: *ODC*.

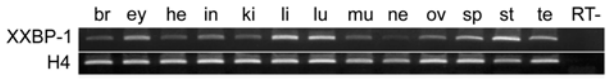


Figure 3.2-5. The expression of *XXBP-1* in different adult tissues revealed by RT-PCR analysis. 1.0 µg of total RNAs extracted from different adult tissues was used for the reverse transcription, and H4 was employed as the loading control. The expression of *XXBP-1* is found in all tested tissues with relatively higher expression level in liver, lung and stomach (br, brain; ey, eye; he, heart; in, intestine; ki, kidney; li, liver; lu, lung; mu, muscle; ne, nerve; ov, ovary; sp, spleen; st, stomach; te, testis).

3.2.4 Down-regulation of XXBP-1 by retinoic acid (RA)

During the embryonic development of *Xenopus laevis*, RA treatment leads to the inhibition of anterior axis, and *XXBP-1* happens to be expressed on the cement gland, it is therefore plausible to investigate the effects of RA on *XXBP-1* expression. Embryos were treated with RA (10^{-6} M) during the stage 8-11 and harvested at the tailbud and neurula stages for whole-mount *in situ* hybridization with *XXBP-1* antisence RNA (Figure 3.2-6), which showed a significant reduction of *XXBP-1* expression in 34 out of 49 RA-treated embryos (87%) compared to the untreated embryos, indicating that RA can down-regulate *XXBP-1* expression in the tailbud and neurula stages. The staining on the cement gland, the sensitive expression domain of *XXBP-1*, was strongly diminished by the RA treatment. This is corresponding to the reduction of anterior axis. In extreme cases shown in Figure 3.2-6A, the staining of *XXBP-1* on the pronephros was also abolished.

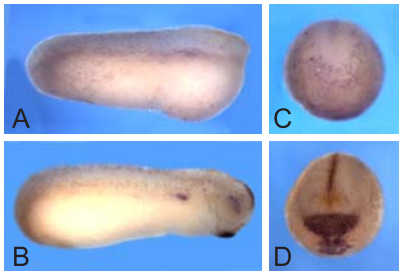


Figure 3.2-6. Effects of retinoic acid (RA) treatment on *XXBP-1* expression revealed by whole-mount *in situ* hybridization. Embryos were treated with RA (10^{-6} M) during the stage 8-11 and harvested at the tailbud and neurula stages for whole-mount *in situ* hybridization with *XXBP-1* antisence RNA. The signals on the treated embryos (A, tailbud and C, neurula) are strongly reduced compared to that on the normal embryos (B, tailbud and D, neurula), indicating that retinoic acid can down-regulate *XXBP-1* expression**.**

3.2.5 Neural inhibitors and MO β-catenin expand the expression zone of XXBP-1 on the dorsal side of embryo

The lack of *XXBP-1* expression on the perspective neural plate during the gastrulation indicates that it may play negative regulatory roles in the neural induction. For this consideration, the effects of anti-neural factors such as *BMP-4*, UV radiation, *Msx1* and *Dlx3* on the expression of *XXBP-1* were investigated by whole-mount *in situ* hybridization. In addition, with respect to the evidences that Wnt/β-catenin pathway play roles in the dorsal ectoderm patterning (Baker *et al.*, 1999; Beanan *et al.*, 2000), whether the morpholino (MO) β-catenin which can block the Wnt/β-catenin signaling pathway has effects on the regulation of *XXBP-1* was also examined. Embryos at the 4-cell stage were injected into two dorsal blastomeres with MO β-catenin (8 ng/embryo), *BMP-4* (1 ng/embryo), *Dlx3* (80 pg/embryo) and *Msx1* (80 pg/embryo) mRNA, respectively. The UV treatment was performed as described in 2.2.2. The treated embryos were grown till the gastrula stages (stage 11-12) for subsequent whole-mount *in situ* hybridization with *XXBP-1* antisense RNA. Figure 3.2-7 shows that all these factors were able to expand the expression region of *XXBP-1* on the dorsal side of the embryos. In the case of *BMP-4* (98%, 42 out of 43 embryos), MO β–catenin (100%, 73 out of 73 embryos), UV (100%, 23 out of 23 embryos) treated embryos, *XXBP-1* was activated in the dorsal marginal zone,

resulting in unrestricted expression of the gene in the entire marginal zone. While *Msx1* (70%, 16 out of 23 embryos) and *Dlx3* (100%, 29 out of 29 embryos) could expand the ventral expression territories of *XXBP-1* to the prospective neural plate. The discernable staining was found covering the perspective neural plate. These results indicated that *XXBP-1* expression could be up-regulated by the known neural inhibitors; therefore it is likely that *XXBP-1* is involved in the differentiation of epidermis. Moreover, *XXBP-1* may also be a negative target of Wnt/β–catenin pathway because the blockage of this pathway by MO β-catenin also led to the expansion of expression territories of *XXBP-1*.

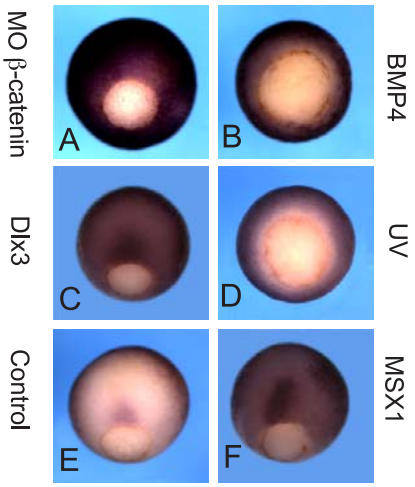


Figure 3.2-7. Neural inhibitors and morpholino β-catenin expand the expression territories of *XXBP-1*. 4-cell stage embryos were injected into two dorsal blastomeres with morpholino β-catenin (8 ng/embryo, A), *BMP-4* (1 ng/embryo, B), *Dlx3* (80 pg/embryo, C) and *Msx1* (80 pg/embryo, F) mRNA, respectively, and were harvested at the gastrula stages for whole-mount *in situ* hybridization with *XXBP-1* antisence RNA. UV-treated and normal embryos were exemplified in (D) and (E), respectively. All these factors can expand the expression area of *XXBP-1*.

3.2.6 Disruption of neural marker genes by overexpression of XXBP-1

The expression pattern of ***XXBP-1*** suggests a role in antagonizing neural induction and in 3.2.5 it has been shown that ***XXBP-1*** expression could be up-regulate by known neural inhibitors. Next it is plausible to ask whether it inhibits neural markers. To address this issue, 200 pg of ***XXBP-1*** mRNA was co-injected with 100 pg of β-***galactosidase*** (***LacZ***) mRNA which was served as lineage tracer into one of dorsal blastomeres of 4-cell stage embryos. Grown to the late neurula stages (stage 18-19), the injected embryos were fixed and stained with X-gal. Embryos exhibiting β-galactosidase activity on one side of the neural tube region underwent whole-mount ***in situ*** hybridization (Figure 3.2-8) using probes for ***XAG2*** (cement gland marker, Aberger ***et al.***, 1998), ***Otx2*** (anterior neural marker, Blitz ***et al.***, 1995), ***Rx2A*** (lens marker, Yoshitake ***et al.***, 1999), ***En2*** (marker for the mid/hindbrain boundary, Hemmati-Brivanlou ***et al.***, 1991), ***Krox20*** (marker for the r3 and r5, Bradley ***et al.***, 1993), ***Pax6*** (neural marker, Hollemann ***et al.***, 1998a), and ***Sox3*** (pan neural marker, Penzel ***et al.***, 1997) respectively. One-side ***XXBP-1*** injection resulted in the reduction of the neural marker ***Otx2*** (60%, 94 out of 156 embryos)***, Rx2A*** (41%, 47 out of 116 embryos)***, En2*** (49%, 31 out of 63 embryos)***, Krox20*** (71%, 34 out of 48 embryos)***, Pax6*** (50%, 28 out of 56 embryos)***,*** and ***Sox3*** (58%, 44 out of 76 embryos). The pronounced suppression was indicated by the diminution or disappearance of the expression territories of these genes. In contrast to the neural markers, no apparent expression variation of ***XAG2*** has been found under the same mRNA injection dose. The inhibition of neural markers suggests that ***XXBP-1*** may play an instructive role in the formation of epidermal tissue. However, it should be considered that one-side injection of ***XXBP-1*** could cause wrong morphogenetic movement revealed by the depression at the injected side or the defect of gastrulation in some cases. Therefore, it cannot be exclude that the interfered morphogenetic movement may also lead to the disruption of neural markers. For this consideration, the animal cap assay was carried out as described in 3.2.10.

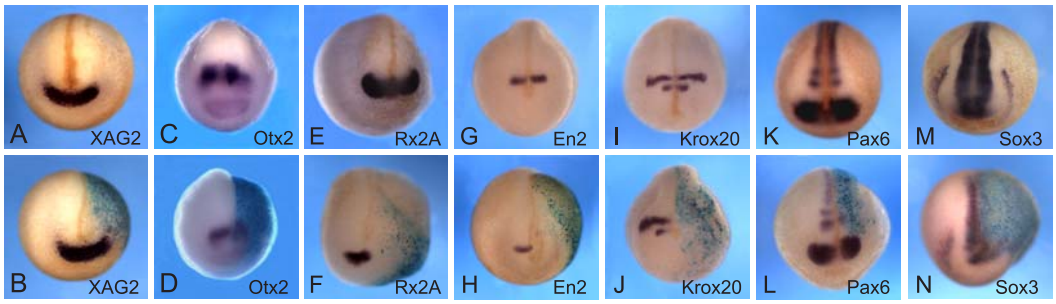
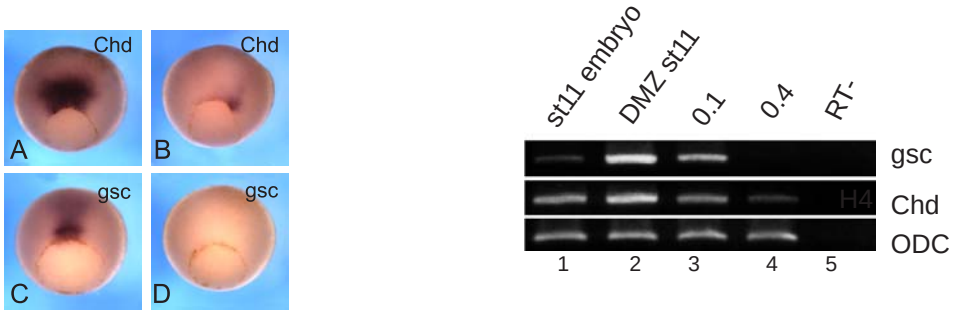


Figure 3.2-8. Overexpression of *XXBP-1* suppresses neural marker genes. The one-side suppression of neural markers was revealed by whole-mount *in situ* hybridization with antisense probes of these neural genes. 200 pg of *XXBP-1* mRNA together with 100 pg of *LacZ* mRNA were co-injected into one dorsal blastomere of 4-cell stage embryos. The surviving embryos developed till the late neurula stages. The injected side was visualized by lacZ staining. (A) The normal expression pattern of the cement gland marker, *XAG2*. (B) *XAG2* was not significantly affected under this dose. (C), (E), (G), (I), (K) and (M): The control expression pattern of *Otx2* (anterior neural marker), *Rx2A* (lens marker), *En2* (marker for the mid/hindbrain boundary), *Krox20* (marker for the r3 and r5), *Pax6* (neural marker) and *Sox3* (pan neural marker), respectively. (D), (F), (H), (J), (L) and (N): The expression of these neural maker genes (see above in order) were inhibited in the injected side of embryos.

3.2.7 XXBP-1 suppresses the dorsal mesoderm markers chordin (chd) and goosecoid (gsc)

Inspection of *XXBP-1* expression within the gastrula stages revealed the staining on the dorsal blastopore lip as described in Figure 3.2-2, which suggests that it may interact with genes affecting the dorsoventral patterning of the marginal zone. I therefore studied the interactions between *XXBP-1* and the dorsal mesoderm marker genes *chordin* (*chd)* and *goosecoid (gsc)* using whole-mount *in situ* hybridization and dorsal marginal zone (DMZ) analysis. The embryos injected dorsally to the equatorial region at the 4-cell stage with *XXBP-1* mRNA (0.1 ng/blastomere) were harvested at the stages 11-11.5 for whole-mount *in situ* hybridization with *chd* and *gsc* antisense RNA. The results showed that the injection of *XXBP-1* led to the suppressions of *chd* (65%, 37 out of 57 embryos) and *gsc* (75%, 42 out of 56 embryos) revealed by their diminished staining on dorsal blastopore lip (Figure 3.2-9I). This was further confirmed by DMZ analyses (Figure 3.2-9II). Four-cell stage embryos were injected into the equatorial region of four blastomeres with 0.1 ng/blstomere or 0.4

ng/blstomere of *XXBP-1* mRNA. DMZs were dissected at the early gastrula stage (stage 10-10.5), and cultured till sibling embryos developed to the stage 11. Using *chd* and *gsc* specific primers, RT-PCR analyses showed that injection of *XXBP-1* acted in a dose-dependent manner to down-regulate the dorsal mesoderm markers *chd* and *gsc* in the DMZ.



(I) (II)

Figure 3.2-9. Overexpression of *XXBP-1* down-regulates the dorsal mesoderm markers *chordin* (*chd*) and *goosecoid* (*gsc*).

(I) Ectopic expression of *XXBP-1* suppresses *chd* and *gsc* at gastrula stages revealed by whole-mount *in situ* hybridization. The embryos were injected into marginal zone at 4-cell stage with 0.1 ng/blastomere of *XXBP-1* mRNA and harvested at stages 11-11.5 for whole-mount *in situ* hybridization using *chd* and *gsc* antisense RNA, respectively. (A) and (C) Normal gastrula embryos. (B) and (D) *XXBP-1*-injected gastrula embryos blotted by c*hd* antisense RNA and *gsc* antisense RNA, respectively. The injection of *XXBP-1* leads to the suppression of *chd* and *gsc*.

(II) Overexpression of *XXBP-1 d*own-regulates *chd* and *gsc* revealed by RT-PCR analysis. Embryos were injected into the equatorial region of four blastomeres at 4-cell stage with 0.1 or 0.4 ng of *XXBP-1* mRNA per blastomere, and DMZ was then dissected and harvested at stage 11 for RT-PCR analyses. st11, stage 11 embryo (lane 1); DMZ st11, *XXBP-1*-unjected DMZ (lane 2); 0.1, DMZ from embryos injected with 0.1 ng/blastomere of *XXBP-1* mRNA (lane 3); 0.4, DMZ from embryo injected with 0.4 ng/blastomere *XXBP-1* mRNA (lane 4); RT-, control without reverse transcriptase (lane 5). The expressinon of *chd* and *gsc* is suppressed.

3.2.8 Overexpression of XXBP-1 leads to embryos ventralization

The expression pattern of *XXBP-1* shows overlaps to that of *BMP-4* and suggests that *XXBP-1* may play a role in dorsoventral patterning of embryonic development, which inspired me to further investigate this hypothesis using gain-of-function approach.

Embryos injected into two dorsal blastomeres at the 4-cell stage with 0.2 ng of ***XXBP-1*** mRNA per embryo showed a range of ventralization phenotype (93%, 81 out of 87 embryos, Figure 3.2-10) according to dorsoanterior index 4-2 (DAI, 4-2) (Kao and Elinson, 1988), which demonstrated the suppression of anterior axis. The most prominent abnormalities were the suppression of anterior-dorsal structures including the cement gland, eyes, otic vesicles and in many cases, the entire heard. With a dose of 0.4 ng of ***XXBP-1*** mRNA per embryo, the injected embryos showed the loss of head structure and axis defect and most of them could be scored as DAI 2 or 1 (100% ventralization, 99 out of 99 embryos). With the highest dose (0.8 ng/embryo) injected into either two dorsal or all four blastomeres of 4-cell stage embryos, the embryos also exhibited ventralization. The extreme cases showed the complete loss of axial structures characteristic of maximally ventralized embryos with DAI of 0 or 1 (93% ventralization for the dorsal injection, 38 out of 41 embryos; 100% ventralization for the radial injection, 21 out of 21 embryos). The representative phenotypes were shown in Figure 3.2-10, whose details were further revealed by histological analyses. Compared to the normal (Figure 3.2-10G), the injected embryos (Figure 3.2-10H) demonstrated the reduction of notochord as well as the indiscernible brain or neural tube. The transversal sections of injected embryos (shown in Figure 3.2-10E) even indicated the loss of notochord (data not shown). The effects of injection were dose-dependent and much less pronounced if ventral blastomeres were targeted. When injected ventrally with 0.2 ng of ***XXBP-1*** mRNA per embryo, 7 out of 56 embryos (12.5%) appeared normal, 8 (14%) showed ventralization (DAI 5-4) and 41 (73%) showed posterior enlargement. While when injected ventrally with 0.8 ng, 17 out of 17 embryos (100%) exhibited mild microcephcephaly.

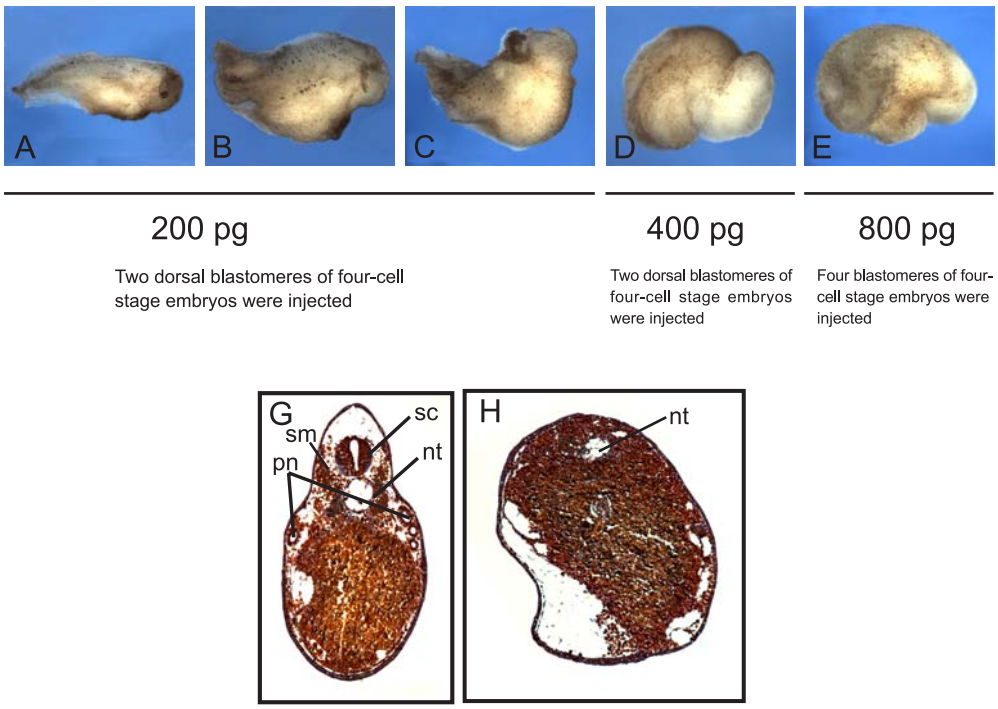


Figure 3.2-10. Phenotypes of *XXBP-1*-injected embryos. (A)—(C) The range of ventralization phenotypes of embryos injected dorsally at 4-cell stage with 200 pg of *XXBP-1* mRNA. (D) and (E) The extreme phenotype of embryos injected at 4-cell stage into two dorsal with 400 pg of *XXBP-1* mRNA, and into four blastomeres with 800 pg of *XXBP-1* mRNA, respectively. The injected embryos completely lost the anterior-posterior and dorsal-ventral axis. (G) The transversal section of sibling control embryo. (H) The transversal section of *XXBP-1* injected embryo shown in (B). Noted that the reduction of notochord as well as the indiscernible brain or neural tube (Abbreviation: nt, notochord; pn, pronephros; sm, somite; sc, spinal cord). Overexpression of *XXBP-1* suppresses dorsoanterior structure of the injected embryos in a dose-dependent manner.

3.2.9 Overexpression of XXBP-1 leads to ventralization of dorsal mesoderm

Ventralization of cell fates was further corroborated by analyses of the expression of marker genes involved in the dorsoventral patterning. Gastrula-stage ventral marginal zone (VMZ) and dorsal marginal zone (DMZ) explants were dissected from embryos microinjected into the equatorial region of four blastomeres at the 4-cell stage with low and high concentrations of *XXBP-1* mRNA, respectively, thereafter cultured till the stage 22, and assayed by RT-PCR for the relative expression level of various maker genes (Figure 3.2-11).

Gsc, a marker for head mesoderm in tailbud embryos, was repressed strongly by ***XXBP-1***. Likewise, ***Xnot***, a marker for notchord, was also suppressed, but in a less sensitive manner compared to ***gsc***. This indicates that the formation of both head mesoderm and notochord is affected by ***XXBP-1***. The suppression of ***Otx2*** was pronounced, which confirms the results shown in Figure 3.2-8D. Unexpectedly, there was no significant decrease in the expression of ***chd***, which appears controversial to the results of whole-mount ***in situ*** hybridization with gastrulae injected with ***XXBP-1*** shown in Figure 3.2-9IB. A similar DMZ analysis was then carried out with mRNA from stage 11 DMZ dissected from ***XXBP-1*** injected embryos (Figure 3.2-9II). The results were consistent with that of whole-mount ***in situ*** hybridization. Considering that 65% of injected embryos showed decreased expression domain of ***chd***, it is likely that RT-PCR may not always reflect the insensitive repression. Alternatively the difference of culture period might account for the indiscernible decrease. The expression of ***ADMP*** varied with different injection doses. It was enhanced when injected with 0.1 ng of ***XXBP-1*** mRNA per blastomere but attenuated when injected with 0.4 ng of ***XXBP-1*** mRNA per blstomere, compared to the untreated DMZ. The loss of dorsal mesoderm marker genes expression paralleled a gain in expression of intermediate and ventro-posterior mesoderm markers. ***Cardiac actin***, a marker for dorsolateral mesoderm, was induced by ***XXBP-1*** at low concentration. However the transcription factor ***MyoDb*** was down regulated, indicating that it may be more sensitive to the induced ventralization. Dose-dependent induction of ventral marker genes by ***XXBP-1*** was varied for the different markers. ***Xven-2*** was steadily induced, while ***Xhox3, Xvent-1, BMP-4*** showed dose-dependent inducible fashion. The expression of ***XAG1*** was kept stably with the low dose injection, but was suppressed with the high dose injection. Taken together, overexpression of ***XXBP-1*** in the explanted dorsal marginal zones led to a down-regulation of dorsal marker genes, and in parallel, up-regulation of ventral marker genes.

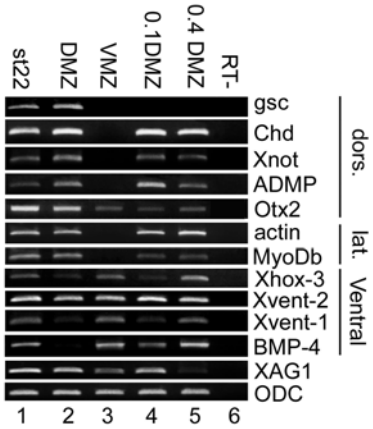


Figure 3.2-11. Overexpression of *XXBP-1* leads to ventralization of dorsal mesoderm. 4-cell stage embryos were either uninjected (st22, DMZ, VMZ, -RT) or microinjected into the equatorial region of four blastomeres of 4-cell stage embryos with increasing amounts of *XXBP-1* mRNA (indicated on the top in ng mRNA per blastomere). Dorsal marginal zone (DMZ) and ventral marginal zone (VMZ) were explanted at the early gastrula stage (stage 10-10.5), subsequently incubated until sibling embryos reached stage 22. 1.0 ng of total RNA was employed for the reverse transcription and analyzed by RT-PCR for the expression of *gsc*, *chd*, *Xnot*, *ADMP*, *Otx2*, *cardiac actin*, *MyoDb*, *Xhox-3*, *Xven-1*, *Xvent-2*, *BMP-4*, *XAG1*. *ODC* served as the loading control. St22, RNA isolated from the whole embryos at stage 22; RT-, control without reverse transcriptase.

3.2.10 XXBP-1 reverses dorsalization induced by dominant negative BMP-4 receptors

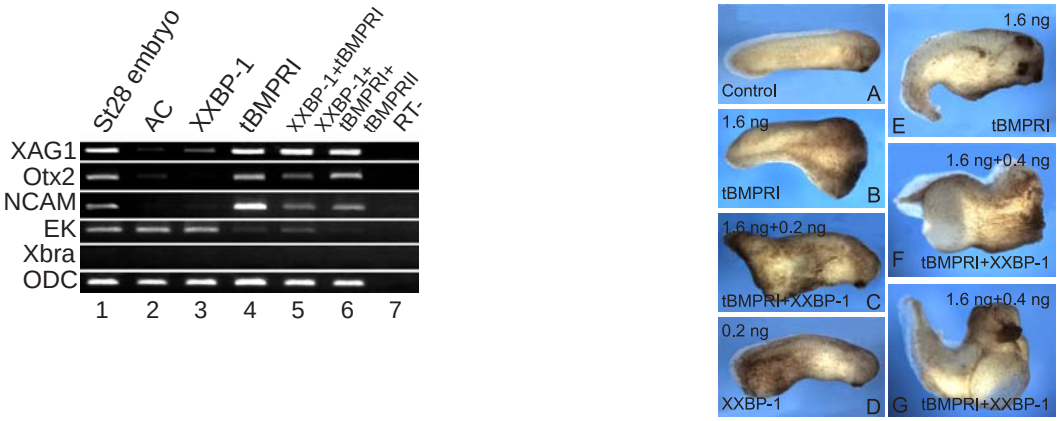
Referred to the expression pattern, both *XXBP-1* and *BMP-4* are excluded from the perspective neural plate during the gastrulation, and their expressions in the neurula stages were quite similar. Moreover, gain-of-function experiments reveal that both of them can induce the ventralization of embryos. This congruence implies that the two factors may act in a common pathway. With respect to this, I next tested the relationship between *XXBP-1* and BMP-4 signaling pathway by using the animal cap assay and gain-of-function assay.

Embryos were injected into four blastomeres at the 4-cell stage with following mRNAs, respectively: 0.4 ng of *XXBP-1*, 1.6 ng of *tBMPRI* (truncate BMP receptor I, Suzuki *et al.*, 1994), a mixture of 1.6 ng of *tBMPRI* and 0.4 ng of *XXBP-1*, and a mixture of 1.6 ng of *tBMPRI,* 0.8 ng of *tBMPRII* (truncte BMP receptor II; Frisch,

1998) and 0.4 ng of ***XXBP-1***. RT-PCR analyses (Figure 3.2-12I) were carried out with RNA extracted from animal caps dissected from these embryos. The epidermis markers ***epidermal keratin*** (***EK***) was strongly expressed in both untreated and ***XXBP-1***-injected animal caps, while a mild increase expression of ***XAG1*** and a down-regulation of ***Otx2*** were found in the ***XXBP-1***-injected animal caps. As described elsewhere (Suzuki ***et al.***, 1994; Sasai ***et al.***, 1995), ectopic expression of ***tBMPRI*** in ectoderm resulted in neural induction which was revealed by a robustly increase in the expression of neural markers ***Otx2*** and ***NCAM***, and a decrease in the expression of ***EK*** in the animal cap assay. In the present study, the expression of ***EK*** was partially restored accompanying with decreases in expressions of ***Otx2*** and ***NCAM*** when ***tBMPRI*** (1.6 ng) and ***XXBP-1*** (0.4 ng) co-injected into the ectoderm. In the case of co-injection of ***tBMPRI*** (1.6 ng), ***tBMPRII*** (0.8 ng), and ***XXBP-***1 (0.4 ng)***,*** the expression levels of ***NCAM*** and ***Otx2*** were moderate in between those obtained with injections of ***tBMPRI*** alone and the combination of ***tBMPRI*** and ***XXBP-1.*** Taken together, the ectodermal cell receiving mixers of ***tBMPRs*** and ***XXBP-1*** adopted a cell fate prior to epidermis. ***XXBP-1*** was able to rescue epidermal fate and block neural induction imposed by ***tBMPRI*** in animal cap assay. Thus, ***XXBP-1*** may act downstream of BMPs receptors.

This was further confirmed by gain-of-function experiments. 4-cell stage embryos were microinjected either ventrally or radially with mRNA of either ***tBMPRI*** alone or a mixture of ***tBMPRI*** and ***XXBP-1***. The ectopic ***tBMPRI*** led to the formation of secondary embryonic axis when injected ventrally (43%, 23 of 53 embryos, Figure 3.2-12IIB) or dorsalization indicated by the enlargement of anterior structure (100%, 27 of 27 embryos, Figure 3.2-12IIE). The phenotype of dorsalization could be efficiently reversed by the co-injection with ***XXBP-1***. The embryo injected ventrally with a mRNA mixture of ***XXBP-1*** and ***tBMPRI*** (Figure 3.2-12IIC) exhibited similar phenotype of ***XXBP-1*** ventral injection (94.3%, 82 out of 87 embryos, Figure 3.2-12IID), showing a slight repression of anterior axis with a significant posterior enlargement. Additionally the embryos injected radially with ***XXBP-1*** showed a range of phenotypes of ventralization revealed by the regression of anterior structure (92%, 11 out of 12 embryos; Figure 3.2-12IIF, G).

In summary, these data support the hypothesis that ***XXBP-1*** is a component of the BMP-4 signaling pathway and acts downstream of BMP-4 receptors.



(I) (II)

Figure 3.2-12. XXBP-1 reverses the dorsalization induced by tBMPRI.

(I) Gene expression in animal caps injected with indicated mRNAs. Animal caps were dissected from stage 9 embryos injected into 4 blastomeres at 4-cell stage with 0.4 ng of *XXBP-1* (lane3), 1.6 ng of *tBMPRI* (lane4), 1.6 ng of *tBMPRI* + 0.4 ng of *XXBP-1* (lane5), 1.6 ng of *tBMPRI* + 0.8 ng of *tBMPRII* + 0.4 ng of *XXBP-1* (lane6), respectively, and were cultured till sibling control reached stage 28. Overexpression of *XXBP-1* rescues the neural induction induced by *tBMPRI* and *tBMPRII*. The absence of *Xbra* signal verified that the animal caps were not contaminated with mesoderm. St28, RNA isolated from the whole embryos at stage 28 (lane1); AC, RNA isolated from control animal cap (lane2); RT- (lane7), control without reverse transcriptase; tBMPRI, truncated BMPs receptor I; tBMPR II, truncated BMPs receptor II.

(II) Microinjection of XXBP-1 rescues dorsalization induced by tBMPRI. (A) The sibling control. (B) Embryo showing the secondary axis which was microinjected into two ventral blastomeres at 4-cell stage with 1.6 ng of *tBMPRI* mRNA. (C) and (D) Embryos microinjected into two ventral blastomeres at 4-cell stage with a mRNA mixture of 1.6 ng of *tBMPRI* and 0.2 ng of *XXBP-1*, and 0.2 ng of *XXBP-1* mRNA, respectively. Phenotypes of embryos microinjected into four blastomeres at 4-cell stage were shown in (E) (1.6 ng of *tBMPRI* mRNA) and (F, G) (an mRNA mixture of 1.6 ng of *tBMPRI* and 0.4 ng of *XXBP-1*). The dorsalization induced by *tBMPRI* was rescued by co-injection with *tBMPRI* and *XXBP-1*.

3.2.11 Noggin antagonizes XXBP-1 in animal cap assay

To confirm that *XXBP-1* plays a role in BMP-4 signaling pathway, the regulation of *XXBP-1* expression by neuralizing factors such as *noggin*, *Zic3* (Nakata *et al*., 1997) and MO β-catenin was studied using the animal cap assay. Embryos were injected into all four blastomeres at the 4-cell stage with *noggin* (160 pg/embryo), *Zic3* (160

pg/embryo), as well as MO β-catenin (16 ng/embryo). The *XXBP-1* expression manner was monitored by RT-PCR analysis using the RNA extracted from animal caps dissected from the injected embryos described above (Figure 3.2-13). The results indicate that *XXBP-1* was in the same way suppressed by *noggin* as *BMP-4* described elsewhere (Gammill *et al.*, 2000), accompanying with the induction of *NCAM.* Both *XXBP-1* and *BMP-4* could be down-regulated by *Zic3* in an insensitive manner. While the injection of MO β-catenin had no significant effects on *XXBP-1* expression as well as *BMP-4* though the expression domain of *XXBP-1* can be expanded to the perspective neural plate with whole-mount *in situ* hybridization. Thus, it is suggested that the induction of *XXBP-1* by MO β-catenin and BMP-4 requires the involvement of other factors. The differences of the two experimental approaches may also account for the variation.

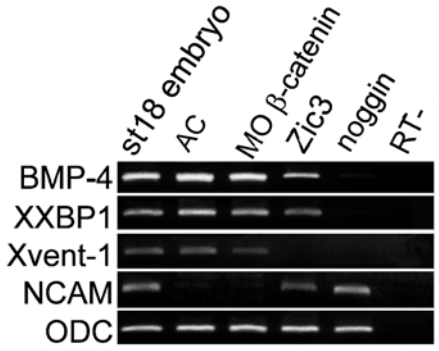


Figure 3.2-13. Down-regulation of *XXBP-1* by ectopic expression of *noggin* in animal cap assay. Animal caps dissected from stage 9 embryos injected into all four blastomeres at the 4-cell stage with 16 ng of morpholino β-catenine, 160 pg of *Zic3*, and 160 pg of *noggin* as indicated, respectively, were assayed by RT-PCR for monitoring the expression of *BMP-4*, *XXBP-1*, *Vent-1 and NCAM*. *ODC was server* as loading control. *Noggin* suppresses simultaneously the expression of *XXBP-1* and *BMP-4*, but induces *NCAM*.

3.2.12 XXBP-1 acts as a transcription activator

The sequence comparison shows that ***XXBP-1*** belongs to basic leucine zipper transcription factors, it naturally gives rise to a question whether it is responsible for activating or repressing transcription. Three repressor and activator fusions were then constructed by fusing the putative DNA–binding domain of ***XXBP-1*** to the previously characterized sequences encoding activating or repressing domain of transcription factors, i.e. ***XXBP1-EnR*** fused with the repressor domain from ***Drosophila engrailed*** (Jaynes and O'Farrell, 1991), ***XXBP1-Eve*** fused with the repressor domain of ***even-skipped*** (Han and Manley, 1993) and ***XXBP1-VP16*** fused with the activator domain of ***VP16*** (Friedman ***et al.***, 1988). The schematic diagram of the XXBP-1 wild type and fusion constructs were shown in Figure 3.2-14I. Theoretically the fusion constructs should phenocopy or at least partially mimic ***XXBP-1*** wild type in the overexpression experiments if they have the same transcription feathers as ***XXBP-1***. Moreover, as ***XXBP-1*** could mediate ***BMP-4*** signaling, it was expected that the loss of ***XXBP-1*** might lead to neural induction in animal cap assay or formation of secondary embryonic axis in overexpression experiments. In addition, based on the expectation that truncated XXBP-1 may sequester the wild-type XXBP-1 and interfere dimerization, a truncated ***XXBP-1*** containing only the sequence encoding basic leucine zipper domain was also constructed.

Overexpression of the fusion constructs with high doses caused apoptosis, the dosages were therefore adjusted to avoid this phenotype. Embryos microinjected dorsally with the same mRNA dose (0.16 ng/embryo) of ***XXBP1-Eve***, ***XXBP1-EnR*** or truncated ***XXBP-1*** showed either gastrulation defect leading to spina bifida or the loss of anterior structure which may also be due to the interference of morphogenetic movement. Embryos with ectopic expression of ***XXBP1-VP16*** (0.04 ng/embryo) exhibited either the strong loss of dorsal-anterior structure as that with overexpression of wild-type ***XXBP-1***, or severe gastrulation blockage called exogastrulation. The results of microinjection were compiled in Table 3.2-1.

The embryos microinjected ventrally with the same dose (0.16 ng/embryo) of ***XXBP1-EnR*** or truncated ***XXBP-1*** also showed spina bifida. However those

microinjected with *XXBP1-VP16* (0.04 ng/embryo) exhibited a similar phenotype of the posterior axis enlargement as wild-type *XXBP-1* (96%, 70 out of 73 embryos), although the reason is still obscure. Overexpression of XXBP1-Eve led to either the secondary axis with a low ratio of 10% or gastrulation defect giving rise to spina bifida (81%, 79 out of 98 embryos) (Figure 3.2-14 and Table 3.2-1). So it is likely that XXBP-1 functions as a transcription activator.

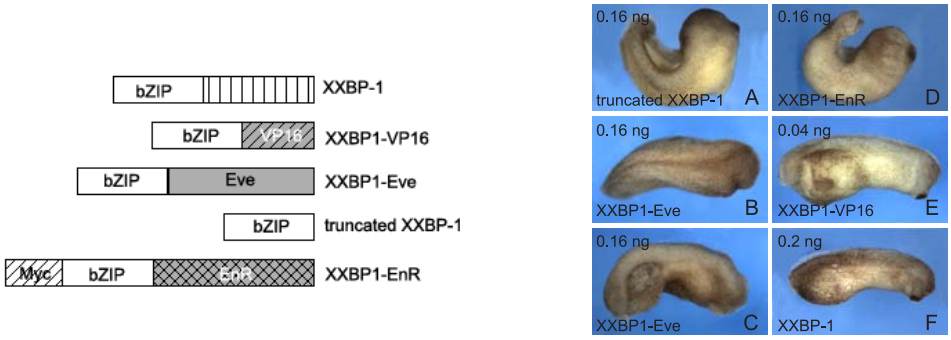


Figure 3.2-14. Overexpression of the fusion constructs and the truncated *XXBP-1*.

(I) Schematic diagrams of the fusion constructs and the truncated XXBP-1. The sequence (amino acids 1-126) containing the basic leucine zipper DNA binding domain (bZIP) of XXBP-1 is shown in white box. The remaining sequence (amino acids 127-350) in carboxyl terminal region is indicated with vertical-dashed box, which was then replaced by VP16 activation domain indicated by dark-dashed box to form XXBP1-VP16, or by even-skipped repressor domain indicated by dark box to form XXBP1-Eve. The sequence encoding the amino terminal region (amino acids 1-126) of XXBP-1 was subcloned into pCS2MTEnR containing coding sequence of engrailed repression domain to obtain XXBP1-EnR. The XXBP1-EnR consists of the Myc domin (write-dashed box), the bZIP domain (write box) and the engrailed domains (dark box with net). Truncated XXBP-1 containing bZIP (amino acids 1-126) was also constructed.

(II) Phenotypes of embryos injected with the different constructs. Embryos were injected into two ventral blastomeres at 4-cell stage with (A) 0.16 ng of truncated *XXBP-1* mRNA, (B, C) 0.16 ng of *XXBP1-Eve* mRNA, (D) 0.16 ng of *XXBP1-EnR* mRNA, (E) 0.04 ng of *XXBP1-VP16* mRNA, (F) 0.2 ng of wild-type *XXBP-1* mRNA. Note that *XXBP1-Eve* induces secondary axis in embryos, but *XXBP1-VP16* induces the phenotype as wild-type *XXBP-1*. The embryos injected with *XXBP1-VP16* and *XXBP-1* wild type show the same enlarged posterior part, suggesting the *XXBP-1* and *XXBP1-VP16* have the same transcription characters.

Table 3.2-1. Phenotypes of ectopic expression of the *XXBP-1* constructs.

mRNA injected	Dose and injection location	Normal	Bent axis	Twin axis	Gastrulation defect	Microcephalic	Posterior enlargent
Truncated XXBP-1	0.08 ng, 2/4V	67 (76%)	11 (13%)		10* (11%)		
Truncated XXBP-1	0.16 ng, 2/4V	24 (27%)	39 (44%)		26* (29%)		
XXBP1-EnR	0.08 ng, 2/4V	5 (8%)	47 (75%)		11* (17%)		
XXBP1-EnR	0.16 ng, 2/4V		22 (35%)		40* (65%)		
XXBP1-Eve	0.16 ng, 2/4V	9 (9%)		10 (10%)	79* (81%)		
XXBP1-Eve	0.08 ng, 2/8V		20 (61%)	1 (3%)	12* (36%)		
XXBP1-VP16	0.08 ng, 4/4					13§ (100%)	
XXBP1-VP16	0.04 ng, 2/4V					3 (4%)	70 (96%)
XXBP1-Eve	0.16 ng, 2/4D	2 (2%)			84* (98%)		
Truncated XXBP-1	0.16 ng, 2/4D	31 (44%)			39* (56%)		
XXBP1-EnR	0.16 ng, 2/4D	13 (18%)			52* (71%)	8 (11%)	
XXBP1-VP16	0.04 ng, 2/4D				27# (54%)	23 (46%)	

The results were scored till injected embryo developed into approximately the stage 32. 2/4D: Two dorsal blastomeres of 4-cell stage embryos were injected; 2/4V: Ventral injection at the 4-cell stage; 2/8V: Two ventral blastomeres were injected at the 8–cell stage; 4/4: all four blastomeres of 4-cell stage embryo were injected. The percentage of the available case is shown in parenthesis.

* the gastrulation defect giving rise to spina bifida.
# the gastrulation defect exogatrulation.
§ severe ventralization with DAI (1-0).

The animal cap assay (Figure 3.2-15) was also carried out to further investigate whether ***XXBP-1*** is transcription activator or not. As expected, neural marker ***NCAM***, ***nrp1*** and ***Otx2*** were induced in the explants by XXBP1-Eve in a dose-dependent manner. However only ***Otx2*** could be slightly induced by truncated ***XXBP-1*** and ***XXBP1-EnR***. Injection of ***XXBP1-VP16*** led to a similar expression manner of the tested markers as ***XXBP-1*** wild type except a weak up-regulation of ***XAG1***. Therefore it supports that ***XXBP-1*** functions as a transcription activator during the embryonic development.

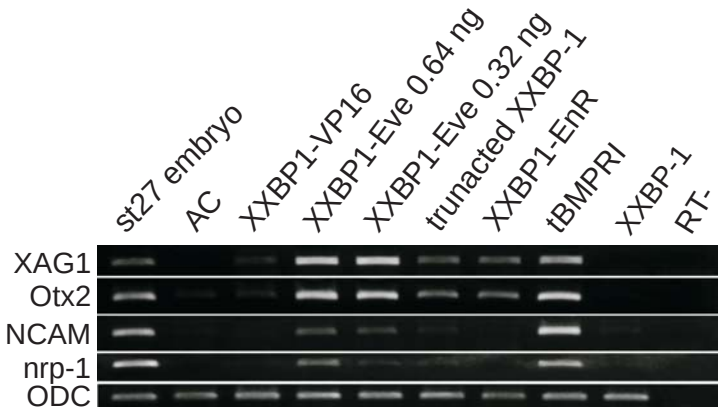


Figure 3.2-15. Animal caps assay with *XXBP-1* constructs. Animal explants were dissected from embryos with mRNAs of *XXBP1-VP16* (0.08 ng/embryo), *XXBP1-Eve* (0.64 ng/embryo), *XXBP1-Eve* (0.32 ng/embryo), truncated *XXBP-1* (0.32 ng/embryo), XXBP1-EnR (0.32 ng/embryo) and *tBMPRI* (1.6 ng/embryo), and *XXBP-1* (0.32 ng/embryo), respectively, and were culture till sibling embryos reached stage 27 for RT-PCR analyses. Note that XXBP1-Eve induces neural marker genes *NCAM* and *nrp1*, suggesting that XXBP-1 funcations as a transcription activator. ODC was employed as a loading control. RT-, samples from the whole uninjected embryo without reverse transcription.

3.3 Isolation and characterization of *XMLP*

3.3.1 Isolation of the gene encoding Xenopus laevis MARCKS like protein (XMLP)

Another clone encoding a predicted polypeptide of 187 amino acids has poor background in the GeneBank. The comparison search indicates that it is most correlated to MARCKS like proteins (MLP), members of MARCKS family (Figure 3.3-1). Although it shares a low identity (32% identity) with the MLPs of mouse, rat, human or rabbit which show a relatively higher identity to each other, the predicted protein sequence of XMLP contains three domains similar to those which are conserved in all other members of MARCKS and MLP families: A myristoylated consensus following the glycine residue at position 2 in the amino terminal region, the site of intron splicing and the putative phosphorylation site domain (PSD). For this reason, it is named as *Xenopus MARCKS like protein* (*XMLP*). The full sequence

was submitted to GeneBank and assigned the accession number: AF187864. At the 5' end, 95 untranslated nucleotides precede the translation initiation site (Kozak, 1986) and at the 3' end the coding region ends with an in-frame stop codon at the position 658, which is followed by a 921-base untranslated sequence. A consensus polyadenylation signal AATAAA is present upstream from the putative poly (A) tail.

The theoretical isoelectric point (pI) of XMLP is 4.38 and that of PSD are 12.06 (http://www.expasy.ch/tools/pi_tool.html), both of which are similar to the common character of this protein family (Blackshear, 1993; Ramsden, 2000).

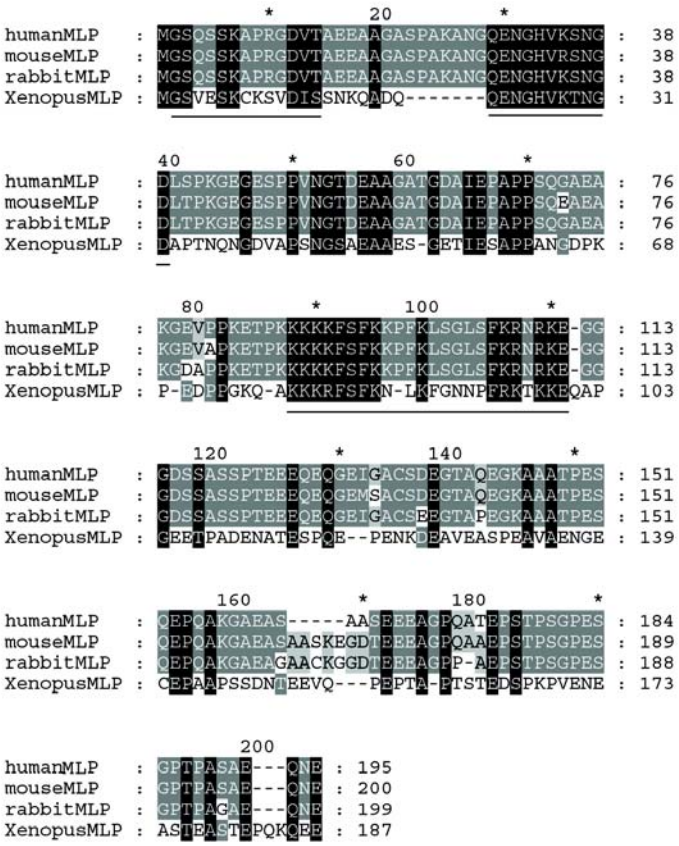


Figure 3.3-1. Comparison of the deduced protein sequence of XMLP with MLPs from human, mouse and rabbit. The black boxes show the conserve amino acids, and the gray boxes, the similar amino acids. The dashes present the gap introduced into the sequence in order to obtain optimal alignment. The similar domains are underlined.

3.3.2 Spatial and temporal expression of XMLP

The whole-mount ***in situ*** hybridization (Figure 3.3-2) revealed that abundant ***XMLP*** maternal transcripts were in the animal halves of embryo during the cleavage stages (Figure 3.3-2A, B). Until the gastrula stages, the signals were detected in almost all regions except the yolk plug (Figure 3.3-2C). Histological analyses showed that ***XMLP*** was predominantly expressed in the ectoderm and mesoderm at the stage 11.5 (Figure 3.3-2D). From the late gastrula stages the transcripts decreased and were restricted to the neural plate (Figure 3.3-2E, F). Transversal section of the neurula (stage 15) depicted the presence of signals in the neuroectoderm (Figure 3.3-2G). Subsequently, the signals have been located in the neural folds and brain area at the early tailbud stage (Figure 3.3-2H, I, M). Sagittal and transversal sections of this stage are shown in Figure 3.3-2J, K and L. At the stage 34 the signals appeared in the head area, somites, gills, pronephros and renal tube (Figure 3.3-2 O, P, Q).

The temporal expression of ***XMLP*** was studied by RT-PCR analyses (Figure 3.3-3), which showed that transcripts could be detected in embryos at any stage. The transcription level was decreased from the uncleaved egg till the stage 23, after which it again rose slightly in the following stages and finally maintained stable. The expression of ***XMLP*** in different adult tissues revealed by RT-PCR analyses (Figure 3.3-4) indicated that ***XMLP*** was abundant in all tested tissues with a relatively low expression level found in intestine and kidney.

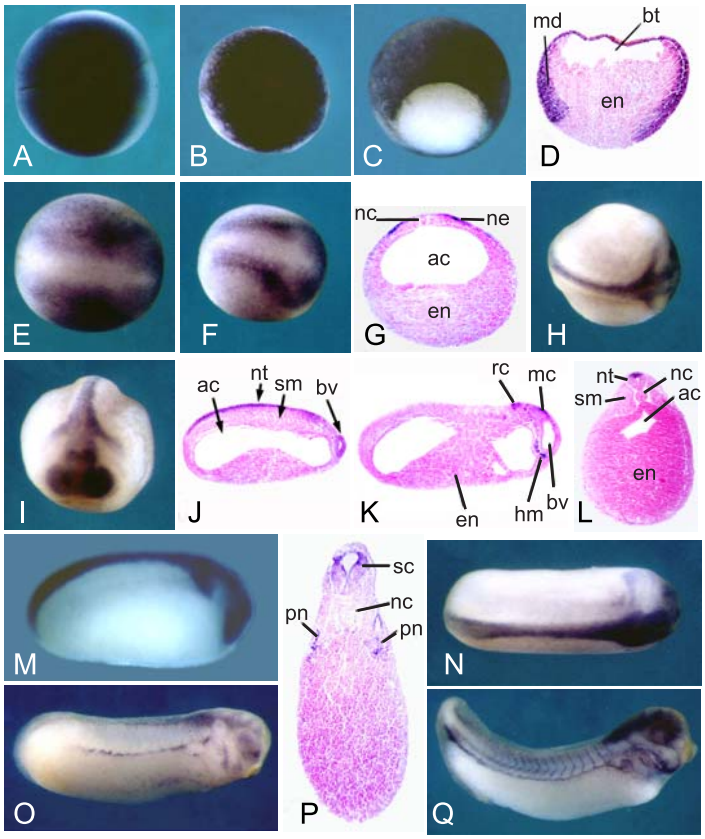


Figure 3.3-2 The spatial expression of *XMLP* revealed by Whole-mount *in situ* hybridization. (A) and (B) Maternally expressed *XMLP* is present in cleave stages. (C) In gastrula stages, signals are detected in the ectoderm and mesoderm. (D) A sagittal section of stage11.5. (E)—(G) At neurula stages, signals are no longer evenly distributed and restricted to neural ectoderm only. (H), (I) and (M) At early tailbud stage, *XMLP* transcripts can only be detected in the neural folds and presumptive brain area. (J) and (K) The sagittal section of (I). (L) A transversal section of stage 21 embryo. (N), (O) and (Q) In later stages *XMLP* is expressed in ectoderm derivatives and pronephros during stage 27—34. (P) A transversal section of stage 34 embryo (ac, archenteron; bt, blastocoel; bv, brain ventricle; en, endoderm; hm, head mesenchyme; mc, mesencephalon; md, mesoderm; nc, notochord; ne, neural ectoderm; nt, neural tube; pn, pronephros; rc, rhombencephalon; sc, spinal chord).

Figure 3.3-3 The temporal expression of *XMLP* in different embryonic stages analyzed by RT-PCR. *XMLP* expression can be detected in embryos at any stage (stage 1—40, Nieuwkoop and Faber, 1975), especially with a high level in the stage 8, when zygotic gene expression just starts. While there is a decrease from the uncleaved egg to the stage 23, after which it again rise slightly in the following stages.

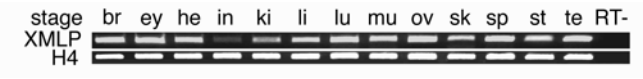


Figure 3.3-4. The expression of *XMLP* in different adult tissues revealed by RT-PCR. *XMLP* transcripts are detected in all tested tissues, however, with a lower level found in kidney and intestine (br, brain; ey, eye; he, heart; in, intestine; ki, kidney; li, liver; mu, muscle; ov, ovary; sk, skin; sp, spleen; st, stomach; te, testis).

3.3.3 Phenotypic effects of XMLP overexpression

XMLP mRNA was injected into one dorsal blastomere of the 4-cell stage embryos. Inspection of gastrula revealed such phenotypes: with the low injection dose, some bulged cells were found to distribute around the former injection site (Figure 3.3-5A). According to the histological analysis (Figure 3.3-5B), these cells contained large nuclei. Such embryos developed quite normally before neurulation, i.e. both dorsal blastopore and yolk plug can be formed correctly. When the injection dose was more than 0.4 ng, bulged cells and mottled surface were shown around the injection area. Some dead cells were found between the vitelline memberane and embryo. The embryos can resilient if this phenotype was not severe. As was true in other studies (Grammer *et al.*, 2000), this is quite similar to the typical phenotype of apoptosis observed in overexpression experiments, i.e. the epithelia disruption and the extrusion of dead cells in the vitelline space in the neurula and tailbud stages. Inspection of tadpoles injected with *XMLP* mRNA revealed two main morphological defects (Table 3.1-1). One was the phenotype of a reduced lens size and abnormal lens shape (Figure 3.3-5C, D, E). Some larvae even showed cyclopia (Figure 3.3-5F). Transversal sections indicated the reduction of neural retina and a part of the diencephalon. In contrast to the normal embryos (Figure 3.3-5G), there was less mesenchyme around the notochord in the forebrain area (Figure 3.3-5H) and smaller or missing eyes (Figure 3.3-5H, I). The other was the phenotype of a bent body axis (Figure 3.3-5D). Both phenotypes were occurred simultaneously when injected with high doses of *XMLP* mRNA. When 0.2 ng of *XMLP* mRNA was injected, 44 % (56 out of 128) of the injected embryos showed lens defects, 1% (1 out of 128) exhibited

bent axis, and 20% (25 out of 128) occurred two phenotypes simultaneously, while when the injection dose was doubled, 27% (59 out of 219) displayed lens defects, 1% (3 out of 219) showed bent axis defects and 33% (71 out of 219) showed lens defects and bent axis defect concurrently. After injection of 0.6 ng, 23% (28 out of 123) showed lens defects, 2% (2 out of 123) exhibited bent axis defects, 70% (86 out of 123) revealed both phenotypes concurrently. In the case of highest dose (0.8 ng), 14% (15 out of 106) showed lens defect, 17% (18 out of 123) exhibits the bent axis defects, 68% (72 out of 108) revealed both phenotypes simultaneous. While control embryos injected with the same quantity of ***LacZ*** RNA developed normally.

Table 3.3-1. Effects of overexpression of *XMLP* in *Xenopus laevis* at the tadpole stage. ***XMLP*** mRNA was injected in one dorsal blastomere of the four-cell stage embryos. The resulting phenotypes were checked at the tadpole stage. The figures in parenthesis show the percentage of available cases.

Injected ***XMLP*** mRNA (ng)	Embryos available (n)	Lens defects	Bent axis defects	Lens defects & bent axis defect
0.2	128	56 (44)	1 (1)	25 (20)
0.4	219	59 (27)	3 (1)	71 (33)
0.6	123	28 (23)	2 (2)	86 (70)
0.8	106	15 (14)	18 (17)	72 (68)

3.3.4 Ectopic expression of XMLP does not change the normal autonomous differentiation of isolated dorsal blastopore lip

It is well known that the isolated dorsal blastopore lip including parts of dorsal ectoderm from stage 10 embryos can autonomously differentiate into notochord, brain structures, and rudimentary eyes (Grunz, 1992, 1999b). This depends on multiple steps including cell interaction, induction and secondary cell interaction. The cooperation of genes expressed in the zone of the Spemann organizer including ***gsc, chd, Xnr3,*** and ***noggin*** drives ectoderm to differentiate into dorsal structures and the central nervous system (for review, see Grunz, 1997). To investigate if lens defects in the ***XMLP*** injected embryos are due to the specific inhibition after overexpression of ***XMLP***, the following experiments were performed. Explants consisting of dorsal blastopore lip and adjacent dorsal ectoderm were isolated from the injected embryos at stage 10 and cultured in Holtfreter's solution until control embryos reached stage

40. Similar to those from uninjected controls, transversal sections showed that these explants had also differentiated into notochord, brain, and eye structure (Figure 3.3-5 J, K), suggesting that *XMLP* does not trigger a specific inhibition of the eye anlage (Figure 3.3-5 K). Therefore the results support the view that the inhibition of eye structures in the injected embryos is the result of disturbed morphogenetic movements rather than a specific inhibition of eye formation.

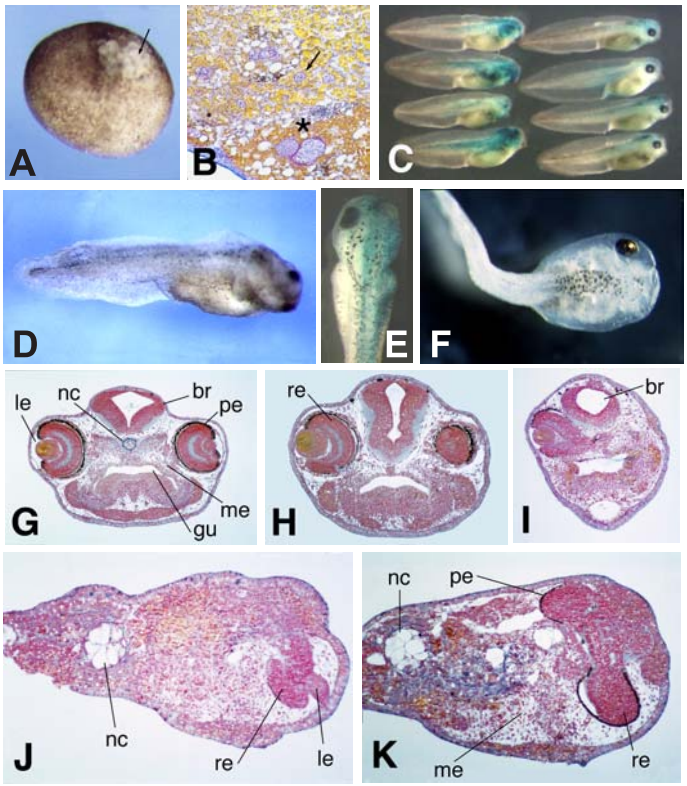


Figure 3.3-5. Phenotypic effects of overexpression of *XMLP*. Embryos were injected into one dorsal blastomere at 4-cell stage. (A) Ectopic *XMLP* results in protrusions of bulge cells distributing around the injection site. (B) The section of an *XMLP*-injected embryo at stage 10 shown in (A). Note that there are some giant nuclei around the injection area (asterisk) and an arrow indicts a normal nucleus. (C) *LacZ* as lineage marker for identifying injected sides of the larva where ectopic *XMLP* caused eye defects. The left column of panel (C) shows the injected sides of the larva and the right column shows the unjected sides of embryos of the same series (compare with larva shown from the dorsal side in (E)). (D) Larva (injected with *XMLP*) with bended axis and one eye. (E) Larva with missing right eye when injected with both *XMLP* and *LacZ*. (F) The injected larvae of stage 40 with one eye only. (G) and (H) The comparison of the histology of notochord in the head area between the normal larvae (G) and larvae with small eye (H). Note that less mesenchyme in (H) are found in the injected embryos compared to the normal ones. (I) Transversal section of the tadpole with one eye shown in (F). (J) and (K) The differentiation of dorsal blastopore lip with adjacent dorsal ectoderm isolated from normal (J) and injected embryos (K). Note that *XMLP*-treated embryos differentiated into notochord and brain structures with a rudimentary eye as the controls. No significant differences have been found in dorsal blastopore lips isolated from either injected or uninjected embryos (abbreviation: br, brain; gu, gut; le, lens; me, mesenchyme; nc, notochord; pe, pigmented epithelium; re, retina).

3.3.5 a morpholino oligo (Mo) against XMLP is effective when injected into cleaving embryos

The loss-of-function of ***XMLP*** was studied by injecting the morpholino oligo (MO) of XMLP. As a new type of antisense oligos, morpholino provides many advantages over phosphorothioates such as excellent sequence specificity, reliable activity in cell and complete resistance to nucleases. MO XMLP was injected into both dorsal blastomeres of 2-cell stage embryos or two dorsal blastomeres of 4-cell stage embryos. Most of the injected embryos showed eye defect, shorten anterior axis and a mild bent axis (Figure 3.3-6 A and Table3.3-2). Up to 24 ng, MO XMLP was introduced into embryos in order to achieve significant results. No significant toxic effects were observed after the injection of morpholino negative control. MO β-catenin was employed as a positive control, injected into two blastomeres of 2-cell stage embryos or two dorsal animal blastomeres of 8-cell stage embryos. Embryos injected in two dorsal animal blastomeres at 8-cell stage with 4 ng of β-catenin MO showed a reduction of the head area and anterior axis (Figure 3.3-6 E). While the injection into each blastomere of two-cell stage embryos with of 8 ng of MO β-catenin prevented any axis formation (data not shown).

Since MO XMLP antisense oligo binds the ***XMLP*** mRNA with high specificity, MO XMLP should strictly compensate the phenotype induced by ***XMLP*** mRNA. To test the specificity of the MO XMLP binding to ***XMLP*** mRNA***,*** synthetic 0.8 ng of ***XMLP*** mRNA together with 16 ng of MO XMLP was co-injected into the two dorsal blastomeres of 4-cell stage embryos. The mRNA did not contain the portion of the 5' UTR of ***XMLP*** so that MO XMLP could not bind the foreign ***XMLP*** mRNA. Inspecting the injected embryos at neurula stage, I found that MO XMLP could significantly rescue the phenotype of apoptosis induced by ***XMLP*** (Figure 3.3-6 B, C)***.*** No embryos out of 48 injected embryos showed apoptosis. 15 normal embryos and 15 embryos with eye defect as well as mild bent axis defect out of 30 survived tadpoles were found in hatched tadpole stages (Figure 3.3-6 D and Table3.3-2). The phenotype induced by MO XMLP was not found any more.

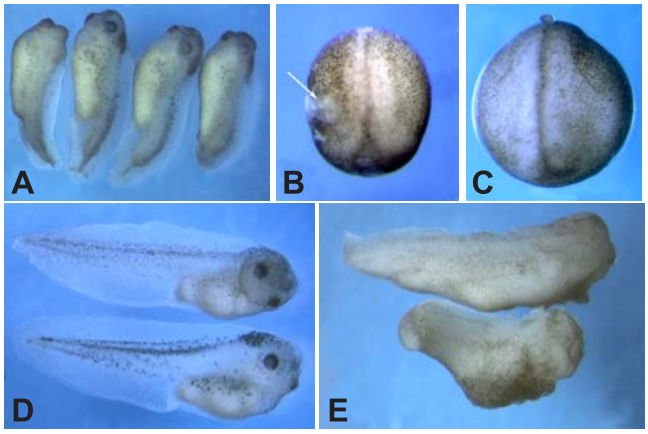


Figure 3.3-6. Effects of injection of Morpholino (MO) XMLP and MO β-catenin. (A) Embryos with shorter anterior axis and eye defects resulted from the injection into both dorsal blastomeres at 2-cell stage with 24 ng of MO XMLP. (B) The phenotype of apoptosis induced by overexpression of *XMLP* (C) The phenotype of apoptosis rescued by MO XMLP (16 ng of MO XMLP and 0.8 ng of X*MLP* mRNA co-injected into both dorsal blastomeres at 4-cell stage. (D) Tadpole co-injected with MO XMLP and *XMLP* mRNA showing relatively normal morphological phenotype or mild bent axis (the upper one). (E) Larvae with reduced anterior axis after microinjecting into two dorsal animal blastomeres at 8-cell stage with 4 ng of MO β-catenin.

Table 3.3-2. Effects of microinjection of Morpholino (MO) XMLP. The results were scored when the embryos developed till around stage 40. 2/2: two blastomeres of 2-cell stage embryos were injected; 2/4d: two dorsal blastomeres of 4-cell stage embryos were injected. The percentage of the available cases is shown in parenthesis.

MO or mRNA injected	Injection location	Phenotypes of the embryos		
		Normal	Mild bent axis only	Shorten trunk, mild bent axis, eye defects, reduced head area
MO XMLP 16 ng	2/2	63 (46)	13 (9)	61(45)
MO XMLP 24 ng	2/2	0 (0)	7 (23)	23 (77)
MO XMLP 8 ng	2/4d	46 (66)	7 (10)	17 (24)
MO XMLP 16 ng	2/4 d	48 (62)	7 (9)	22 (29)
MO XMLP 24 ng	2/4d	4 (8)	0 (0)	47 (92)
XMLP 0.8 ng + MO XMLP 16 ng	2/4d	15 (50)	15[a] (50)	–

[a]The 15 injected tadpoles with XMLP 0.8 ng together with MO XMLP 16 ng showed lens defect and mild bent axis defect simultaneously.

3.3.6 The glycine residue at position 2 and the PSD (ED) domain are correlated with apoptosis function in overexpression experiments

To gain insight into the role of functional domains of XMLP, four mutants were constructed (Figure 3.3-7, see 2.9.3) in which (1) glycine 2 was replaced with alanine (G2A) in order to introduce adverse effect to the myristoylation consensus; (2) serine 83 was changed into alanine (S83A) to prevent phosphorylation of serines in putative ED (PSD) since phosphorylation plays a functional role in ***in vitro*** experiments of other members of MARCKS family; (3) the splicing domain from position 22 to 27 was deleted to yield XMLP SD deletion mutant (SD); and (4) the region between lysine 79 and serine 83 was deleted to yield XMLP ED deletion mutant containing truncated ED domain (PSD). The mRNAs of ***XLMP*** wild type and these mutants were microinjected into two dorsal blastomeres of 4-cell stage embryos with different doses. Because apoptosis significantly starts at neurula stages in the overexpression experiments, these embryos were subject to the inspection at late neurula stages (Figure 3.3-8 A to F and Table 3.3-3). For ***XMLP*** wild tpye, the phenotype of apoptosis as described above was significant when 0.4 ng of mRNA per embroys was injected into embryos (75 out of 85 injected embryos, Figure 3.3-8 A). The mutants S83A, G2A, and ED did not significantly cause apoptosis when injected the same dose (10 out of 85 for S83A, 8 out of 116 for G2A, and 0 out of 60 for ED), while all embryos (91 out of 91) injected with 0.4 ng of mutant SD mRNA showed apoptosis. It is therefore suggested that the mutants S83A, G2A and ED can reduce apoptosis in overexpression experiments. When injected with the doubled dose (0.8 ng), 40 out of 55 embryos injected with mutant S83A showed the phenotype of apoptosis, in contrast, only 5 out of 44 embryos injected with mutant G2A and 0 out of 59 embryos injected with ED mutant showed this phenotype. Moreover, only 7 out of 69 embroys injected with the 1.6 ng of ED mutant displayed apoptosis. The phenotypes of tadpole embryos are shown in Figure 3.3-8H to M and Table 3.3-3. The common features were eye defects, bent axis and in a few cases the severe reduction of the anterior axis. There was no significant difference between the mutants and ***XMLP*** wild type with exception of apoptosis. However, when 1.6 ng of ED mutant RNA was injected, the ectoderm in the hind brain area of tailbud stage

remained open, presumably due to an incomplete closure of neural folds (Figure 3.3-8L). This phenotype is quite similar to F52 knockout mice that showed exencephaly and postnatal anencephaly.

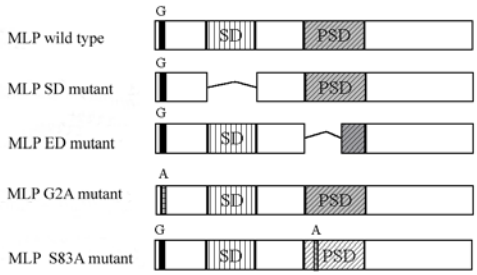


Figure 3.3-7. Schematic diagrams of XMLP wile tpye and mutants. Functional PSD (ED) is indicated as a dark-slashed box, the site of intron splicing as a vertical dashed box, the glycine at position 2 as black box and the substituted alanine by box with grid (A in both G2A and S83A mutants). The white-slashed box in XMLP S83A mutant represents the mutant ED domain with serine replaced by alanine (A).

3.3.7 Ectopic XMLP expression blocks normal rhombomere formation

It has been shown that *XMLP* transcripts were restricted to the presumptive neural system during neurulation (3.3.1 and Figure 3.3-2), for which the effect of ectopic XMLP expression on Krox20, a neural marker, whose expression is used to identify rhombomere 3 and 5 in the development of hindbrain (Figure 3.3-9A) was investigated. Overexpression of *XMLP* was carried out by injecting into one dorsal blastomere of 4-cell stage embryos with *XMLP* mRNA synthesized in vitro and LacZ as a lineage tracer. Ectopic expression of the *XMLP* resulted in a decreased *Krox20* signal in the rhombomere 3 and 5 areas. The stripes appeared irregular and the first one even disappeared on the injected side (28 out of 48 injected embryos, Figure 3.3-9B).

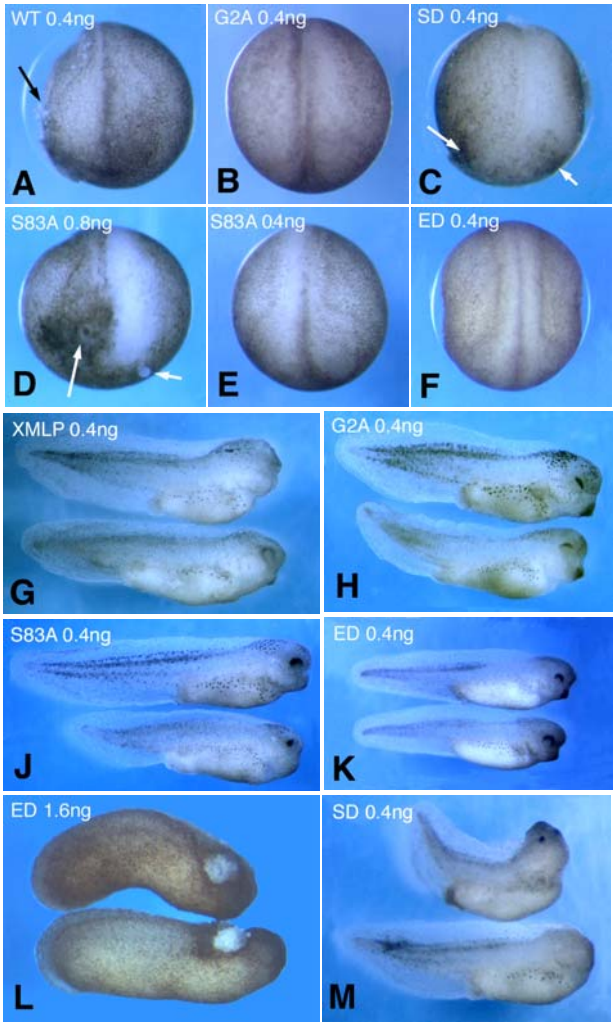


Figure 3.3-8. Comparison of phenotypes induced by the overexpression of wild-type and mutants *XMLP*. Embryos were injected into two dorsal blastomeres at 4-cell stage with mRNA of *XLMP* wild type and mutants, respectively. At neurula stage, the phenotype induced by 0.4 ng of wild-type *XMLP*, G2A, SD, S83A or ED are shown in (A), (B), (C), (E) and (F), respectively. Note the apoptosis were induced by 0.4 ng of *XMLP* wild type (A) and SD (C), but not by G2A (B), S83A (E) and ED (F). However embryos (D) showed apoptosis when 0.8 ng of S83A was injected. Apoptosis areas are shown by the arrows in (A), (C), and (D). Larva injected with 0.4 ng of *XMLP* wild type is shown (G). (H), (J), (K), and (M) indicate the phenotypes induced by G2A, S83A, ED, and SD, respectively, such as eye defects and a reduced anterior region. (M) The extreme case of injected embryos with SD mutant. (L) The phenotype induced by the injection of 1.6 ng of ED, which is quite similar to the phenotype of exencephaly in which the neural tube was not closed completely, and the epidermis did not cover the internal yolk-rich tissue either.

Table 3.3-3. Effects of *XMLP* wild type and mutants, and the combination with MO XMLP. The injected embryos were scored at early tadpole stage, while the results of overexpression of 1.6 ng of ED mutant were collected at middle tailbud stage. ***XMLP*** wild type and mutant mRNAs and MO XMLP were injected into two dorsal blastomeres of 4-cell stage embryos. n indicates the number of injected embryos available. The percentage of the available cases is shown in parenthesis. Embryos with apoptosis were inspected at late neurula***.***

RNA injected per embryos	Normal (n)	Lens defect (n)	Bent body axis (n)	Phenotype showing lens defect, reduced anterior axis and bent axis defect simultaneously (n)	Apoptosis
XMLP 0.4 ng	0 (0)	0 (0)	0 (0)	41 (100)	75 (88)
XMLP + MO XMLP 0.8 ng+16 ng	15 (50)	0 (0)	0 (0)	15 (50)	0 (0)
ED 0.4 ng	29 (48)	21 (35)	10 (17)	0 (0)	0 (0)
ED 0.8 ng	5 (7)	39 (53)	12 (16)	18 (24)	0 (0)
ED 1.6 ng	23 (42)	0 (0)	3 (5)	29[a] (53)	7 (10)
G2A 0.4 ng	12 (23)	0 (0)	0 (0)	40 (77)	8 (7)
G2A 0.8 ng	0 (0)	0 (0)	0 (0)	26 (100)	5 (11)
S83A 0.4 ng	16 (40)	8 (20)	16 (40)	0 (0)	10 (12)
S83A 0.8 ng	4 (12)	0 (0)	0 (0)	29 (88)	40 (73)
S83A 1.6 ng	0 (0)	0 (0)	1 (3)	31 (97)	48(98)
SD 0.2 ng	0 (0)	15 (45)	0 (0)	18 (55)	18 (49)
SD 0.4 ng	0 (0)	18 (30)	0 (0)	43 (70)	91(100)

[a] The phenotype is quite similar to exencephaly. The result was scored at middle tailbud stage when the eye rudiment has not yet clearly formed, so the embryos not showing exencephaly were scored as normal.

3.3.8 Retinoic acid hydroxylase (XCYP26) changes the expression pattern of XMLP

Retinoic acid (RA) is known to participate in the development of the nervous system and the formation of the limb buds (for review, see Maden ***et al.***, 1998; Zile, 1998). To determine whether the RA metabolites affect the expression pattern of ***XMLP*** (Figure 3.3-9C), whole-mount ***in situ*** hybridization were performed on stage 20 embryos co-injected into one blastomere at 2-cell stage with 2 ng of ***XCYP26*** (Hollemann ***et al.***, 1998b) and 100 pg ***LacZ*** mRNA, which revealed that the signal stripes on the injected side were weaker or even disappeared (57%, 31of 54 cases) in contrast to the uninjected side (Figure 3.3-9D).

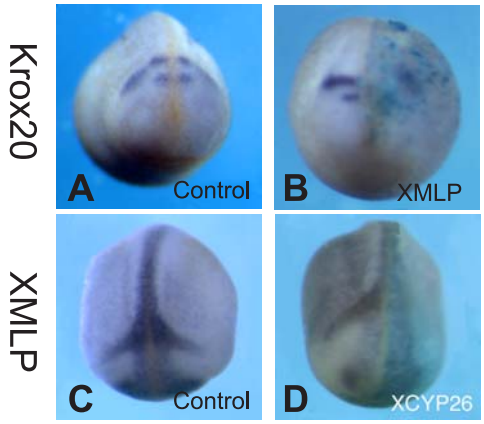


Figure 3.3-9. Effect of ectopic expression of *XMLP* on *Krox20* and the expression alternation of *XMLP* induced by *XCYP26*. 0.8 ng of *XMLP* mRNA was injected into one dorsal blastomere of 4-cell stage embryos. *LacZ* mRNA was co-injected as a lineage tracer indicated in light blue. Signals were detected by whole-mount *in situ* hybridization performed on *XMLP*-injected embryos at stage 20 with *Krox 20* antisense RNA. (A) The expression of *Krox 20* in the control embryos. (B) The expression of *Krox20* disturbed on the injected side. 2 ng of *XCYP26* mRNA was injected into one blastomere of 2-cell stage embryos, which was visualized by *LacZ* staining. Signals were detected by whole-mount *in situ* hybridization performed on *XCYP26*-injected embryos at stage 20 with *XMLP* antisense RNA. (C) The expression of *XMLP* in the normal embryos. (D) The altered expression of *XMLP* indicated by *LacZ* staining (signal stripes vanished or got smeared) at injected side caused by overexpression of *XCYP26*.

3.4 Isolation of *XAT* and the expression pattern

The clone encoding the *Xenopus* homologue of arginine:glycine amidinotransferase (XAT) consists of an open reading frame encoding a protein of 422 amino acids with a predicted molecular weight of 48572.88 Da (GenBank accession number: AF187863). XAT shares 83% identity of amino acids with its homologue in chicken, 85% identity to rat AT, and 84% identity to human AT (Figure 3.4-1).

The spatial expression pattern of XAT was analyzed by whole-mount in situ hybridization (Oschwald et al., 1991; Harland, 1991), which indicated that the signals were barely detected before the gastrula stages, and successively were around the yolk plug. The stronger signals were found on the dorsal blastpore zone, but no transcripts were detected in the animal halve (Figure 3.4-2C, D). At the stage 15, the signals were restricted to the midline of the neural plate (Figure 3.4-2E, F) and the corresponding transversal sections showed that the transcripts were located in the

notochord (Figure 3.4-2G). The signal increased at the ventral-posterior part of the trunk at the early of tailbud stage together with strong expression in the notochord (Figure 3.4-2H). At the later tailbud stages and early larvae signals could be seen in the trunk, especially at the presumptive gut and notochord area (Figure 3.4-2I, J, K). The temporal profile of *XAT* expression analyzed by RT-PCR showed that the expression of this gene started after the stage 8, and was slightly increased during the gastrula and neurula stages, then maintained stable in the tailbud and later stages (Figure 3.4-3).

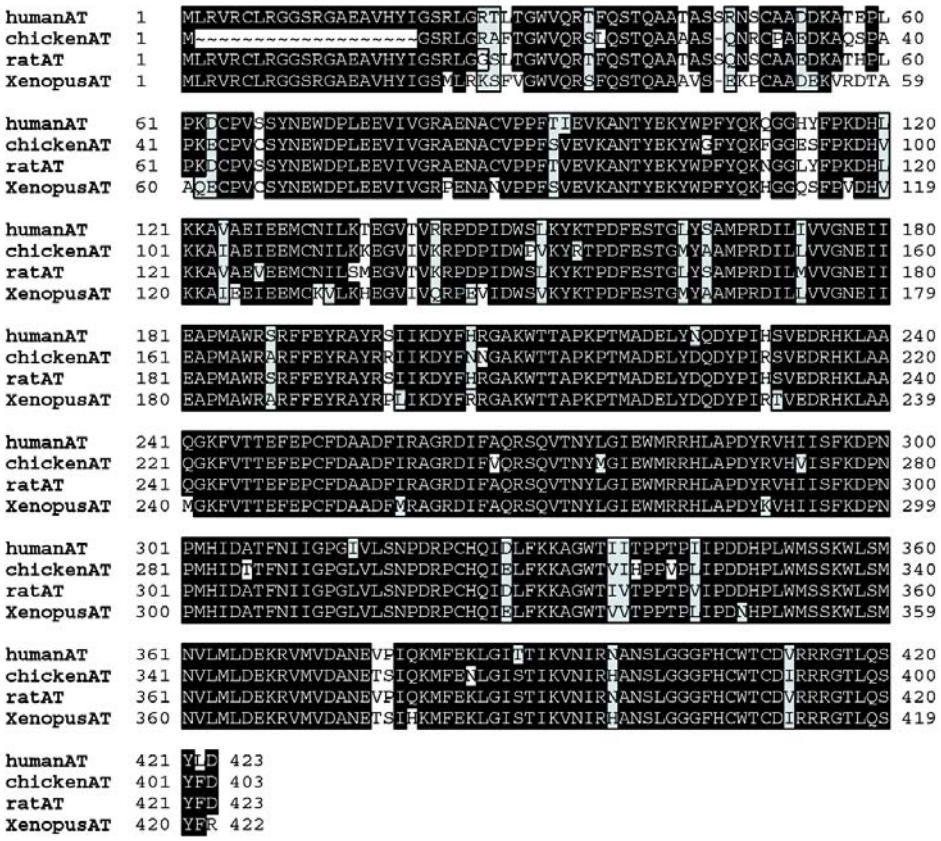


Figure 3.4-1. Alignment of the deduced protein sequence of AT in *Xenopus*, chicken, rat and human. Numerical figures indicate amino acid positions starting with the initiation of methionine. Hyphen represents gaps introduced into the sequences in order to obtain optimal alignment. The black boxes indicate the conserved amino acids, and the gray box, the similar amino acids. The accession number for the *Xenopus laevis AT* (*XAT*) cDNA sequence is AF187863.

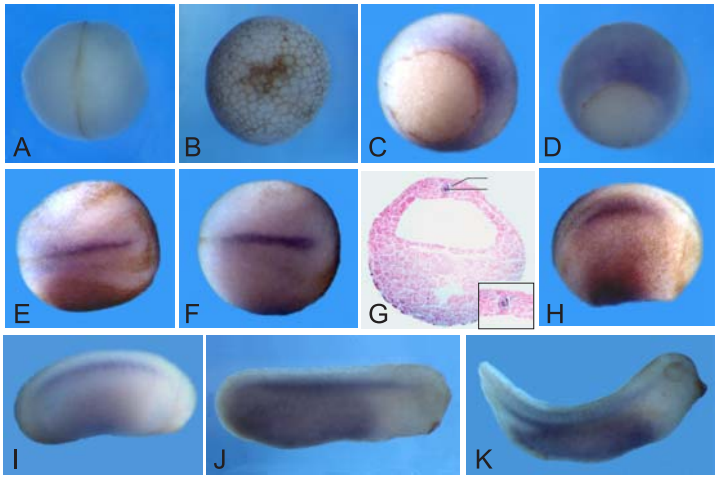


Figure 3.4-2. The expression of *XAT* revealed by whole-mount *in situ* hybridization. *XAT* transcripts are barely found from the 2-cell stage to the stage 8 (A, B). In the middle gastrulae stages, signals are detected around the yolk plug (C, D). In the neurula stage *XAT* is expressed in the midline of the medullary plate (E, F). Transversal sections show that the signals are located in the notochord (G). In the tailbud and tadpole stages transcripts are mainly found in the notochord and the trunk area (H to K) (Abbreviations: at, archenteron; ed, endoderm; nt, notochord; sm, somite).

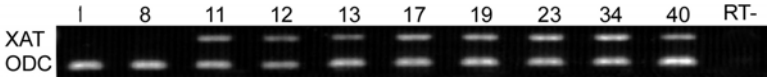


Figure 3.4-3. The temporal expression of *XAT* in different embryonic stages analyzed by RT-PCR. The stage definition is according to Nieuwkoop and Faber (1975), ODC is indicated as internal standard control.

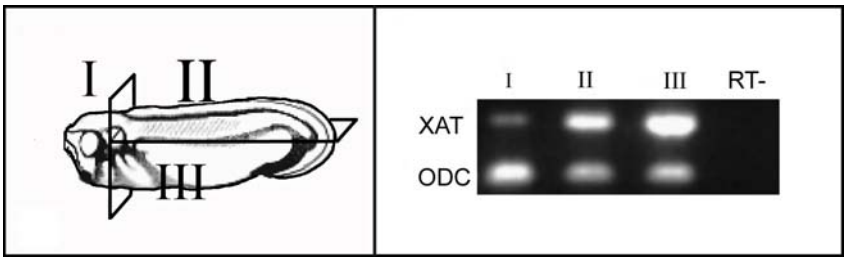


Figure 3.4-4. The expression of *XAT* shown in explants dissected at the stage 28. Signals in the head area (I) are much lower than that in dorsal zone of trunk (II) and the remaining ventral parts including the presumptive gut area (III).

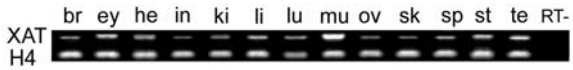


Figure 3.4-5. The expression of *XAT* in different adult tissues analyzed by RT-PCR. *XAT* transcripts are detected in all tested tissues, however, with large abundance in eye, muscle, stomach and testis. H4 is indicated as internal standard control (Abbreviations: br, brain; ey, eye; he, heart; in, intestine; ki, kidney; li, liver; mu, muscle; ov, ovary; sk, skin; sp, spleen; st, stomach; te, testis).

By whole-mount in situ hybridization few transcripts of *XAT* could be found in the head in contrast to abundant signals in the trunk area. To determine the expression level in different parts of embryo by RT-PCR, the stage 28 embryos were dissected into three parts: head (containing cement gland), dorsal zone of trunk (containing notochord and somites) and the remaining ventral part including the presumptive gut area as shown in Figure 3.4-4A. The results shown in Figure 3.4-4B revealed that the expression of *XAT* in the head area was much lower than in the other two zones, which was consistent with the data of whole-mount *in situ* hybridization. RT-PCR analyses of adult tissues showed a higher expression level in eye, muscle, stomach and testis (Figure 3.4-5).

4. Discussion

The discovery of Mendel's laws of heredity sparked a scientific quest to understand the content of genetic information in the last hundred years, especially the past decades when we have advanced from having very little information about the genetic details of biology to possessing an immense amount of structural information about individual genes. Currently, the complete genome sequences of humans and of more than 60 other species are available in databases. The complete of human genome program will lay the foundation for a broad range of functional genomic tools to facilitate analyses of individual gene functions. However, it only provides a new starting point for understanding the basic genetic makeup and variations. Our ability to deciphering such information remains woefully inadequate. Step by step, the scientific progresses will naturally focus on gene functional studies which may depend on the integration of unlimited information from different model organisms. The use of a combination of different animal models has been proved to be practicable to complement the deficiency of individual systems. ***Xenopus laevis*** is one of these well-established experimental model system. Screening a cDNA library of ***Xenopus*** by the large-scale whole-mount ***in situ*** hybridization is a good approach to isolate novel genes because the expression pattern often gives important clues about the functions of a gene and makes readily testable prediction. So it is a way for gene identification using characters of functional judgments directly and in this aspect it is on the further way than the genome project itself. Moreover, for most of all cDNA-generating ESTs, partially sequencing identified sequence homologies instead of sequencing full inserts is an efficient compromise in a large-scale approach to assign potential functions of a gene.

4.1 Why not many endoderm-specific genes in our cDNA library?

The primary goal of this study was to identify novel endoderm genes involved in the early embryogenesis of ***Xenopus laevis*** and subsequently to study their functions. However most of the new sequences look like ectoderm or mesoderm-correlated genes though some early endoderm determinants such as ***Sox17***α and ***Sox17***β have also been isolated from this cDNA library. The tissue prepared for the construction of this cDNA library was activin-treated ectoderm, showing strong positive signals when

it was analyzed by whole-mount ***in situ*** hybridization with the endodermal marker ***endodermin*** (***Edd***) (Sasai, 1996). However there were some mesodermal and neural derived tissues found by the histological inspection. The expression of ectoderm or mesoderm specific genes in the tissues was also detectable using sensitive approaches such as RT-PCR. Some endodermal determinants start to be activated very early. For example, ***mixer*** (Henry and Melton, 1998) is turned on and off specifically during the gastrulation; ***milk*** (Ecochard ***et al.***, 1998) is first detected at the stage 9 in the dorsal marginal zone, as the gastrulation proceeds it becomes undetectable at the late-gastrula stages; ***Sox17***α and ***Sox17*** β (Hudson ***et al.***, 1997) are initiated even earlier; ***Edd*** expression begins just before the gastrula stages. While most of the endoderm specific genes initiate expression approximately a day after the gastrulation ends and just before the conversion of ectoderm to epidermis from the stage 25 to 30 (Henry and Melton, 1998), such as the ***liver fatty acid binding protein*** (***LFABP***), an anterior small intestine and liver marker (Gordon ***et al.***, 1983), ***Xlhbox-8*** (Wright ***et al.***, 1988), a pancreatic homobox gene (homologous to ***Pdx1***), and the ***intestinal fatty acid binding protein*** (***IFABP***), a marker of small intestine (Henry ***et al.***, 1996; Shi ***et al.***, 1994). Therefore, the period of culture after activin treatment may be too short to activate most endoderm genes. Alternatively, it also should be considered that the number of clones analyzed may be inadequate due to generally low ratio of endoderm determinants during the embryonic development.

4.2 XXBP-1 participates BMP-4 signaling pathway

It is demonstrated that ***XXBP-1***, a leucine zipper transcription factor, is involved in BMP-4 signaling pathway and functions as a transcription activator. The overexpression of ***XXBP-1*** in early embryos leads to the ventralization and can rescue neural induction of ectodermal explants as well as dorsolization of intact embryos imposed by the dominant negative BMPs receptor I (tBMPRI), suggesting that ***XXBP-1*** acts downstream of BMP-4 receptor and mediates BMP-4 signaling in the epidermal induction and the inhibition of neural differentiation.

4.2.1 Expression locations of XXBP-1

According to the whole-mount ***in situ*** hybridization, ***XXBP-1*** is expressed in the dorsal blastopore lip and the ventral ectoderm but not in the perspective neural plate.

Moreover, it leads to ventralization of the injected embryos in overexpression experiments and directs epidermal fate in ectoderm in the animal cap assays when co-injected with tBMPRI. These evidences support that ***XXBP-1*** acts as an epidermal inducer or a neural inhibitor. Although ***XXBP-1*** is expressed in the dorsal blastopore lip, it plays a negative role for the dorsalization and neural induction of embryos. This is reminiscent of ***ADMP*** (***anti-dorsaling morphogenetic protein***, Moos Jr ***et al.***, 1995) which, demonstrating very strong ventralization activities, is also expressed in the dorsal blastopore lip. They are then unexpected genes in the Spemann organizer in light of classical embryological evidences that associate dorsalization influences with this region in the embryos. I also found that ***XXBP-1*** could suppress ***gsc*** and ***chd***, and up-regulate ***BMP-4***, ***vent-1***, ***vent-2*** and ***Xhox3***. Thus, it seems there are negative regulatory signal pathways affecting on the dorsal mesoderm differentiation. Ectopic ***noggin*** ablates the native expression of ***XXBP-1*** in the animal cap assays, suggesting that ***XXBP-1*** could be a direct or indirect target gene for ***noggin***. At the late gastrula and neurula stages the signal staining of ***XXBP-1*** is intensified on the cement gland primordium and mature cement gland, which is similar to that of ***BMP-4*** (Fainsod ***et al.***, 1994; Hemmati-Brivanlou and Thomsen, 1995; Grammill ***et al.***, 2000). This, together with the fact that the signal is excluded from the neural plate at the gastrula stages and its activity for ventralization, implies that ***XXBP-1*** may involve in ***BMP-4*** signaling pathway. At the late neurula and early tailbud stage, the staining strip extends from cement gland to the ventral side (Figure 3.2-2N) and the corresponding sagittal sections show that the staining lies in the mesoderm closely adjacent to the epidermis of embryos (Figure 3.2-3B, B1). The expression domain of ***XXBP-1*** is not the liver primordium (Hausen and Riebesell, 1991) although the knockout mouse lacking the mouse ***XBP-1*** showed the disruption of liver development (Reimold ***et al.***, 2000). Nevertheless, the dramatic shift of its expression domain during the embryonic development and its presence in all tested adult tissues indeed indicate that ***XXBP-1*** must play multiple roles for cell fate determination and embryogenesis.

4.2.2 XXBP-1 lies the downstream of BMP-4 receptors and up-regulates BMP-4

Bone morphogenetic proteins (BMPs) are typical members of the transforming growth factor β (TGF-β) family with diverse roles in the embryonic development. They elicit wide biological responses in different biological contexts, which are partially due

to intracellular cofactors that participate in BMPs and other signaling pathways (for review, see von Bunoff and Cho, 2001). Signaling pathway involving BMP-4 is implicated in both the mesoderm induction and the dorsoventral pattering. The binding of organizer antagonists such as chordin and noggin to BMP-4 appears to be responsible for the establishing a morphogen gradient of BMP-4 activities, which specifies different dorsoventral fates in the early gastrulae (for review, see Dale and Jones, 1999, De Robertis ***et al***., 2000). Moreover, BMPs were strongly involved in the ectoderm differentiation. In the absence of BMP-4, the ectoderm will give rise to neural ectoderm. Conversely the ectoderm will differentiate into epidermis (for review, see Weinstein and Hemmati-Brivanlou, 1997).

In this study, I show evidences that ***XXBP-1*** is involved in ***BMP-4*** pathway. First, the expression pattern of ***XXBP-1*** at the gastrula stages indicates that it may play a negative role in the neural induction. And the intensified signal staining on the cement gland at the neurula stages overlaps that of ***BMP-4***. Second, ectopic expression of ***BMP-4*** up-regulates the expression of ***XXBP-1*** on the dorsal marginal zone (Figure 3.2-7A) and vice versa (Figure 3.2-11). Third, overexpression of each of them leads to ventralization of the injected embryos in a dose-dependent manner, resulting in phenotypes ranging from micorcephaly to Bauchstück. Fourth, both ***BMP-4*** and ***XXBP-1*** can suppress ***gsc*** (Figure 3.2-9I) while are down-regulated by ***noggin*** in animal cap assay (Figure 3.2-13). Finally, co-injection with ***XXBP-1*** and truncated BMP receptor I (tBMPRI) can reverse the dorsalization induced by injection with tBMPRI alone, and direct the epidermal fate in the animal cap assay (Figure 3.2-12).

With respect to the hierarchy, the evidences support that ***XXBP-1*** acts downstream of the BMPs receptor. tBMPRs are able to interfere with BMPs signaling pathway. Overexpression of tBMPRs in the ectoderm explants induces neural tissue as indicated by neural markers ***NCAM*** and ***Otx2***. However, the expression of ***NCAM*** and ***Otx2*** is suppressed and that of ***epidermal keratin*** is partially restored by the co-injection with tBMPRs and ***XXBP-1***. This means the treated ectoderm adopts a cell fate prior to the epidermis and ***XXBP-1*** is sufficient to attenuate the neural induction imposed by tBMPRI. In overexpression experiments, ***XXBP-1*** can also reverse the dorsalization induced by tBMPRI. Thus it is suggested that ***XXBP-1*** acts downstream of BMP-4 receptors, yet the induction of ***BMP-4*** by ***XXBP-1*** in the DMZ analyses raises the possibility that ***XXBP-1*** is required for the maintenance of ***BMP-4*** expression.

4.2.3 *The correlation between XXBP-1 and Wnt/β-catenin pathway*

BMP-4 is repressed at the dorsal side of embryo, including the dorsal mesoderm and the prospective neural plate during the gastrulation. There are some evidences that Wnt/β-catenin signaling pathway is involved in this process. In this signaling pathway, the signaling is mediated by the blockage of activity of glycogen synthase kinase 3 (Gsk3). The inhibition of Gsk3 stabilizes β-catenin so that it can form complexes with members of the family of LEF/TCF DNA-binding proteins. Consecutively the complexes facilitate the nuclear localization of these factors and therefore activate the target genes (for review, see Moon and Kimelman, 1998). Wnt/β-catenin signaling cooperates with the neural inducers to specify posterior versus anterior neural identity (McGrew ***et al.***, 1995) and the maternal β-catenin is required for dorsal mesoderm development between the stage 7 and 9 (Wylie ***et al.***, 1996). In ***Xenopus laevis***, the activation of the Wnt/β-catenin singaling pathway inhibits ***BMP-4*** transcription in ectodermal explants at the gastrula stages and results in the induction of neural marker (Baker ***et al.***, 1999). The treatment of ***Xenopus*** embryos with LiCl leads to a phenotype of dorsalization with greatly enhanced forebrain structures (Kao and Elinson, 1988). The treatment also leads to the differentiation of neurons as well as neural crest in ectodermal explants. LiCl inhibits the activity of glycogen synthase kinase β (GSK- 3β), preventing the degradation of β-catenin. In this study, I also made attempt to investigate the relations between Wnt/β-catenin and ***XXBP-1***. The results indicate that the expression locations of ***XXBP-1*** are expanded to the whole DMZ of embryo in which Wnt/β-catenin signaling pathway is blocked by morpholino β-catenin oligos. And this phenotype can be mimicked by the dorsal overexpression of ***BMP-4*** and the UV treatment. Thus it is shown here another evidence that there exists crosstalk between BMPs and Wnt/β-catenin signaling pathways, and also suggested that the Wnt/β-catenin pathway is involved in the neural induction and the neural plate patterning. The blockage of Wnt/β-catenin signaling path way will lead to the failure of the formation of the Spemann organizer, which in turn will not emanate the BMPs antagonists. The patterning of neural plate therefore are interfered. This may be the possible reason for the expansion of ***XXBP-1*** expression territories on the dorsal marginal zone of the embryo injected with MO β-catenin. Alternatively, ***XXBP-1*** may be a direct negative target of Wnt/β-catenin or a indirect target regulated via BMP-4 signaling

pathway, repressed from the neural plate by the activation of this pathway as BMP-4. Similarly, the regression of ***Dlx3***, another anti-neural factor from the prospective neural plate, is also mediated by β-catenin (Beanan ***et al.***, 2000). Neither ***BMP-4*** nor ***XXBP-1*** is up-regulated in the ectoderm explants injected with MO β-catenin, suggesting the down-regulation of ***BMP-4*** by Wnt signaling in the prospective neural plate needs the cooperation with other factors. In agreement with our hypothesis that Wnt/β-catenin is involved in the neural induction and the neural plate patterning, the neural induction in the absence of mesoderm can be initiated by the β-catenin dependent expression of secreted BMP antagonists at the blastula stage in ***Xenopus*** (Wessely ***et al.***, 2001).

4.2.4 *XXBP-1 functions as a transcription activator*

The predicted amino acids sequence reveals that XXBP-1 belongs to the basic leucine zipper family. In this protein family, the region adjacent to the leucine repeats is highly basic in each of the members and could comprise a DNA-binding site. The two leucine zippers in effect form a Y-shaped structure in which the zippers comprise the stem and the two basic regions bifurcate symmetrically to form the arms that bind to DNA. This is known as the bZIP. Zippers may be used to sponsor formation of homodimers or heterodimers (Lewin, 2000).

With the context of the embryos, the constructs fused with protein domains known to have repressive or activating transcriptional activities—the repressor domains of the ***Drosophila*** engrailed (EnR) and even-skipped proteins (Eve) and the activator domain of VP16, were employed to characterize the transcription activity of ***XXBP-1***. This approach has been used successfully for characterization of the activity of several other transcriptional activators involved in ***Xenopus*** development (Conlon ***et al.***, 1996; Horb and Thomsen, 1997; Onichtchouk ***et al.***, 1998; Gómez-Skarmeta ***et al***., 2001)***.*** XXBP-1 is demonstrated to have activator activity with several criteria. First, when fused with the activator domain of ***VP16***, the construct of ***XXBP1-VP16*** showed similar biological activities as the wild-type ***XXBP-1***, i.e. ventralization of the embryos when injected dorsally and the phenotype of a posterior enlargement when injected ventrally. Second, both ***XXBP1-VP16*** and ***XXBP-1*** wild type cannot induce neural markers ***NCAM*** and ***nrp1***. Third, when fused with the repressor domain of ***Eve***, the ***XXBP1-Eve*** is able to induce twin axis when ventrally overexpressed though the ratio was relatively low. Moreover, in contrast to the wild-type ***XXBP-1***, ***XXBP1-Eve***

can induce neural markers ***NCAM*** and ***nrp-1***. Thus ***XXBP1-Eve*** up-regulates genes that would ordinarily be repressed by ***XXBP-1.*** The results shown by ***XXBP1-EnR*** are a blur. It cannot induce neural markers and twin axis but leads to the gastrulation defect giving rise to spina bifida. The obscure results may be due to the addition of 6 Myc domains NH_2-terminal flank to the bZIP domain, which may interfere DNA binding activity of the fusion constructs. It's worth noting that the gastrulation defect was observed in the overexpression of each construct. However, the results must be interpreted with respect to the likely mechanism of the action. The strong transcriptional activator or repressor domain will act wherever there is an accessible XXBP-1 binding site and not just in cells that express ***XXBP-1***. This will inevitably lead to pleiotropic effects on differentiation by altering transcription level in the vicinity of ***XXBP-1*** biding sites. Alternatively, the fusion constructs may have toxic effects and interfere with the normal embryonic development.

In the study of the truncated mutant of ***XXBP-1*** containing the first 126 amino acids (including bZIP), the truncated construct has weak effects on the neural induction as indicated by the increased expression of ***Otx2*** and ***NCAM***. The overexpression of this truncated mutant also leads to gastrulation defect giving rise to spina bifida. These suggest that ***XXBP-1*** forms homodimers to carry out its functions.

4.3 XMLP is involved in the process of morphogenetic movement.

The present data about ***XMLP*** shows that ***XMLP*** is involved in the normal development of neural tube and the primordium of the central nervous system. It also induces apoptosis in overexpression experiments. Data of other members of the MARCKS family together with my results suggest that ***XMLP*** is involved in the morphogenetic movement during early embryonic development. The disturbance of morphogenetic movement and induced apoptosis by overpression of ***XMLP*** could be reasons for the reduction of the anterior axis and eye defects.

4.3.1 XMLP and XMARCKS show different temporal expression patterns

The expression pattern of ***XMLP*** is different from its homologue ***MARCKS*** in ***Xenopus*** (***XMARCKS***). For ***XMARCKS***, there is a clear decreased level from 4-cell stage to mid blastula transition (MBT), which reflects the decay of maternal transcripts. Thereafter, zygotic transcription of the ***MARCKS*** appeared to be switched

on and maintained permanently active throughout development (Shi ***et al***., 1997). In contrast, the reduced level of ***XMLP*** is not as significant as that of ***XMARCKS***, i.e. ***XMLP*** is expressed strongly at stage 8. The lowest expression level is reached at the early tailbud stage and followed by an increase of the ***XMLP*** expression and maintenance of a constant level. The differences between the two homologues may reflect the expressions of redundancies regulated by different mechanisms. It should be noted that the primary sequence of XMLP shows a very low similarity to its homologue in human, mouse and rabbit. Alternatively, it could be a member of other protein families, which may account for the expression difference. The mice homologue of ***XMLP*** mRNA (F52) is expressed with a high level from 8.5 days post conception (dpc) through 14.5 dpc. At 17.5 dpc, the mRNA level remains high in the brain only, and not in other parts of embryos. Thus, F52 mRNA is abundantly expressed during the period of neurulation (Wu ***et al.***, 1996). Similarly, ***XMLP*** is also abundantly expressed in the neural folds in neurula stages, and shows a high expression level in the brain area of tadpole stage embryos. The signal location indicates that it plays an important role in the development of the central nervous system.

4.3.2 The blockage of XMLP can disturb normal anterior axis formation

Embryos with the blocked ***XMLP*** by the injection with morpholino (MO) XMLP show head reduction and eye defects, suggesting that ***XMLP*** is involved in anterior axis formation. This phenotype can be rescued by the injection with ***XMLP***, which indicates that MO XMLP specifically binds ***XMLP*** mRNA. Data from ***in vitro*** studies of other members of this protein family show that they are very important for cell shape and cell motility. MARCKS is found associated with the plasma membrane and in the cytosol. The translocation happened prior to changes in cell morphology (Ramsden, 2000). I also suppose that the disturbance of normal morphogenetic movement cause the abnormal phenotype observed with injection of MO XMLP. If XMLP has a similar function as other members of this protein family, it is reasonable that the elimination of XMLP will change the cell morphology inevitably followed by a change of incident processes downstream in the embryonic development.

4.3.3 The function of XMLP is correlated with its ED domain and glycine at position 2— overexpression of XMLP can induce apoptosis

Apoptosis is a part of the developmental program of an organism. At the beginning

it could not be surely distinguished if the phenotype of overexpression experiments was due to RNA toxicity or specific functions of ***XMLP***. ***LacZ***, therefore, was microinjected as a control strictly under the same experimental conditions as that for ***XMLP*** capped mRNA. Since such phenotypes were not found in ***LacZ*** injected embryos, I could exclude the possibility of toxic effects (data not shown). The results from other studies also support my view with regard to this phenotype (Grammer ***et al.***, 2000). Many cDNAs encoding factors which cause cell damage or inhibit normal cell cycle progression (transcription inhibitors, translation inhibitors and mitosis blocking agents) can lead to the phenotype of apoptosis described above. Although there exist some reliable methods to show apoptosis in ***Xenopus***, e.g. TUNEL or ELISA, it is important to focus the interest on the apoptosis observed in overexpression experiments. This is beneficial in identification and characterization of cell death inducers and effectors, and in distinction it from phenotypes caused by direct inhibition or cell death.

The results here indicate that the ED domain (PSD) and the glycine at position 2 of XMLP are important for its function properties. The substitution of alanine for serine at the position 83 can reduce but not abolish ***XMLP*** apoptosis inducing activity in overexpression experiments because significant apoptosis is found only when the injection dose of S83A is increased as shown in Table 3.3-3. However, the intact ED domain is essential for this function. No significant differences were found between the SD mutant and ***XMLP*** wild type in overexpression experiments. But this highly conserved domain contained in MARCKS family must presumably have a certain function though so far no experimental evidence exists. Overexpression of MARCKS in tumor-derived choroidal melanoma cells, where the amount of the endogenous protein is very low, can significantly decrease cell proliferation (Manenti ***et al.***, 1998). Ectopic apoptosis may be one of the reasons for eye defects and the reduction of anterior in overexpression experiments, since normal embryonic development could be inhibited by severe ectopic apoptosis (Grammer ***et al.***, 2000). Overexpression of ***XMLP*** alone without any apoptosis does not inhibit the histolotypic differentiation of isolated dorsal blastopore lip.

4.3.4 Overexpression of mutant ED inhibits the closure of neural tube

Interestingly, embryos injected with 1.6ng of ***XMLP*** ED mutant mRNA showed a phenotype which is quite similar to exencephaly in mice. In contrast to wild-type

XMLP, it does not cause apoptosis, but aberrant tissue morphogenesis including abnormalities in eye formation and neural tube closure. These data are in agreement with observations in the mice that knockout ***XMLP*** homologues leads to embryonic exencephaly and postnatal anencephaly. Probably XMLP acts like F52, MARCKS and other PKC substrates such as GAP43 by mediating cytoskeletal changes and controlling of morphogenesis in different tissues. I suppose that adverse ED mutant molecules have a strong influence on cellular architecture. The introduced ED mutant molecules can compete with the native XMLP ***in vivo.*** They could be adulterated the cellular architecture during embryonic development, therefore must presumably alter the cellular architecture, influence the motility, and alter the mutual cell affinity to the surrounding cells resulting in a failure of neural tube closure. In contrast, MO XMLP does not cause such significant phenotype. This may be due to the partial compensation by other redundant molecules. It is also possible that abundant XMLP is already present in the embryos when MO XMLP was injected. The data support the view that ***XMLP*** participates in morphogenetic movements. Morphogenesis including neurulation requires multiple cellular processes such as cell proliferation, cell shape change and cell migration (Schoenwolf, 1990). All these processes depend on the reorganization of the cytoskeleton, cell-cell signaling and mutual cell affinity. Ectopic expression of ***XMLP*** and morpholino injection had no obvious effects on morphogenetic movements during gastrulation since injected embryos showed normal blastopore and yolk plug formation. It could then be argued that non-significant effects on embryonic development may be partially correlated with still high levels of maternal ***XMLP*** transcripts and their long half-life.

4.3.5 Ectopic expression of XCYP26 affects the expression pattern of XMLP

Several experiments have shown that RA can alter the positional values of cells in limb development and in the anterior-posterior axis formation (Conlon, 1995). The effects of RA are mediated at different levels, but mainly by gene transcription and interactions with multiple nuclear receptors (RARS and RXRs). RAR-RXR heterodimer binds to RA response elements (RARES) in the promoter regions of target genes and regulate transcription in a ligand-dependent manner. XCYP26 is a RA hydroxylase, which could rescue RA induced developmental defect and alter the expression patterns of some other molecular markers for neural development (Hollemann ***et al.***, 1998b). In this study, I also find that ***XCYP26*** can influence the

XMLP expression pattern. The signal stripe in stage 20 embryos on the injected side in contrast to that on the uninjected side gets diffused or even disappears. Therefore I suggest that RA participates in the regulation of ***XMLP*** and may be involved in anterior-posterior axis formation.

4.4 Expression of *XAT* in dorsal blastopore lip

Our results show that during embryogenesis, ***XAT*** is preferentially expressed around the blastpore and later in the notochord of neurula and tailbud stages. These data of the spatial and temporal expression of a gene encoding an enzyme close related to energy metabolism confirms classical experiments on the molecular level, which suggested that certain enzymes are active in form of a gradient-like pattern especially around the blastopore and in those parts of the embryo, where cell activities including cell division are most prominent (Sáxen and Toivonen, 1962). In addition, ***XAT*** was posteriorly intensified in the tailbud stage, showing much strong expression than the cranial part. This implies ***XAT*** may correlated with the FGF pathway because FGF is known to be essential for posterior patterning. Further functional experiments especially on the protein level will be essential to get a better understanding about the temporal and spatial control mechanisms of this enzyme.

5. Summary

In this study, the large-scale whole-mount ***in situ*** hybridization was employed to screen an activin-treated ectoderm cDNA library of ***Xenopus laevis***, leading to the isolation of 51 novel cDNA sequences of ***Xenopus***. So this approach turned out to be practicable for identification of new genes from a cDNA library in a large scale. ***XXBP-1***, ***XMLP*** and ***XAT*** were selected for further functional studies based on their suggestive expression pattern.

XXBP-1 is a novel basic leucine zipper transcription factor in ***Xenopus***. It is a maternal factor and expressed on the dorsal blastopore lip and ventral ectoderm with exception of perspective neural plate in gastrula stages. RT-PCR indicated that ***XXBP-1*** is weakly expressed before gastrula stages, afterwards is up-regulated and kept in a persistent level during the embryonic development. Overexpression of ***XXBP-1*** leads to ventralization of the injected embryos as described for ***BMP-4***. Moreover, ***XXBP-1*** and ***BMP4*** interact in a positive feedback loop. Consistent with mediating ***BMP-4*** signaling, the ectopic expression of ***XXBP-1*** partially recovers the expression of ***epidermal keratin*** in animal cap assay and converses the dorsolizaiton imposed by truncate BMP receptor I. Thus, we propose that the ***XXBP-1*** is downstream of BMP receptors, and plays roles in the inhibition of neural differentiation.

XXBP-1 functions as a transcriptional activator. When overexpressed ventrally, the effect of wild type ***XXBP-1*** can be phencopied by the XXBP1-VP16 containing the activator domain VP16 and the DNA binding domain of ***XXBP-1***. Another fusion construct, XXBP1-Eve containing the DNA binding domain of ***XXBP-1*** and even-skipped repression domain induce neural markers ***NCAM*** and ***nrp1*** in animal cap assay and leads to the secondary axis when overexpressed ventrally in a low ratio (10%). This confirms that ***XXBP-1*** acts as an activator, playing negative roles in neural induction.

The XMLP may be a new number of the small MLP protein family. Using whole-mount ***in situ*** hybridization and RT-PCR, ***XMLP*** maternal transcripts were detected during the cleavage stages. After MBT the signals are restricted to the neural plate. Subsequently ***XMLP*** is expressed predominantly in the brain, somites, and

pronephros. Ectopic expression of ***XMLP*** results in eye and axis defects and as well as a change of the expression pattern of ***Krox 20***. Apoptosis was induced by the injection of ***XMLP***. Overexpression of mutant ***XMLPs*** indicated that this phenotype is correlated with its putative PSD domain and glycine at position 2. The loss-of-function of ***XMLP*** was studied by injection with a morpholino oligo complementary to ***XMLP*** mRNA, which revealed the malformations of anterior axis and eye defects. According to extirpation experiments the phenotypes might be correlated with disturbed morphogenetic movements rather than an inhibition of induction process. Overexpression of ***XCYP26*** resulted in a shift of the expression pattern of ***XMLP***, showing the signal stripe of ***XMLP*** in injected half of the embryo getting diffuse or even disappeared. This observation suggests that retinoic acid plays an important role in the ***XMLP*** regulation. Taken together, XMLP may participate in pattern formation of the embryonic axis and the central nervous system.

XAT encodes ***Xenopus*** amidinotransferase which shares a highly conserved sequence with the homologues of human, chick and rat. Characterization of embryonic expression indicates that ***XAT*** is differentially expressed around the yolk plug including the dorsal blastopore area at early gastrula stages and is extensively expressed in the midline of the neural plate of early neurula stages. Sections reveal that its transcripts are located in the notochord. In the tailbud stage signals are found both in the notochord and the trunk area, whereas faint signals can be found in the cephalic part only.

6. Zusammenfassung

In dieser Arbeit wurde in großem Maßstab mittels Random Screening und whole-mount ***in situ*** Hybridisierung von Genen einer Bibliothek von aktivin-behandelten Ektoderm durchgeführt. Auf diese Weise wurden 51 neuen cDNA-Sequenzen von ***Xenopus laevis*** isoliert. Aufgrund ihres sehr interessanten Expressionsmusters wurden dann ***XXBP-1***, ***XMLP*** und ***XAT*** für weitere funktionelle Studien ausgewählt.

XXBP-1 ist ein neuer basischer Leucine Zipper Transkriptionsfaktor bei ***Xenopus laevis***. Es handelt sich um einen maternalen Faktor, der auf der dorsalen Seite der Urmundlippe und ventralen Ektoderm exprimiert wird mit Ausnahme der zukünftigen Neuralplatte im Gastrula-Stadium. RT-PCR Analyse konnte zeigen, dass ***XXBP-1*** vor Beginn der Gastrulation schwach, während der weiteren Embryonalentwicklung aber dann kontinuierlich exprimiert wird. Eine Überexpression von ***XXBP-1*** führt zur Ventralisierung der injizierten Embryonen wie es von ***BMP-4*** bekannt ist. Weiterhin interagiert ***XXBP-1*** und ***BMP-4*** in Form einer positiven Rückkoppelung. Im Rahmen der BMP-4 Signalkette bewirkt die ektopische Expression von ***XXBP-1*** eine partielle Rekonstitution von epidermalem Keratin im Animalen Kappen-Assay (animal cap assay) und inhibiert die Dorsalisierung hervorgerufen durch Rumpf (truncated)-BMP-Rezeptor I. Die Ergebnisse deuten daraufhin, dass XXBP-1 downstream vom BMP-Rezeptor wirkt und eine bedeutsame Rolle in der Hemmung der neuralen Differenzierung spielt.

XXBP-1 funktioniert als Transkriptionsaktivator. Bei einer ventralen Überexpression kann sein Effekt durch eine VP 16 Aktivatordomäne, die mit der DNA-bindenden Domäne des ***XXBP-1*** fusioniert ist, simuliert werden. Ein Konstrukt, das die DNA-bindende Domäne ***XXBP-1*** und even-skipped Repressionsdomäne enthält, induziert den neuralen Marker ***NCAM*** and ***nrp1*** im Animalen Kappen-Assay und führt zur Bildung einer sekundären Achse bei einer ventralen Überexpression. Diese Ergebnisse bestätigen, dass XXBP-1 als ein Aktivator fungiert und eine Hemmung der neuralen Induktion bewirkt.

Das von uns ebenfalls charakterisierte Gen ***XMLP*** gehört zu der kleinen Proteinfamilie MLP. Durch whole-mount ***in situ*** Hybridisation und RT-PCR konnte nachgewiesen werden, dass ***XMLP*** maternal während der frühen Teilungsstadien

exprimiert wird. Nach der MBT (**m**id**b**lastula **t**ransition) sind die Signale beschränkt auf die Neuralplatte. In der weiteren Entwicklung ist ***XMLP*** exprimiert vor allen Dingen im Gehirn, Somiten und Vornieren (pronephros). Ektopische Expressionen von ***XMLP*** führt zu Augen- und Achsendefekten und zu einer Veränderung des Expressionsmusters von ***Krox20***. Injiziertes ***XMLP*** mRNA führt zur Apoptosis. Ergebnisse aus Überexpressionsexperimenten mit ***XMLP***-Mutanten weisen darauf hin, dass dieser Phänotype auf die putativen PSD Domäne und dem Glycin in Position 2 zurückzuführen ist. Injizierte Morpholino Oligos komplementär zu ***XMLP*** mRNA bewirken Deformationen der anterioren Achse und Augendefekte. Extirpationsexperimente weisen darauf hin, dass dieser Phentotyp eher auf gestörte morphogenetische Bewegungen als auf eine Inhibition des Induktionsprozesses zurückgeht. Eine Überexpression von ***XCYP26*** ergab eine Veränderung des Expressionsmusters von ***XMLP***; der Signalstreifen von ***XMLP*** in der injizierten Hälfte des Embryos wurde diffus oder verschwand sogar. Diese Beobachtung deutet darauf hin, dass Retinsäure eine wichtige Rolle spielt in der ***XMLP*** Regulation. Zusammenfassend kann man sagen, dass XMLP eine wichtige Rolle in der Musterbildung der embryonalen Achsenbildung und des zentralen Nervensystems spielt.

Ein weiteres untersuchtes Gen ***XAT*** weist Sequenzen auf, die hochkonservierte Bereiche zu menschlichen, Hühner- und Ratten- Amidinotransferase enthält. Wir konnten zeigen, dass ***XAT*** differenziell in der Zone des Dotterpfropfes einschließlich der dorsalen Urmund-Region in der frühen Gastrula exprimiert wird. In frühen Neurula-Stadien finden sich Signale in der Mittellinie der Neuralplatte. Histologische Schnitte zeigten, dass das Gen in der Chorda exprimiert wird. Im Schwanzknospenstadien können die Signale in der Chorda und in der Rumpfregion nachgewiesen werden. Schwache Signale fanden sich in der Kopfregion.

7. Synopsis

Nach der Sequenzzierung des Genoms des Menschen und anderer Organismen ist es nun von Interesse die Funktionen der Gene während der Embryonalentwicklung besser zu verstehen. Dieses Verständnis wird große Auswirkungen haben auf die biomedizinische Forschung und die angewandte Medizin.

Xenopus laevis ist ein exzellentes Vertebraten-Modellsystem, dessen Embryonen in großer Zahl das ganze Jahr über zur Verfügung stehen. Es erlaubt die Analyse der zeitlichen und räumlichen Wirkungsweise von Genen und ihren Signalketten.

Die Untersuchungen in der Entwicklungsbiologie haben sich vom zellulären zum molekularbiologischen Level verschoben. Eine große Zahl von Genen konnten mittlerweile während der frühembryonalen Entwicklung identifiziert und charakterisiert werden. Die Embryonalprozesse können einfach bezeichnet werden als an- oder abschalten von Genen. In der frühen Embryonalentwicklung von ***Xenopus*** findet sich zunächst eine animale-vegetative Polarität. Nach der Befruchtung kommt es zur differenziellen Aktivierung des Wnt/β-catenin Signalweges. auf der zukünftigen Dorsalseite des Embryos. Die Kooperation mit TGF-β führt zur Spezifizierung der dorsalen und ventralen Achse und zur Ausbildung des Spemannschen Organisators. TGF-β ist bei der Ausbildung des Entoderms beteiligt und bei der Bildung des Mesoderms in der marginalen Zone. Das in der animalen Hälfte lokalisierte Ektoderm differenziert sich in Abwesenheit von BMP-4 in neurale Richtung und zu Epidermis in seiner Anwesenheit.

Aktivin spielt eine wichtige Rolle bei der mesodermen und entodermen Musterbildung. Disaggregierte Zellen der animalen Kappe, welche mit niedrigen Konzentrationen von Aktivin behandelt werden, differenzieren sich zu Mesoderm-Derivaten, während sie sich bei höheren Konzentrationen von Aktivin zu entodermen Derivaten differenzieren (Grunz 1983, Ariizumi ***et al***., 1991). In diesem Labor wurde ausgehend von RNA prepariert, von Aktivin-behandelten Ektoderm, eine cDNA-Bibliothek konstruiert, die eine effiziente Isolierung von Genen erlauben sollte, die für die Bildung der mesodermalen/entodermalen Musterbildung verantwortlich sind. Mit random screening wurde die cDNA-Bibliothek analysiert. 51 neue Gene wurden isoliert und bei der Gen-Bank mit entsprechender Accession-Number angemeldet.

Mit 3 Genen ***XXBP-1***, ***XMLP*** und ***XAT*** mit besonders ausgeprägten Expressionsmuster wurden weitere funktionelle Studien durchgeführt.

7.1 XXBP-1: Ein Transkriptionsaktivator beteiligt am BMP-4 Signalweg

Identifzierung des *Xenopus X-box binding protein 1* (*XXBP-1*)

Die Sequenz des ***XXBP-1*** cDNA (GeneBank accession number: AF358133) weist einen offenen Leserahmen auf der für ein Polypeptide von 350 Aminosäuren codiert und eine basische leucine zipper (bZIP) Domäne enthält. Die abgeleitete Aminosequenz ist weitgehend identisch mit dem Transkriptionsfaktor X-box binding protein 1 beim Menschen (hXBP-1, NM_005080, 66% amino acids identity; Liou ***et al***., 1990), Ratte (JC4857, 66% amino acids identity), Maus (NP_038870, 66% amino acids identity) und Zebrafisch (AY029577, 59% amino acids identy).

Zeitliche und räumliche Expression von *XXBP-1*

Mittels whole-mount ***in situ*** Hybridisierung (Fig. 3.2-2) konnten wir zeigen, dass ***XXBP-1*** in der animalen Hälfte des Embryos vor der Gastrula nur schwach exprimiert ist. Während der Gastrulation finden sich Signale in der dorsalen Urmundlippe und im ganzen ventralen Ektoderm, aber nicht in der zukünftigen Neuralplatte. Während der mittleren Neurula-Stadien wird ***XXBP-1*** schwach ventrolateral exprimiert. Die Signale werden in anterior/posterioren Richtung entlang der Neuralleisten- und in der Haftdrüsen (cement gland)-Region gefunden. Im Schwanzknospenstadium finden sich Signale in der Hafttdrüse und den Vornieren (hauptsächlich im Glomerulus-Bereichl). Die RT-PCR Analyse zeigt, dass ***XXBP-1*** ein maternaler Faktor ist, der vor der Gastrulation schwach exprimiert wird, dann aber während der weiteren Embryonalentwicklung kontinuierlich vorhanden ist (Fig. 3.2-4).

Down-Regulation der *XXBP-1* mittels Retinsäure (RA)

In den Stadien von 8-11 wurde der Effekt einer RA-Behandlung (10^{-6} M) auf die Expression von ***XXBP-1*** mittels whole-mount ***in situ*** Hybridisierung durchgeführt getestet. Im Vergleich zu unbehandelten Kontrollen wiesen RA-behandelte Embryonen eine verringerte ***XXBP-1*** Expression in Neurula- und Schwanzknospen-

Stadien auf (87%, 34 von 49 Embryonen zeigten signifikante Signal-Reduktion; Fig. 3.2-6).

Neurale Inhibitoren und MO β-catenin können die Expressionsbereiche von *XXBP-1* auf der Dorsalseite des Embryos vergrößern

Es wurden die Effekte von neuralen Inhibitatoren, wie BMP-4, UV-Bestrahlung, Msx1 und Dlx3, und morpholino (MO) β-catenin auf die Expression von ***XXBP-1*** mittels whole-mount ***in situ*** Hybridisierung untersucht (Fig. 3.2-7). Es ergab sich, dass ***XXBP-1*** in der dorsalen Marginalzone durch ***BMP-4*** (98%, 42 von 43 Embryonen), MO β-catenin (100%, 73 von 73 Embryonen) oder UV (100%, 23 von 23 Embryonen) aktiviert wird. Das Ergebnis ist eine Expression in der gesamten Marginalzone. ***Msx1*** und ***Dlx3*** führten zu einer Ausdehnung der ventralen Expressionen des ***XXBP-1*** über die gesamte prospektive Neuralplatte. Zusammenfassend kann man feststellen, dass all diese neuralen Inhibitoren und MO β-catenin die Expressionsregion des ***XXBP-1*** ausweiten.

Hemmung von neuralen Markergenen induziert durch Mikroinjektion von *XXBP-1*

Einseitige Injektion von ***XXBP-1*** (1 Blastomere im 2-Zellstadium) ergab die Reduktion der neuralen Marker wie ***Otx2*** (60%, 94 von 156 Embryos), ***Rx2A*** (41%, 47 von 116 Embryos), ***En2*** (49%, 31 von 63 Embryos), ***Krox20*** (71%, 34 von 48 Embryos), ***Pax6*** (50%, 28 von 56 Embryos), and ***Sox3*** (58%, 44 von 76 Embryos) (Abb. 3.2-8). Der Effekt der ***XXBP-1*** Injektion wurde sichtbar durch das Verschwinden der Expression dieser Gene. Anderseits wurde die Expression von ***XAG2*** (Haftdrüsen [cement gland] spezifisches Gen) nicht beeinflusst.

Hemmung der dorsalen mesodermalen Marker *chordin* (*chd*) und *gooescoid* (*gsc*) durch *XXBP-1*

Whole-mount ***in situ*** Hybridisierung ergab, dass die Injektion von ***XXBP-1*** mRNA zur Unterdrückung von ***chd*** (65%, 37 von 57 Embryos) und ***gsc*** (75%, 42 von 56 Embryonen) führt (Abb. 3.2-9I). Die Analyse der dorsalen Marginalzone ergab Hinweise, daß diese down-regulation dosisabhängig ist (Abb. 3.2-9II).

Überexpression von *XXBP-1* führt zur Ventralisierung der Embryonen

Besonders ausgeprägte Abnormalitäten ergaben sich durch Überexpression von ***XXBP-1*** in Form der Unterdrückung der anterioren-dorsalen Strukturen einschließlich der cement gland, Augen, Ohrvesicle und in vielen Fällen des gesamtes Kopfbereiches. Die histologische Analyse dieses Phänotypes der Ventralisierung (Abb. 3.2-10) ergab eine Reduktion der Chorda und der Gehirnstrukturen (Abb. 3.2-10H).

Überexpression von *XXBP-1* ventralisiert dorsales Mesoderm

Ventrale Marginalzone (VMZ) und dorsale Marginalzone (DMZ) früher Gastrulastadien von ***XXBP-1*** injizierten Embryonen wurden isoliert und bis zum Stadium 22 in Kultur gehalten. Danach erfolgte die RT-PCR Analyse verschiedener Markergene (Abb. 3.2-11). ***Gsc*** and ***Xnot***, aber nicht ***chd***, werden inhibiert, was darauf hinweist, dass die Bildung von Kopfmesoderm und Chorda durch ***XXBP-1*** beeinflusst wird. Der Verlust der Expression der dorsalen mesodermen Markergene geht einher mit dem Anstieg der Expression intermediärer und ventro-posteriorer mesodermaler ***Markerwie Herzaktin*** (c***ardiac actin***). Der ventrale mesoderme Marker, ***Xvent-2*** wurde bei allen Knzentrationen, ***Xhox3***, ***Xvent-1*** und ***BMP-4*** in einer dosisabhängigen Weise induziert. Zusammenfassend kann man sagen, dass die Überexpression von ***XXBP-1*** in der isolierten dorsalen marginalen Zone zu einer down-regulation von dorsalen Markergenen und parallel zu einer up-regulation von ventralen Markergenen führt.

XXBP-1 **hebt die Dorsalisierung auf, die durch dominant negative BMP-4 Rezeptoren induziert wird**

Das Animale Kappen-Assay wurde durchgeführt mittels RNA isoliert aus animalen Kappen von Embryonen nach Injektion von mRNAs von 0,4 ng ***XXBP-1***, 1,6 ng tBMPRI (Suzuki ***et al***., 1994), eine Mischung von 1,6 ng tBMPRI und 0,4 ng ***XXBP-1*** und eine Mischung von 1,6 ng tBMPRI, 0,8 mg tBMPRII (Frisch, 1998) und 0,4 ng von ***XXBP-1*** (Abb. 3-12l). Im Gegensatz zu Ektodermzellen mit tBMPRI alleine, differenzieren sich Ektodermzellen nach Injektion einer Mischung von tBMPR und ***XXBP-1*** in epidermale Richtung. Dies deutet darauf hin, dass ***XXBP-1*** die durch tBMPRs hervorgerufene Neuralinduktion inhibiert. Somit dürfte ***XXBP-1*** downstream

des BMP-Rezeptor wirksam sein. Dies wurde weiterhin bestätigt durch Überexpressions-Experimente, in denen ***XXBP-1*** die durch Injektion von tBMPRI bewirkte Dorsalisation aufhebt (Abb. 3.2-12II).

Noggin ist ein Antagonist von _XXBP-1_ im Animalen Kappen-Assay

Im Animalen Kappen-Assay wurde ***XXBP-1*** in der gleichen Weise durch Noggin unterdrückt wie ***BMP-4***. ***XXBP-1*** und ***BMP-4*** konnten durch ***Zic3*** inhibiertwerden, während MO β-catenin keinen signifikanten Effekt hatte.

Zusammenfassend kann man sagen, dass ***XXBP-1*** beteiligt ist am BMP-4 Signalweg aus folgenden Gründen: Erstens, das Expressionsmuster von ***XXBP-1*** in der Gastrula deutet darauf hin, dass es eine negative Rolle spielt in der neuralen Induktion (Abb. 3.2-2). Zweitens, ektopische Expression von ***BMP-4*** steigert die ***XXBP-1*** Expression in der dorsalen marginalen Zone (Abb. 3.2-7) und vice versa (Abb. 3.2-11). Drittens, Überexpression von beiden führt zur Ventralisierung der Embryonen in einer dosisabhängigen Weise, was in den Phänotypen von Microcephalie bzw. Bauchstücken resultiert (Abb. 3.2-10). Viertens, ***XXBP-1*** kann gsc unterdrücken (Abb. 3.2-9), während beide—***BMP-4*** und ***XXBP-1*** durch ***Noggin*** im Animalen Kappen-Assay inhibiert werden (Abb. 3.2-13). Viertens, Koinjektion von mRNA von ***XXBP-1*** und tBMPRI kann die Dorsalisierung der Embryonen verhindern, die durch Injektion von tBMPRI alleine hervorgerufen wird (Abb. 3.2-12II), und bewirkt weiterhin eine direkte Epidermalisierung im Animalen Kappen-Assay (Abb. 3.2-12I).

Die Ergebnisse deuten darauf hin, dass ***XXBP-1*** downstream der BMP-4 Rezeptoren wirkt. Überexpression von tBMPRs in ektodermalen Explantaten induzieren neurale Gewebe, nachgewiesen durch neurale Marker wie ***NCAM*** und ***Otx2***. Andererseits wird die Expression von ***NCAM*** und ***Otx2*** unterdrückt und epidermales Keratin durch Koinjektion von tBMPRI und ***XXBP-1*** induziert. Das bedeutet, dass XXBP-1 in der Lage ist, die neurale Induktion, die durch tBMPR hervorgerufen wird, zu inhibieren. In Überexpressions-Experimenten kann ***XXBP-1*** die Dorsalisierung, die durch tBMPRI hervorgerufen wird, inhibieren. Diese Ergebnisse zeigen, dass ***XXBP-1*** downstream der ***BMP-4*** Rezeptoren wirkt. Die Induktion von ***BMP-4*** durch ***XXBP-1*** in DMZ ergibt Hinweise darauf, dass ***XXBP-1*** notwendig ist für die Aufrechterhaltung der ***BMP-4*** Expression.

XXBP-1 wirkt als ein Transkriptionsaktivator

Die putative DNA-Bindungsdomäne des ***XXBP-1*** wurde fusioniert mit der vorher charakterisierten Sequenz, die entweder den Repressor oder den Aktivatordomäne – wie die Repressordomäne von Drosophila engrailed (Jaynes and O' Farrell, 1991) oder die Repressordomäne von even-skipped (Han and Manley, 1993) bzw. die Aktivatordomäne von VP16 repräsentiert (Friedmann ***et al***., 1988). Die schematischen Darstellung des Wildtypes XXBP-1 und der Fusionskonstrukte sind in Abbildung 3.2-14I gezeigt. Überexpressionsexperimente mit XXBP1-V16, das die Aktivatordomäne enthält, imitiert den Effekt des XXBP1-Wildtyp. Es resultierte eine Ventralisierung des Embryos nach einer dorsalen Injektion bzw. eine posteriore Vergrößerung des Embryos nach ventraler Injektion. Andererseits bewirkt XXBP1-Eve mit der Repressionsdomäne die Bildung einer Doppelachse nach ventraler Injektion (Fig. 3.2-14II). Im Gegensatz zu XXBP-1-Wildtyp und XXBP1-VP16 kann XXBP1-Eve die neuralen Marker ***NCAM*** und ***nrp1*** induzieren (Abb. 3.2-15). Deshalb kann XXBP1-Eve Gene aktivieren, die normalerweise durch ***XXBP-1*** reprimiert werden und folglich ***XXBP-1*** als ein Transkriptionsaktivator fungiert. Die Rumpfmutante von ***XXBP-1***, die die ersten 126 Aminosäuren (einschließlich bZIP) enthalten, hat einen schwachen Effekt auf die neurale Induktion wie es sich in der erhöhten Expression von ***Otx2*** äußert. Die Überexpression dieser Rumpfmutante führt während der Gastrulation zu Defekten, insbesondere zu spina bifida. Diese Ergebnisse deuten darauf hin, dass XXBP-1 Homodimere bildet, um seine Funktionen auszuüben.

7.2 *XMLP* ist bei morphogenetischen Prozessenbeteiligt

Identifizierung eines *Xenopus MARCK verwandten Proteins* (*XMLP*)

Der isolierte Klon kodiert für ein Protein mit 187 Proteinen, das mit der MARCKS-ähnlichen Proteinfamilie (MLP)verwandt ist. In Hormologie-Vergleichen konnte eine 32%ige Identität mit MLP der Maus, Ratte, Mensch und Kaninchen, nachgewiesen werden (Abb. 3.3-1). Die abgeleitete Protein-Sequenz enthält drei konservierte Domänen, die auch bei den anderen Mitgliedern der MARCKS und MLP-Familie zu finden sind; es handelt sich dabei um eine myristilierte Consensus-Sequenz, die einem Glycine-Rest in Position 2 in der Amino-terminalen-Region folgt, und zwar in der Region des intron splicing und der putativen Phosphorylierungsdomäne (PSD).

Der theoretisch isoelektrische Punkt (pI) von XMLP (4.38) und der PSD-Domaine (12.06) sind typisch diese Proteinfamilie. Die volle Sequenz wurde eingereicht unter der Accession-Number: AF187864 in der Gen-Bank.

Räumliche und zeitliche Expression von *XMLP*

Maternale Transkripte von ***XMLP*** wurden während der Furchungsstadien in der animalen Hälfte des Embryos mittels whole-mount ***in situ*** Hybridisierung nachgewiesen (Abb. 3.3-2A, B). In der Gastrula wurden Signale in fast allen Regionen mit Ausnahme der Dotterpfropfregion gefunden (Abb. 3.3-2C). Im Stadium 11.5 wurde ***XMLP*** hauptsächlich im Ektoderm und Mesoderm exprimiert (Abb. 3.3-2D). Ab der späten Gastrula fanden sich Transkripte nur im Bereich der präsumptiven der Neuralpatte(Abb. 3.3-2E-G). In der weiteren Entwicklung fanden sich Signale in der Neuralfalten und in der Gehirnregion des Schwanzknospenstadiums (Abb. 3.3-2H, I, M). Im Stadium 34 wurden Signale in der Kopfregion, Somiten, Vornierenkanälchen und Harnleiter gefunden (Abb. 3.3-2O-Q). Transkripte konnten in allen Stadien mittels RT-PCR nachgewiesen werden. Der Transkriptionslevel nahm vom ungefurchten Ei und während der Furchungsstadien bis zum Stadien 23 ab, während in den folgenden Stadien leicht anstieg und schließlich eine stabile Höhe erreichte (Abb. 3.3-3). Mittels RT-PCR wurde die ***XMLP***-Expression in allen untersuchten adulten Geweben gefunden. Eine relativ niedrige Expression wurde im Darm und in der Niere nachgewiesen (Abb. 3.3-4).

Phänotypische Effekte durch *XMLP* Überexpression

Embryos zeigten nach Injektion von ***XMLP*** mRNA in eine dorsale Blastomere des 4-Zell-Stadiums den Phänotyp Apoptose (Abb. 3.3-5A, B). Zwei Haupttypen morphologischer Defekte wurden nach Injektionen bei der Inspektion der Larven beobachtet. Ein Typ wies Reduktion und eine abnormale Struktur der Linse auf (Abb. 3.3-5C, D, E). Einige Larven zeigten sogar Cyclopie (Abb. 3.3-5F). Andere Embryonen wiesen eine gebogene Körperachse auf. Bei höheren Injektionsdosen zeigten die Embryonen eine Kombination beider Phänotypen. Die Ergebnisse der Injektionsversuche sind in Tabelle 3.3-1 dargestellt.

Ektopische Expression von *XMLP* änderte nicht die normale autonome Differenzierung von isolierter dorsaler Urmundlippe

Explantate bestehend aus dorsaler Urmundlippe und daran anschließendes dorsales Ektoderm wurden von injizierten Embryonen und Kontrollen im Stadium 10 isoliert und anschließend bis zum Stadium 40 aufgezogen. Wie die uninjizierten Kontrollen entwickelten sich die Explantate injizierter Embryonen in gleicher Weise in Chorda, Gehirn und Augenstrukturen (Abb. 3.3.-5 J, K). Dies deutet darauf hin, dass die Augendefekte nicht auf eine spezifische Inhibition der Augenanlage durch XMLP (Abb. 3.3-5) sondern auf eine Störung der morphogenetische Bewegungen zurückzuführen sind.

Der Effekt von morpholino oligo (MO) XMLP nach Injektion in Furchungsstadien

Der Verlust der Funktion ***XMLP*** (loss-of-function) wurde durch Injektion von Morpholino Oligos (MO)- XMLP in eine Blastomere des 2-Zell-Stadiums oder zwei dorsale Blastomeren im 4-Zell-Stadium untersucht. Keine signifikanten toxischen Effekte wurden nach der Injektion von Morpholino bzw. Negativkontrollen beobachtet. MO-XMLP injizierte Embryonen zeigten Augendefekte, verkürzte anteriore Achse und eine schwache Körperachsenkrümmung (Abb. 3.3-6A und Tabelle 3.3-2). Weiterhin konnten MO-XMLP signifikant den Phänotyp der Apoptose, induziert durch XMLP, rückgängig machen (Abb. 3.3-6B, C).

Das Glycine in Position 2 und die PSD (ED) Domäne sind korreliert mit der Apoptose in Überexpressionsexperimenten

Zum Studium der Rolle der funktionellen Domäne von ***XMLP*** wurden vier Mutanten SD, S83A, G2A, ED konstruiert (Abb. 3.3-7, siehe auch 2.9.3) und in Überexpressionsexperimenten eingesetzt. Im Gegensatz zum Wildtyp XMLP, riefen die Mutanten S83A, G2A und ED keine signifikante Apoptose hervor. Die Phänotypen der Larven nach Injektion in zwei dorsale Blastomeren des 4-Zell-Stadiums sind in Abb. 3.3-8H bis M gezeigt. Alle zeigten Augendefekte, gebogene Körperachse und in wenigen Fällen eine starke Reduktion der vorderen Körperachse. Insbesondere wenn 1,6 ng ED-Mutanten RNA injiziert wurde, blieb der ektodermale Bereich der Hinterhirnregion der Schwanzknospenstadien offen, wahrscheinlich zurückzuführen auf einen unvollständigen Verschluß der

Neuralwülste (Abb. 3.3-8L). Dieser Phänotyp ist vergleichbar mit dem der F52 Knockout-Maus, die Exencephalie und postnatale Anencephalie zeigt. Die Ergebnisse sind in Tabelle 3.3-3 dargestellt.

Ektopische *XMLP* Expression inhibiert die normale Rhombomerenbildung

Überexpression von ***XMLP*** wurde realisiert durch Injektion in eine dorsale Blastomere des 4-Zell-Stadiums und gleichzeitig von ***LacZ*** mRNA als Lineage tracer. Die anschließende whole-mount ***in situ*** Hybridisierung deutet darauf hin, dass ektopische Expression des ***XMLP*** eine Auslöschung des ***Krox20*** Signals in den Rhombomeren 3 und 5 bewirkt (Abb. 3.3-9B).

Retinsäure Hydroxylase (*XCYP26*) verändert das Expressionsmuster von *XMLP*

Nach Co-Injektion von 2 ng XCYP26 und 100 pg LacZ capped RNA in eine Blastomere des 2-Zell-Stadiums erfolgte die Analyse mit XMLP antisense im Stadium 20 mittels whole-mount ***in situ*** Hybridisierung. Die Signalstreifen auf der injizierten Seite waren schwächer oder waren überhaupt nicht mehr vorhanden (31 von 54 Fällen) im Gegensatz zur uninjizierten Seite (Abb. 3.3-9D).

Zusammenfassend kann festgestellt werden, dass ***XMLP*** beteiligt ist an der normalen Entwicklung des Neuralrohres und der Anlage des Zentralen Nervensystems. Weiterhin induziert ***XMLP*** Apoptose in Überexpressionsexperimenten. Im Vergleich zu anderen Mitgliedern der MARCKS-Familie kann man annehmen, dass XMLP bei morphogenetischen Bewegungen während der embryonalen Entwicklung beteiligt ist. Die gestörten morphogenetischen Bewegungen und die induzierte Apoptose könnten Gründe für eine Reduktion der anterioren Körperachse und der Augendefekte sein. Weiterhin ist das Expressionsmuster von ***XMLP*** durch die ektopiche Expression von ***XCYP26*** betroffen. Dies deutet darauf hin, dass RA an der Regulation von XMLP und bei der anterior/posterioren Körperachsenbildung beteiligt ist.

7.3 Zeitliche und räumliche Expression von *XAT*

XAT besitzt eine hoch konservierte Sequenzregion einer Amidinotransferase, die auch beim Menschen, Huhn und Ratte vorhanden ist (Abb. 3.4-1). ***XAT*** wird in der frühen Gastrula in der Region des Dotterpfropfes einschließlich der dorsalen

Urmundlippenregion differenziell exprimiert. Signale finden sich dann in der Mittellinie der Neuralplatte der frühen Neurulastadien (Abb. 3.4-2). Die Auswertung transversaler histologischer Schnitte (Abb. 3.4-2G) ergab, dass die Transkripte in der Chorda lokalisiert sind. Im Schwanzknospenstadium werden die Signale sowohl in der Chorda als auch in der Rumpfregion gefunden, während nur schwache Signale in der Kopfregion vorhanden sind (Abb. 3.4-2 und 3.4-4). Die RT-PCR Analyse (Abb. 3.4-3) ergab die Expression des Gens ab Stadium 8, gefolgt mit einem leichten Anstieg während der Gastrula und Neurula bzw. gleichbleibendem Niveau im Schwanzknospenstadium und späteren Embryonalstadien. RT-PCR adulter Geweben zeigt eine höhere XAT Expression vor allem im Auge, Muskel, Magen und Hodengewebe (Abb. 3.4-5).

8. References

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Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Functional studies of novel genes involved in embryogenesis of the vertebrate ***Xenopus laevis***" zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Herrn Hui Zhao befürworte.

Essen, den 15.04.2002

Professor Dr. Horst Grunz

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbstständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den 15.04.2002

Hui Zhao

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Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Essen, den 15.04.2002

Hui Zhao