Identification and Functional Characterization of Novel Genes Involved in Primary Neurogenesis in *Xenopus laevis*

Dissertation
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultäten
der Georg-August-Universität zu Göttingen

vorgelegt von
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Göttingen 2002

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Tag der mündlichen Prüfung: 20 . 06 . 2002

Herewith I certify, that I prepared the Ph.D thesis on "**Identification and Functional Characterization of Novel Genes Involved in Primary Neurogenesis in *Xenopus laevis***" on my own and with no other sources and aids than quoted.

Göttingen, 13. 05. 2002

Jacob Souopgui

To my mother, Ermine
and my wife, Dorothée

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ABBREVIATIONS

APS	ammonium peroxodisulfate
APB	alkaline phosphatase buffer
ATP	adenosintriphosphate
BCIP	5-bromo-4-choro-3-indolyl-phosphate
BMB	Boehringer block
bp	base pairs
BSA	bovine serum albumin
cDNA	complementory DNA
CHAPS	3-((3-cholamidopropyl)dimethylammonio)-1-propansulfate
CTP	cytosintriphosphate
dATP	desoxyriboadenosintriphosphate
°C	degree Celsius
dCTP	desoxyribocytosintriphosphate
dH_2O	distilled H_2O
dGTP	desoxyriboguanosintriphosphate
DEPC	diethylpyrocarbonate
DIG-	Digoxigenin-11-2′-deoxyuridin-5′-triphosphate
DMSO	Dimethylsulfoxide
DNA	desoxyribonucleic acid
Dnase	Deoxyribonuclease
dNTP	desoxyribonucleotidtriphosphate (dATP, dCTP, dGTP, dTTP)
DTT	dithiotreitol
dTTP	desoxyribotymidintriphosphate
EDTA	ethylendiamintetraacetate
EGTA	ethylenglycol-bis(2-aminoethylether)-N,N'-tetraacetate
F	forward (primer)
GTP	guanosintriphosphate
h	hour
HEPES	N-(-hydroxymethyl)piperazin,N'-3-propansulfoneacid
HCG	human chorionic gonadotropin
IPTG	isopropyl-ß-D-thiogalactopyranoside

kb	kilobase
LB	Luria-Bertrani medium
M	mol
m	milli
MBT	midblastula transition
min	minute
µ	micro
MAB	Malic acid buffer
MEM	MOPS-EGTA-MgSO4-Buffer
MEMFA	MOPS-EGTA-MgSO4-Formaldehyde-Buffer
MOPS	3-(N-Morpholino)propanesulfonic acid
MO	morpholino oligonucleotide
mRNA	Messenger-RNA
myr	myristylation
NBT	nitro-blue-tetrazolium
NTP	ribonucleotidetriphosphate (ATP, CTP, GTP, TTP)
PVP	polyvinyl pyrrolidon
PCR	polymerase chain reaction
pH	Prepondirance of hydrogen ions
R	reverse (primer)
RNA	Ribonucleic acid
RT	room temperature
rpm	revolution per minute
RT-PCR	Reverse transcriptase –PCR
SSC	Standard Saline Citrate Buffer
Taq	*Thermus aquaticus*
TBE	Tris-Borate-EDTA-Electrophoresis buffer
SDS	sodiumdodecylsulfate
TEMED	N,N,N'N'-tetramethyl-ethylenediamine
Tris	trihydroxymethylaminomethane
TTP	thymidintriphosphate
U	units
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

I. INTRODUCTION

Developmental biology is a multidisciplinary field of scientific investigations whose main objective is to understand the formation of a complex adult organism from a single cell. During the last few decades, intensive research work using molecular biology techniques, has focused on elucidating the molecular events occuring during development. Investigations at the level of individual genes are currently guiding developmental biologists towards a better understanding of the genetic network underlying the process of development. Interestingly, these studies have set additional relevant goals, including definition of experimental protocols for the generation of specific cell types from pluripotent precursor cells *in vitro* to be used in medical applications for the treatment of diseases associated with cell degeneration. Furthermore, developmental biology can provide a foundation for targeted drug development and a better understanding of diseases associated with genetic disorders. The use of appropriate animal models has been one of the foremost preoccupations in this field.

The African claw-toed frog *Xenopus leavis*, whose life cycle is shown in figure 1, is one of the systems that can be reliably used to illustrate the principle mechanisms of vertebrate development. There are several advantages of using *Xenopus* for the study of embryonic development. Embryos are relatively large, easy to cultivate and survive after experimental manipulations. Initially, the egg has a single animal-vegetal axis of asymmetry. Sperm entry induces the microtubule-driven rotation of the egg cortex by 30° relative to the inner cytoplasm (Gerhart et al., 1989). This cortical rotation displaces the vegetal maternal determinants to the equatorial region opposite the sperm entry point and defines the future dorso-ventral axis (Harland and Gerhart, 1997). Inhibition of this rotation by UV light treatment leads to a range of ventralization phenotypes (Kao and Elinson 1988).

The fertilized egg undergoes a series of synchronized divisions under the control of

maternal regulators, including cyclins and cyclin-dependent kinases. These cleavages lead successively to the morula, a mass of cells without an internal cavity, and the blastula, different from the previous by the presence of the blastocoel. The morphology of the embryo remains

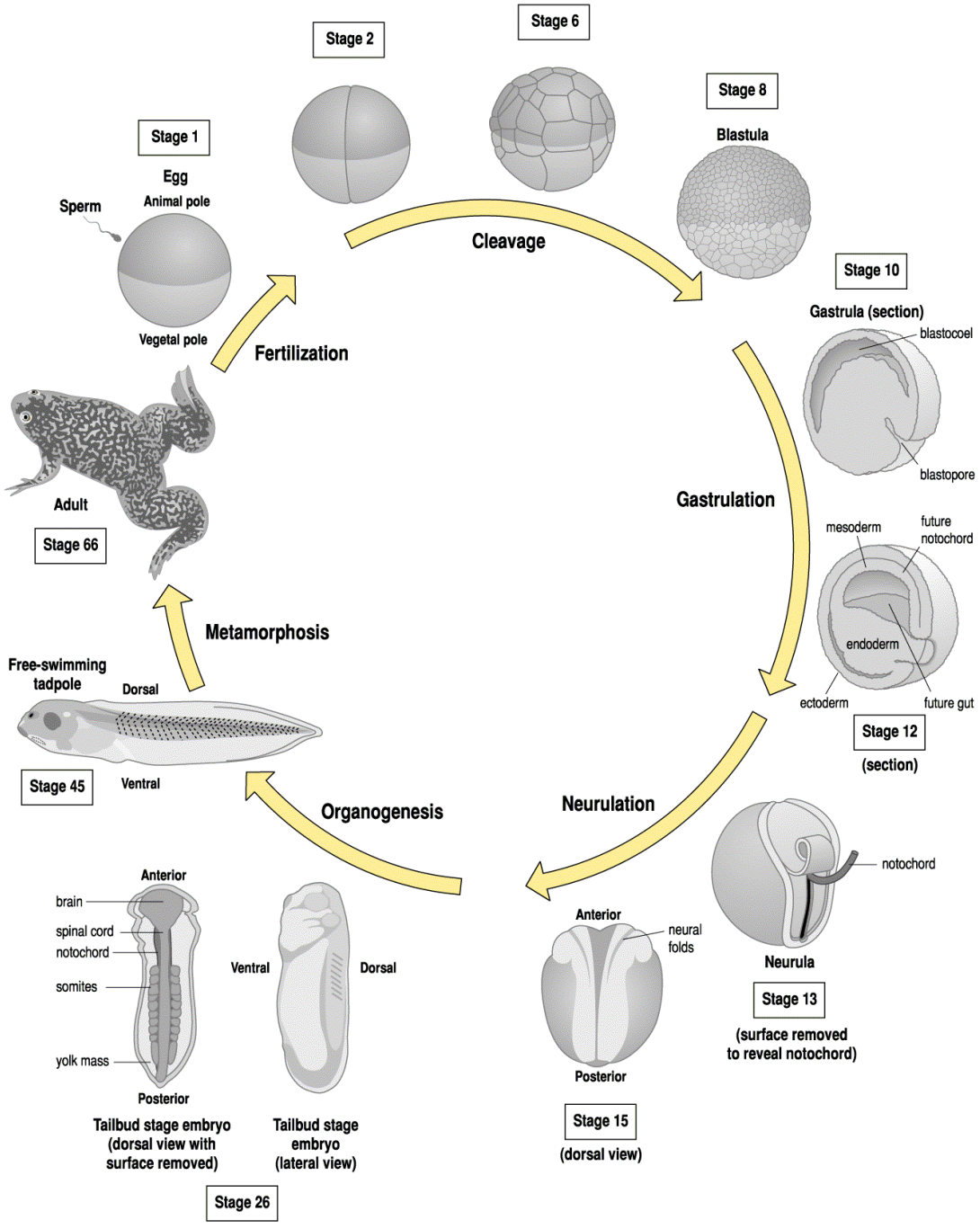


Figure 1: **Basic stages of *Xenopus laevis* development** (Wolpert, 1998)

At 25° C, the gastrulation process occurs within the next 10 to 15 hours after fertilization while neurulation is completed after about 20 hours. The tailbud embryo is obtain after one day and the free-swimming tadpole after 4 days. Depending on the type of food, an adult can be raised after one year or more.

radially symmetrical until late blastula stage. Following the onset of zygotic transcription at midblastula, the Spemann organizer is generated in the future dorsal side of the embryo (Gerhart et al., 1989). Cells from this center invaginate, converge, and extend internally in a process known as gastrulation, a key event in the context of vertebrate development.

During gastrulation, the characteristic three germ layers, ectoderm, mesoderm and endoderm, are created. The ectoderm corresponds to the animal hemisphere, the mesoderm arises from the equatorial or marginal zone, and the endoderm to the vegetal hemisphere. By the completion of gastrulation, the anterior-posterior axis is established (Gerhart et al., 1989).

I. 1 Neural induction and patterning in *Xenopus*

During gastrulation, the ectoderm lying along the dorsal midline of the embryo differentiates into neural tissue. This neural development is viewed as progressing in four major steps. Firstly, cells gain the ability to become neural precursors in response to the appropriate signal (competence). Secondly, such neural precursors go through a phase where they can still respond to signals that repress neuralisation (specification). Thirdly, cells lose this same ability (commitment) and, in the fourth step, they exit the cell cycle to become post-mitotic neurons (differentiation). Neural induction is a term that defines the step when ectodermal cells become specified as neural precursor cells (reviewed by Wilson and Edlund, 2001).

Transplantation experiments have been informative in understanding the process of neural induction in *Xenopus* (Mangold, 1933; Philips and Doniach, 1990). A graft of the organizer region from an early gastrula to the ventral side of another gastrula stage embryo results in the formation of nervous system from the host ectoderm that would normally have formed epidermis (Spemann and Mangold, 1924; Mangold, 1933; Smith and Slack, 1983; Recanzone and Harris, 1985; Shih and Keller, 1992). Ectoderm from a prospective epidermal

area, transplanted before gastrulation to the dorsal side of another gastrula, develops into neural tissue (Smith and Slack, 1983; Phillips, C. R., 1991). In addition, explants of ectodermal tissue from blastula or gastrula stage embryos grafted onto oocytes pre-injected with RNA encoding noggin reconstitute neural induction (Lustig and Kirschner, 1995). This suggest that neural tissue can be induced from as yet unspecified ectoderm by signals emanating from the mesoderm of the organizer region. Kelly and Melton (1995) proposed a two-step model to support this inductive activity (fig. 2).

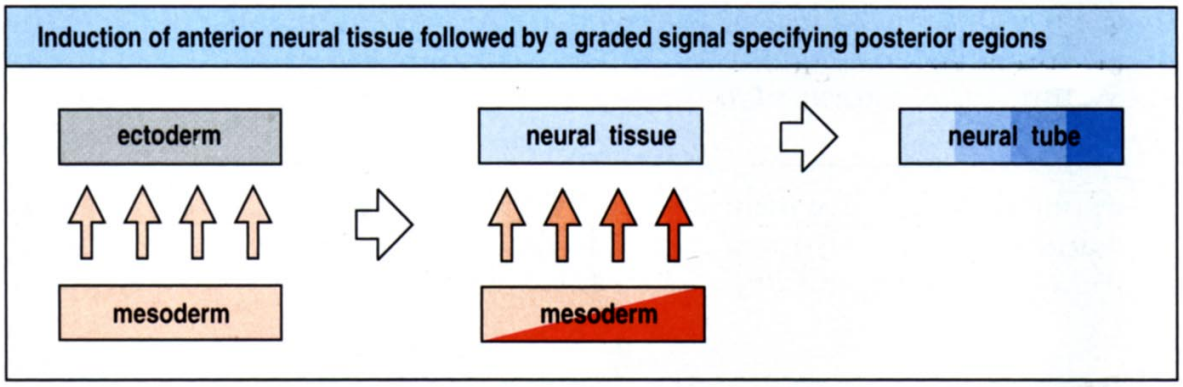


Figure 2: **Two-step model of neural patterning by induction**. (Wolpert, 1998)

In this model, one signal from the mesoderm first induces anterior tissue throughout the corresponding ectoderm. A second, graded signal from the mesoderm then specifies more posterior regions.

It has also been shown that a graft from the dorsal lip region of an early gastrula induces the development of an additional complete anterior-posterior axis, whereas a graft from the dorsal lip region of late gastrula only induces the formation of an additional tail structure. Mesoderm grafts from different positions along the antero-posterior axis of a newt neurula have been transplanted into the blastocoel of an early gastrula; transplants of anterior mesoderm induce a head with eye and forebrain, while posterior mesoderm generates trunk and tail (Mangold., 1933). In a more recent analysis using embryological and molecular techniques, Zoltewicz and Gerhart (1997) divided the earliest gastrula organizer, a region

measuring 20 cells high by 25 cells wide, into anterior (vegetal) and posterior (animal) halves. They observed that each half, not only has a distinct fate and state of specification, but also induces a unique set of region-specific neural genes. When wrapped in animal cap ectoderm, the anterior half induces only anterior specific genes (XAG-1 and OtxA), while the posterior half induces anterior (OtxA and reduced levels of XAG-1) as well as posterior (Hox B9) neural markers. These studies demonstrate that the activities of Spemann organizer in *Xenopus* embryos are already regionalized at early gastrula stages.

Like the amphibian organizer, the chick and mouse node/organizer can induce ectopic neural cells (Storey et al., 1992; Beddington, 1994). However, although such transplantation studies demonstrate that the node/organizer is sufficient to induce ectopic neural cells, it was not addressed whether the organizer region is required during the normal process of neural induction. To address this question, the node/organizer was surgically removed in chick, frog, zebrafish, and mouse embryos, and the resulting transformants also develop a neural plate (reviewed in Wilson and Edlund, 2001). Collectively, these findings suggest that the neural inducing signals may derive from tissues other than or in addition to the node/organizer.

I. 2 The molecular nature of neural induction in *Xenopus*

Since the discovery of the inducing activities of the Spemann organizer, enormous efforts have been made to characterize the molecular events involved in the process of neural induction. These efforts have been guided by the observation that dead tissue was still competent in neural induction, thereby, raising the possibility that the inducing signals are diffusible molecules. Proteins encoded by specific genes have been the main candidate molecules. A secreted member of the transforming growth factor-beta (TGF-ß), the bone morphogenetic protein-4 (BMP-4), has been shown to induce epidermis at the expense of

neural tissue in *Xenopus* embryos (Wilson and Hemmati-Brivanlou, 1995). The temporal and spatial expression pattern of this gene is highly relevant: ubiquitous throughout the late blastula, BMP-4 is no longer expressed in the dorsal region when the gastrulation proceeds (Hawley et al., 1995). BMP-4 loss-of-function using a dominant negative receptor results in direct neural induction (Hawley et al., 1995). These results have raised the hypothesis that neural inducers might act by blocking BMP-4 binding signals. Several BMP antagonists identified in Spemann Organizer and other tissues contribute to both dorsal mesodermal and neural identities; these include Noggin (Smith and Harland 1992; Lamb et al., 1993; Smith et al., 1993; Zimmerman et al., 1996), Chordin (Saisi et al., 1995, 1995; Piccolo et al., 1996), follistatin (Hemmati-Brivanlou et al., 1994; Fainsod et al., 1997), cereberus (Bouwmeester et al., 1996; Hsu et al., 1998), and ventroptin (Sakuta et al., 2001).

Whereas neural induction in *Xenopus* appears relatively well described, little is known about signals that mediate neural induction in other vertebrates. In other species, structures homologous to the organizer express BMP antagonists, however BMP antagonists do not have potent inducing activity in chick epiblast (Streit et al., 1998), suggesting that additional or alternative mechanisms are used for neural induction. Early expression of murine Wnt8, Xwnt8, ß-catenin, or dominant-negative GSK3 was found to induce the expression of neural-specific genes and inhibited the expression of BMP-4 in *Xenopus* ectoderm (Baker et al., 1999). Inhibition of ß-catenin activity in the neural ectoderm of whole embryos by a truncated TCF results in a decrease in neural development, suggesting that a cleavage-stage Wnt signaling normally contributes to early repression of BMP-4 on the dorsal side of the embryo and sensitises the ectoderm to respond to neural inducing signals from the organiser (Baker et al., 1999). The antagonists of Wnt signalling, dnXwnt8 and Nxfrz8, inhibit Wnt mediated induction of Xnr3 and siamois, known to have neuralizing activity, but without influencing

neural induction, suggesting an alternative mechanism. Conversely, dominant-negative TCF blocks both Wnt-mediated induction of Xnr3 and neural induction, providing evidence that downstream components of the classical Wnt-induced signal transduction pathway are required for neuralization. This positive implication of the wnt signaling in the neural induction however seems not to be conserved in evolution, and the situation appears to be completely different in chick (reviewed in Wilson and Edlund, 2001).

The lack of requirement of the node for the generation of neural cells in chick and mouse raise the possibility that the specification of neural fate is initiated before the formation of the node. Consistent with this idea, the early neural markers Sox3, SoxD and Geminin, which induce neural differentiation if ectopically expressed, are already present in the ectoderm before gastrulation in late-blastula embryos, and Sox3 expression becomes restricted to dorsal ectoderm before the onset of gastrulation in *Xenopus* (Penzel et al., 1997; Mizuseki et al., 1998; Kroll et al., 1998). Moreover, by use of an antibody that recognizes the activated (phosphorylated) form of the BMP effectors Smad1, 5 and 8, which are indicators of active BMP signaling, it was shown that BMP signaling starts to be restricted ventrally by late blastula, before the organizer has formed (Kurata et al., 2001). Finally, a recent study demonstrated that neural induction can occur in the absence of mesoderm (Wessely et al., 2001). Collectively, these studies indicate that blastula stages animal caps contain both prospective neural and epidermis cells. One explanation that the predisposition to neural differentiation is not detected in animal cap explants may be attributed to BMPs, secreted by epidermal prognitor cells in the same explants. Therefore, embryonic ectoderm cells may be exposed to signals that specify neural fate before the formation of the organizer. In this model, BMP antagonists would not induce neural fate, but rather prevent the suppression of a previously specified neural character.

I. 3 Neuronal differentiation in *Xenopus* embryos

The central nervous system (CNS) is one of the earliest organs formed during the development of vertebrates and invertebrates. The differentiation of this organ from the neurogenic epithelium in vertebrates involves a complex interplay between factors that regulate gene transcription and those that mediate cell-cell interactions. Two main genetic pathways, comprising proneural and neurogenic signalings, are evolutionarily conserved and have been well defined in this context.

I. 3. 1 Proneural signaling

Determination and differentiation of neuronal cells result from a cascade of genes whose activities define the proneural pathway. The main function of this genetic network is to transform pluripotent neural cells into fully committed neuronal cells (reviewed in Jan and Jan, 1993; Chitnis, 1999).

In all animal species, basic helix-loop-helix (bHLH) type transcription factors have been shown to function in a two-step model to induce the neuronal fate in the first step, and to promote the fate commitment in the second step. In *Drosophila*, proteins encoded by the *achete-scute* gene complex and *atonal* (Campos-Ortega, 1998; Modolell, 1997) initiate neurogenesis in a selected precursor cells. Similarly in vertebrates, the *atonal* homologs *Neurogenin1*, *Neurogenin2* and *NeuroD* drive the early "determination" and the late "differentiation" phases of neurogenesis, respectively (Ma et al., 1996; Lee, 1997; Tomita et al., 2000). Consistent with the temporal and spatial expression of these genes, the findings that the downstream bHLH genes, *NeuroD* or *NSCL* are not activated in subsets of differentiating neurons in mice that lack *Ngn1* or *Ngn2* (Fode et al. 1998; Ma et al., 1998) support the idea of a two-step model. However, some non-bHLH proteins including HLH proteins and zinc-finger proteins are required to regulate successive stages of neuronal

differentiation in the developing neural tissues (Bellefroid et al., 1996; Dubois et al, 1998; Lamar et al., 2001b; Pozzoli et al., 2001).

In *Xenopus*, expression of the bHLH gene neurogenin-related-1(X-Ngnr-1) defines three bilateral territories of primary neurogenesis (fig. 3) in the developing neural plate (Ma et al., 1996). The lateral stripes of these neuronal progenitors differentiate into sensory neurons while the intermediate stripes give rise to interneurons, and the medial stripes to motorneurons during late neurogenesis (Chitnis et al., 1995). As neurulation proceeds, the open neural plate folds to give rise to the neural tube (fig. 3).

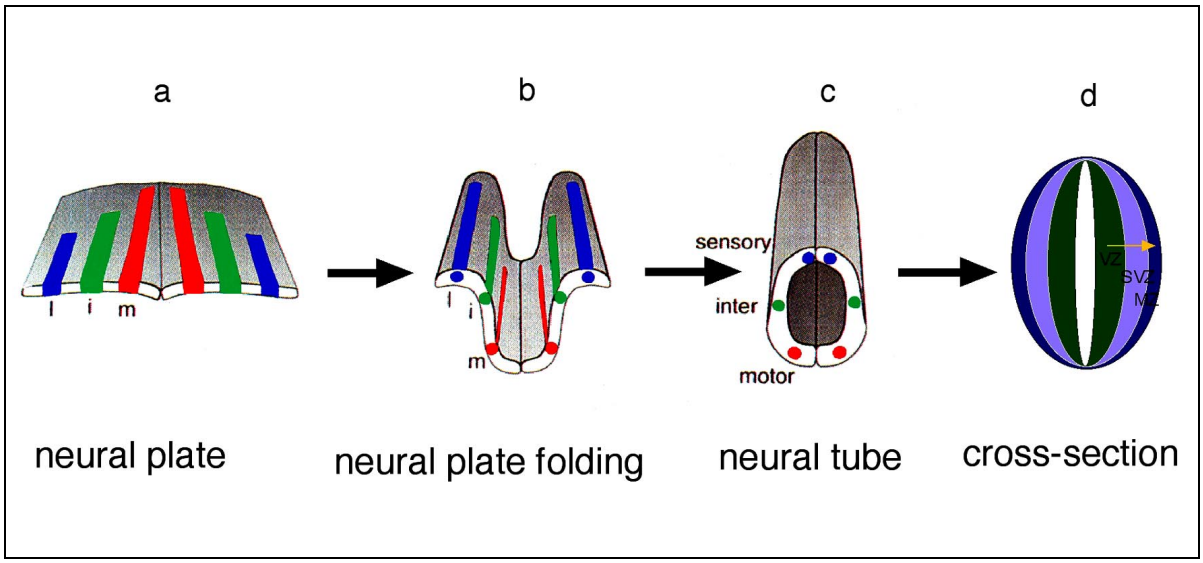


Figure 3: **Model highlighting the steps of neurogenesis in *Xenopus laevis*** (After Chitnis et al., 1995)

(a) In the open neural plate, three bilateral stripes of cells, the lateral (l), intermediate (i), and medial (m) ones define the territory of primary neurons.
(b) The neural plate folds to give rise to the neural tube.
(c) By completion of the neural tube, neuronal precursors from the lateral stripes differentiate into sensory neurons, while those from the intermediate stripes give rise to interneurons and those from the medial stripes differentiate into motor neurons.
(d) In a cross-section of the neural tube, three main layers can be distinguished. Mitotically active neural cells are stored in the ventricular zone (vz); postmitotic neurons are found in the subventricular zone (svz) and marginal zone (mz). Fully committed neurons are located in the marginal zone. Neuronal differentiation progresses from the ventricular zone towards the marginal zone as indicated by the arrow. Genes involved in neuronal fate determination are actively expressed in the ventricular zone, while those required for the fate commitment are expressed in the subventricular and/or marginal zones.

Ectopic expression of X-Ngnr-1 initiates a proneural gene program which leads to formation of ectopic neurons in both the whole embryo and in animal cap explants (Ma et al., 1996). To date, based on microinjection experiments, the HLH protein Coe2 (Dubois et al., 1998) could be placed immediately downstream of X-Ngnr-1 and upstream of X-NeuroD, as this gene is activated by X-Ngnr-1 and in turn activates X-NeuroD (Lee et al., 1995). Howevever, NeuroD was also shown to be directly activated by X-Ngnr-1 (Perron et al., 1999b). Downstream in the cascade, another HLH protein, Xebf3 (Pozzoli et al., 2001), appears to be activated by X-NeuroD and is found to induce N-tubulin, a neuronal specific marker (Oschwald et al., 1991). A parallel program, mostly made of non bHLH/HLH proteins includes successively X-Ngnr-1, NKL (Lamar et al., 2001b), X-MyT1 (Bellefroid et al., 1996), and N-tubulin (fig. 4).

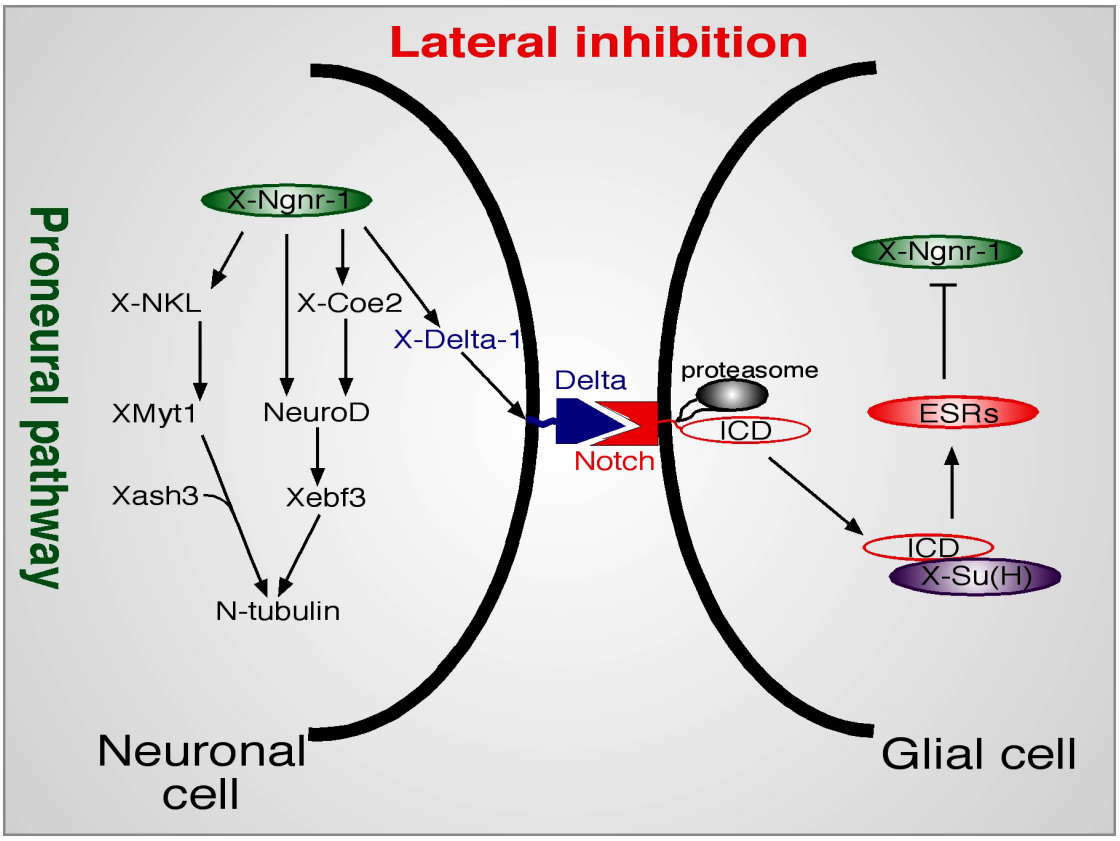


Figure 4: Model showing the different pathways involved in neuronal fate commitment

X-Ngnr-1 induces two proneural cascades of gene activities, including the bHLH/HLH (NeuroD/X-Coe2, Xebf3) cascade and the zinc finger cascade (X-NKL, XMyT1). Simultaneously, X-Ngnr-1 induces the expression of X-Delta-1 which in turn binds to X-Notch-1 and activates a cascade of neurogenic genes in a process called lateral inhibition. In sum, only cells expressing high levels of proneural genes eventually commit to a neuronal fate (Heitzler and Simpson, 1991; Ghysen et al., 1993; Lee et al., 1995; Heitzler et al., 1996; Bellefroid et al., 1996; Dubois et al., 1998; Pozzoli et al., 2001).

Although these proneural transcription factors are individually able to induce ectopic neurogenesis, as marked by N-tubulin gene expression, their proneural activity appears to be amplified when additional factors are present. For example, XMyT1 synergizes with the bHLH, Xash3 (Ferreiro et al., 1994) to induce a strong ectopic expression of N-tubulin (Bellefroid et al., 1996). This suggests that the above parallel proneural programs may function in a co-operative manner. Consistent with this, suppression of each of these genes blocks neuron formation. Finally, activation of X-Ngnr-1 also leads to the activation of an antagonizing pathway whose activity is known as lateral inhibition, a process governed by Delta/Notch signaling.

I. 3. 2 Lateral inhibition in neuronal differentiation

In both vertebrates and invertebrate species, not all neural cells differentiate into neurons. Some neuronally specified cells may loose their identity through local cell-cell interaction called lateral inhibition (reviewed in Ghysen et al., 1993). During development, this process regulates the differentiation of specific cell types, i.e. neuronal and glial cells in the neural tissues (Beatus and Lendahl, 1998). Lateral inhibition is mediated by the neurogenic genes, in particular the receptor Notch, and its putative ligand Delta. Activation of Notch inhibits the activity of the proneural genes by inducing the expression of inhibitory bHLH transcription factors in the *Enhancer of split* E(spl) complex (Jennings et al., 1994; Wettstein et al., 1997; reviewed in Kadesh, 2000; Lamar et al., 2001a)

In *Xenopus*, activation of the determination factor, X-Ngnr-1, promotes the expression of X-Delta-1(Chitnis and Kintner, 1996). Upon translocation of X-Delta-1 to the cell surface (fig. 4), it interacts with the receptor X-Notch-1 (Coffman et al., 1990), thereby activating a signal transduction pathway which leads to the repression of X-Ngnr-1 in the neighbouring cell (Chitnis and Kintner, 1996; Lamar et al., 2001a). In the molecular context, Delta-Notch

interaction promotes the proteolytic cleavage of the intracellular domain (ICD) of Notch (De Strooper et al., 1999). ICD translocates to the nucleus and associates with a member of the CSL family of DNA-binding proteins, i.e. X-Su(H) (Wettstein et al. 1997). Association with ICD changes the properties of CSL proteins as transcription factors, by displacing one set of interacting proteins that repress transcription, while recruiting others that activate (reviewed in Kadesch, 2000). Recent studies provide evidence for Notch regulating an ankyrin repeat protein (Nrarp) that is required to form a ternary complex with ICD and X-Su(H), thereby modulating the activity of these proteins (Lamar et al., 2001a).

Thus expression of the proneural factor X-Ngnr-1 is repressed in those cells where Notch signaling is active. The question remains of how one cell within the cluster gains sufficiently high levels of proneural activity in order to escape lateral inhibition. It has been proposed that the proneural genes become refractory to lateral inhibition when their levels of expression exceed some threshold value (Van Doren et al., 1992). Furthermore, it has been shown that some downstream targets of X-Ngnr-1, including XNeuroD, XMyT1, and Xebf3 confer insensitivity to lateral inhibition.

I. 4 Cell cycle regulation and neuronal differentiation

In order for normal development to proceed, cell cycle withdrawal and differentiation must be tightly coordinated. In the context of neurogenesis, recent studies dealing with detailed molecular mechanisms involved in neuronal differentiation reveal that one key decision in cell fate commitment lies at the level of the cell cycle regulation. Cell cycle regulators and cell fate determination/differentiation factors have been shown to cross-regulate each other (reviewed in Ohnuma et al., 2001).

In *Xenopus*, the spatial and temporal patterns of cell division in the early embrogenesis reveal differential mitotic activities, including more pronounced cell division in the neural

plate than in nonneural ectoderm (Saka and Smith, 2001). How could neuronal precursors exit from this intensive proliferative activity and commit to the specified fate? Previous studies have shown that ectopic activation of Delta/Notch signaling, as regulator of neuronal fate specification leads to the expansion of the neural tube (increased proliferation) and disorganisation of the brain (Coffman et al., 1993). However the molecular mechanisms underlying these effects are presently unknown in *Xenopus*.

Although experimental evidence reveals that constitutively active alleles of Notch are involved in malignant transformation of cell lines (Rohn et al., 1996; Capobianco et al., 1997; Bellavia et al., 2000; Soriano et al., 2000), it has been shown only recently, that ICD-Notch directly induces Cyclin D1 and Cdk2, thereby promoting S-phase entry (Ronchini and Capobianco, 2001). However, if this mechanism is conserved in the context of neurogenesis and how the proneural pathway promotes cell cycle exit is not fully understood.

Recent studies have identified a regulatory circuit that is involved in proliferation control within the neural plate, but which does not seem to be directly linked to Notch signaling. XBF-1, an anterior neural plate-specific winged helix transcription factor, has been reported to promote proliferation of neuroectodermal cells at a high dose, while low doses inhibit ectodermal proliferation and induce neural cell fate. The effect of XBF-1 on cell proliferation seems to be mediated by the cyclin-dependent kinase (Cdk) inhibitor $p27^{XIC1}$, which is a direct target gene for XBF-1 (Hardcastle and Papalopulu, 2000). $p27^{XIC1}$ had previously been demonstrated to exert a function in cell fate determination in the retina; overexpression of $p27^{XIC1}$ in retinoblasts increases the number of Müller glial cells at the expense of bipolar neurons, while its inhibition reduces the number of Müller glial cells (Ohnuma et al., 1999). However, expression characteristics of both, XBF-1 and $p27^{XIC1}$, are not compatible with their functioning as cell cycle regulators in the context of primary

neurogenesis. Thus, if and how neurogenin is linked to proliferation control remains to be defined.

I. 5 Goals and experimental approaches

Our general interest in this project was to identify novel genes involved in primary neurogenesis in *Xenopus*. The strategy adopted was an expression pattern screen of an embryonic brain cDNA library. Among the neural specific genes, those expressed in the territory of primary neurons, as marked by the expression pattern of N-tubulin in three bilateral stripes of cells in the open neural plate, were our first targets for further characterization. Genes identified as being novel after sequencing and database analysis were selected for functional investigation.

Three previously unknown genes encoding proteins with a putative regulatory function were selected for further studies. These candidate genes include XPak3, a serine/threonine protein kinase, X-Mxi1, a basic helix-loop-helix leucine zipper protein, and XSeb4, a RRM-like RNA-binding protein. Expression patterns of these genes were defined by use of RT-PCR and wholemount *in situ* hybridisation. Embryo microinjection experiments were used to analyse the response of the specific genes upon overexpression of neuronal regulators, including X-Ngnr-1 and some of its downstream targets as well as X-Notch-1, in whole embryos an in the animal cap explants. This analysis allowed to place XPak3 in a position within the cascade of genes that regulate primary neurogenesis in *Xenopus*.

In the last step, we studied the role of XPak3 and XSeb4 in neuronal determination/ differentiation. For this purpose, capped-mRNAs were prepared from their coding regions and injected into embryos for the gain-of-function assays. Conversely, specific morpholino antisense oligonucleotides were designed upstream of each open reading frame and injected for the loss-of-function experiments.

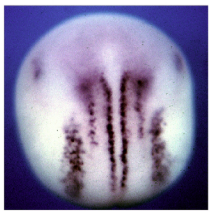
II RESULTS

II. 1 Identification of novel genes with function in neurogenesis

II. 1. 1 Expression pattern screen

To identify novel genes with a potential role in *Xenopus* neurogenesis, single phage or plasmid clones were randomly picked from a tadpole head cDNA library (kindly provided by Thomas Hollemann). This library named Head Fr. 1-38, was 99% recombinant with a diversity of $5x10^4$ clones and a concentration of $2x10^7$ phages/ml. Fluorescein-labelled antisense RNAs were synthesized from PCR-amplicons prepared from individual clones and used in wholemount *in situ* hybridization. Three embryonic stages, including late gastrula, open neural plate, and tailbud, were combined per probe. The five hundred genes analysed showed a variety of expression patterns, including neural specific (17 clones), and neuron specific ones (5 clones). We have defined two synexpression groups to classify our five genes of interest: firstly, the N-tubulin synexpression group which, by definition, comprises a set of neural specific genes involved in neuronal differentiation with an expression pattern similar to that of N-tubulin. Secondly, the Delta synexpression group, that includes a set of neural specific genes which have a role in the lateral inhibition pathway and whose expression pattern mimics the one of X-Delta-1 (Table 1).

Table 1: Results of expression pattern screen. Genes written in green are functionally characterized in this thesis.

Group	Clone	Name	previously known in Xenopus	Function
 N-tubulin synexpression group	JS5.42	N-tubulin	yes	- structural protein, molecular marker of neuronally differentiated cells (Oschwald et al., 1991)
	JS464	**XPak3**	no	- actin cytoskeleton reorganisation leading to neurites outgrowth in cell lines (Bagrodia and Cerione, 1999) - axonal guidance in Drosophila (Hing et al., 1999)
	JS5.22	Tau/MAP-2	yes	- regulation of organelles, neurofilaments, and APP vesicles trafficking in neurons (Stamer et al., 2002). -Alzheimer's disease marker (Liu et al., 2002)
	JS097	MAX interactor 1 (Mxi1) also known as Mad2	no	- antagonizes the oncogenic activity of Myc in cell culture assays (review, Zhou and Hurlin, 2001)
	JS4.3	RBP Elav-type (erlC)	yes	- involved in posttranscriptional regulation in neuronally specified cells (Perron et al., 1999a)
 Delta synexpression group	JS124	**XSeb4**	no	RRM type protein

Based on sequence identity with homologs described in other species, the candidate genes were identified as XPak3, XSeb4, N-tubulin, XelrC, Tau, and X-Mxi1. Interestingly, the isolation of N-tubulin and XelrC, known neuronal markers in *Xenopus*, testifies the reliability of our screening system.

II. 1. 2 Sequence analysis of XPak3

One of the novel genes identified in the N-tubulin synexpression group was clone JS464. Its nucleotide sequence, 2758 base pairs (bp) in size, contains an open reading frame (ORF) encoding for a protein of 564 amino acids. The ORF is flanked upstream by a 132 bp untranslated region (UTR), containg two in frame stop codons. Downstream of the ORF, a 731 bp UTR is flanked by a poly(A) tail (fig. 5). These structural features testify that JS464 contains the complete ORF. The gene bank accession number AF485330 was assigned to this gene. Comparative analysis of the predicted amino acid sequence reveals that JS464 represents the *Xenopus* homolog of the p21-activated kinase isoform 3 (XPak3). Members of this serine/threonine protein kinase family are structurally characterized by a regulatory domain which modulates the activity of the kinase domain (Lei et al. 2000). The previous known *Xenopus* isoforms, namely XPak1 (Faure et al., 1997), and XPak2 (Cau et al., 2000), were cloned from an oocyte library.

The regulatory domain of XPak3 is located in the amino terminal half of the protein and contains several proline-rich motifs (PXXP) (Manser et al., 1994). One of the most conserved of these motifs (fig. 6) is located in the N-terminus and is known to mediate protein-protein interaction between Pak proteins, and the adaptor protein Nck in vertebrates, or Dock in *Drosophila* (Bokoch et al., 1996; Lu et al., 1997). More internally, another proline-rich motif is found (fig. 6), that is thought to promote the interaction of Pak proteins with a guanine nucleotide exchange factor Pix (Manser et al., 1998).

The regulatory domain also contains a conserved Cdc42/Rac1 interacting binding (CRIB) and an autoinhibitory domain (AID) which can interact in *trans* with the kinase domain and inhibits the kinase activity (Frost et al, 1998; Zhao et al., 1998; Zenke et al., 1999; Lei et al., 2000). An additional evolutionarily conserved G-beta/gamma (Gbg) subunit binding domain is located in the C-terminus of the protein (Leberer, E., 1998; Leeuw et al., 1998). Although these domains are highly conserved in the three *Xenopus* isoforms, XPak3 reveals a strong homology (96% identity) with the mouse and human Pak3 proteins (fig. 6). In these species, the kinase domain of Pak3 shows a high sequence homology both in number

```
                GAT AAG CTA TGG CAG TTG TGC AGA TGG ATT AAG CCG GGG CTT CCC TTT CAT TAG   54
GAA AAG CAC ACG GCG GAA GGG GAA GAG CTG ATG CAG CAT GCC AGG GTG GAT GCC AGG AGG   114

                            M   S   D   S   V   D   I   E   E   K   P   P   A   P   14
CTG TGA GGA GTG CTG AAG ATG TCC GAT AGT GTG GAT ATT GAA GAG AAA CCT CCA GCT CCC   174
P   L   R   M   N   S   N   N   R   D   S   S   A   L   N   H   C   S   K   P   34
CCT CTG AGA ATG AAT AGT AAC AAC CGC GAT TCT TCT GCA TTG AAT CAC TGC TCG AAA CCA   234
L   P   M   A   P   E   E   K   N   K   K   A   R   L   R   S   I   F   P   G   54
CTT CCT ATG GCC CCA GAA GAG AAG AAC AAG AAA GCC AGG CTG CGC TCC ATT TTT CCA GGA   294
G   G   D   K   T   N   K   K   K   E   K   E   R   P   E   I   S   L   P   S   74
GGA GGG GAT AAA ACC AAC AAG AAG AAA GAA AAG GAA CGC CCT GAA ATA TCG CTC CCT TCA   354
D   F   E   H   T   I   H   V   G   F   D   A   V   T   G   E   F   T   P   D   94
GAT TTT GAA CAC ACC ATC CAC GTT GGA TTT GAT GCT GTT ACT GGA GAA TTC ACT CCA GAT   414
L   C   G   S   Q   M   G   T   G   K   L   P   E   G   I   P   E   Q   W   A   114
CTC TGT GGT TCA CAG ATG GGC ACA GGG AAA CTC CCA GAG GGG ATC CCG GAA CAA TGG GCA   474
R   L   L   Q   T   S   N   I   T   K   L   E   Q   K   K   N   P   Q   A   V   134
CGA CTA CTC CAA ACC TCA AAT ATA ACC AAA CTG GAG CAG AAA AAG AAC CCT CAG GCA GTA   534
L   D   V   L   K   F   Y   D   S   K   E   T   V   N   N   Q   K   Y   M   S   154
TTA GAC GTC CTT AAA TTT TAT GAT TCC AAA GAG ACC GTG AAC AAC CAG AAG TAC ATG AGC   594
F   T   S   G   D   K   S   A   H   G   Y   I   A   A   H   S   L   N   A   K   174
TTT ACA TCT GGA GAT AAA AGT GCA CAT GGA TAC ATA GCT GCC CAC TCG CTG AAT GCA AAG   654
T   A   S   E   P   P   L   A   P   P   V   S   E   E   E   D   E   E   E   E   194
ACT GCA TCT GAG CCT CCC CTG GCA CCT CCA GTT TCA GAG GAA GAG GAT GAA GAA GAG GAA   714
E   E   E   D   D   N   E   P   P   P   V   I   A   P   R   P   E   H   T   K   214
GAG GAG GAA GAT GAC AAT GAG CCC CCG CCT GTT ATT GCT CCT CGC CCT GAG CAT ACA AAA   774
S   I   Y   T   R   S   V   I   E   P   V   A   L   T   A   P   A   K   E   A   234
TCG ATC TAC ACC CGA TCT GTT ATC GAA CCA GTT GCA TTA ACT GCA CCA GCT AAA GAA GCC   834
S   T   S   P   V   T   P   Q   P   E   N   S   N   S   S   T   S   T   L   Y   254
TCC ACC TCT CCT GTG ACC CCC CAG CCA GAA AAC TCA AAC TCA AGC ACG AGC ACT TTG TAT   894
R   N   T   D   R   Q   R   K   K   S   K   M   T   D   E   E   I   L   E   K   274
AGG AAC ACC GAC CGG CAA CGT AAG AAG TCC AAG ATG ACA GAT GAA GAG ATC CTG GAG AAA   954
L   R   S   I   V   S   V   G   D   P   K   K   K   Y   T   R   F   E   K   I   294
TTA CGA AGT ATT GTC AGT GTG GGG GAT CCA AAA AAG AAA TAC ACC AGA TTT GAA AAG ATT  1014
G   Q   G   A   S   G   T   V   Y   T   A   I   D   I   A   T   G   Q   E   V   314
GGC CAA GGG GCA TCA GGT ACA GTG TAC ACA GCA ATA GAC ATC GCT ACA GGA CAG GAG GTA  1074
A   I   K   Q   M   N   L   Q   Q   Q   P   K   K   E   L   I   I   N   E   I   334
GCG ATT AAG CAA ATG AAC CTG CAA CAG CAG CCC AAA AAG GAA TTA ATT ATT AAC GAG ATC  1134
L   V   M   R   E   N   K   N   P   N   I   V   N   Y   L   D   S   Y   L   V   354
TTG GTC ATG AGG GAA AAT AAA AAT CCC AAC ATT GTG AAT TAT TTA GAC AGT TAT CTG GTT  1194
G   D   E   L   W   V   V   M   E   Y   L   A   G   G   S   L   T   D   V   V   374
GGG GAT GAA CTC TGG GTA GTG ATG GAA TAC CTG GCT GGT GGT TCT CTG ACA GAT GTT GTT  1254
T   E   T   C   M   D   E   G   Q   I   A   A   V   C   R   E   C   L   Q   A   394
ACA GAG ACT TGC ATG GAT GAA GGA CAG ATT GCA GCC GTC TGT CGA GAG TGC CTA CAA GCC  1314
L   D   F   L   H   S   N   Q   V   I   H   R   D   I   K   S   D   N   I   L   414
CTG GAC TTC TTG CAT TCA AAC CAG GTC ATC CAC AGA GAT ATA AAA AGT GAC AAC ATT CTG  1374
L   G   M   D   G   S   V   K   L   T   D   F   G   F   C   A   Q   I   T   P   434
CTT GGC ATG GAT GGA TCA GTA AAG CTT ACT GAT TTT GGT TTC TGT GCT CAG ATT ACA CCT  1434
E   Q   S   K   R   S   T   M   V   G   T   P   Y   W   M   A   P   E   V   V   454
GAG CAG AGT AAA CGA AGC ACA ATG GTT GGA ACA CCC TAC TGG ATG GCG CCA GAA GTA GTA  1494
T   R   K   A   Y   G   P   K   V   D   I   W   S   L   G   I   M   A   I   E   474
ACA AGG AAA GCC TAT GGG CCA AAA GTG GAC ATC TGG TCT TTA GGC ATT ATG GCC ATA GAA  1554
M   V   E   G   E   P   P   Y   L   N   E   N   P   L   R   A   L   Y   L   I   494
ATG GTA GAA GGA GAA CCC CCT TAC CTT AAT GAA AAT CCT CTC AGA GCA TTG TAT CTG ATA  1614
A   T   N   G   T   P   E   L   Q   N   P   E   R   L   S   A   I   F   R   D   514
GCC ACC AAT GGC ACC CCT GAA CTT CAG AAC CCA GAA AGA CTG TCG GCC ATA TTT CGA GAT  1674
F   L   N   R   C   L   E   M   D   V   D   R   R   G   S   A   K   E   L   L   534
TTC CTT AAC CGA TGT TTA GAG ATG GAT GTG GAC AGG CGA GGC TCT GCT AAG GAG CTT TTA  1734
Q   H   P   F   L   K   I   A   K   P   L   S   S   L   T   P   L   I   I   A   554
CAG CAT CCA TTT CTA AAA ATA GCC AAG CCG CTC TCT AGC CTG ACC CCA CTG ATC ATA GCA  1794
A   K   E   A   I   K   N   S   S   R                                           564
GCA AAG GAA GCC ATT AAG AAT AGC AGC CGC TAG CCG TGC CCT CCA CTC CCT ACA GAA GAA  1854

CCC GGC AAA CTC CAT AAA ACC TCA TCT CTT CAC ACC GCA GGA GCA GAG CTG ATT AAC TGG  1914
GAC TTC CAG CGC TAT GGC GAC AAA GCT CGC TAC GAG GCA CTT GTG TCT TCC TGC CCT CCT  1974
CGG ATC CCT TAA ACT CCT TTT ATT CTT TTT TTT AAT GTA GGG TCG GAT CTG CAA ATC TTG  2034
GGG GTG TTG GAA TCT GGT GCT GTG AAC GCG CAC CCT TAT AAT GTG CAG AAA CGT TCC TCG  2094
GAC TGG TTA CTT TCA TAA CAG ACA GTT CAC TTT CTG GTA TCT CGT TTG CTT TGT ATT AAA  2154
AGA AAA ACT TAA AAG GGT ACT ACT GCA TTG TCA AAC ATG CAA CAA CCG TAA ATG ACT CCA  2214
GAA GGT CCG TGT AGA GCG CAT AGG CCA CCG TTC TAA TTC TAG TCT CAG TCA GTT GTC TGT  2274
TTC AGC ACA GGG TCT GTT TAC ATT CAT TCA TTG AAT TAA GAT CTG CAT GTA AAC ACA ACC  2334
ATT CTT TAG GGG GGT ATT AAG GTT GTT GAG GTG ATG TCC AGC CTT AAG AAC AGA GCA AAT  2394
GAA GCA TTT GAG TGG AAA GAG GCC CAT TTG TTG CTG AAC GAT CCT GGC ACC TTG TGC TTT  2454
GCT TTC CTG GCA GTG AAG AGG TTA GCA GCC TGA ATC TAT GGC ACA AAG TCT AAC ATT GTG  2514
GAT CTG TTT TTT TGT TAA ATG GTT AGA ATT TTA CTG ACC CAT GTC CCC ACA AAG CCT TCA  2574
TAC TTT CTC CTG CGC TCA AAT GAT GGC TGC AGG TAT GGT ACA GCC AAG GAT GGA CTC TAG  2634
CTA TTT ATT TTG CTA TTA CAG GTA TGG GAT CCG TTA TCC AGA AAC ACA TTA TAC AGA GGG  2694
CTT CGA ATT ACA GAA AGG CCT TCT CCA ATA GAC TCC ATT TTT TTT AAA AAA AAA AAA AAA  2754
AAA A                                                                           2758
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Figure 5: **Prediction of XPak3 amino acids sequence from the cDNA**

The longest coding region of XPak3 DNA sequence was translated into the corresponding amino acid sequence, numbered in bold characters. The 5'UTR, upstream to the start codon ATG, contains two stop codons indicated in bold. The 3'UTR possesses 931 bp. Here, the poly(A) tail is bold. The amino acid sequence contains the characteristic kinase domain marked in bold characters. Its N-terminal half comprises the regulatory domain.

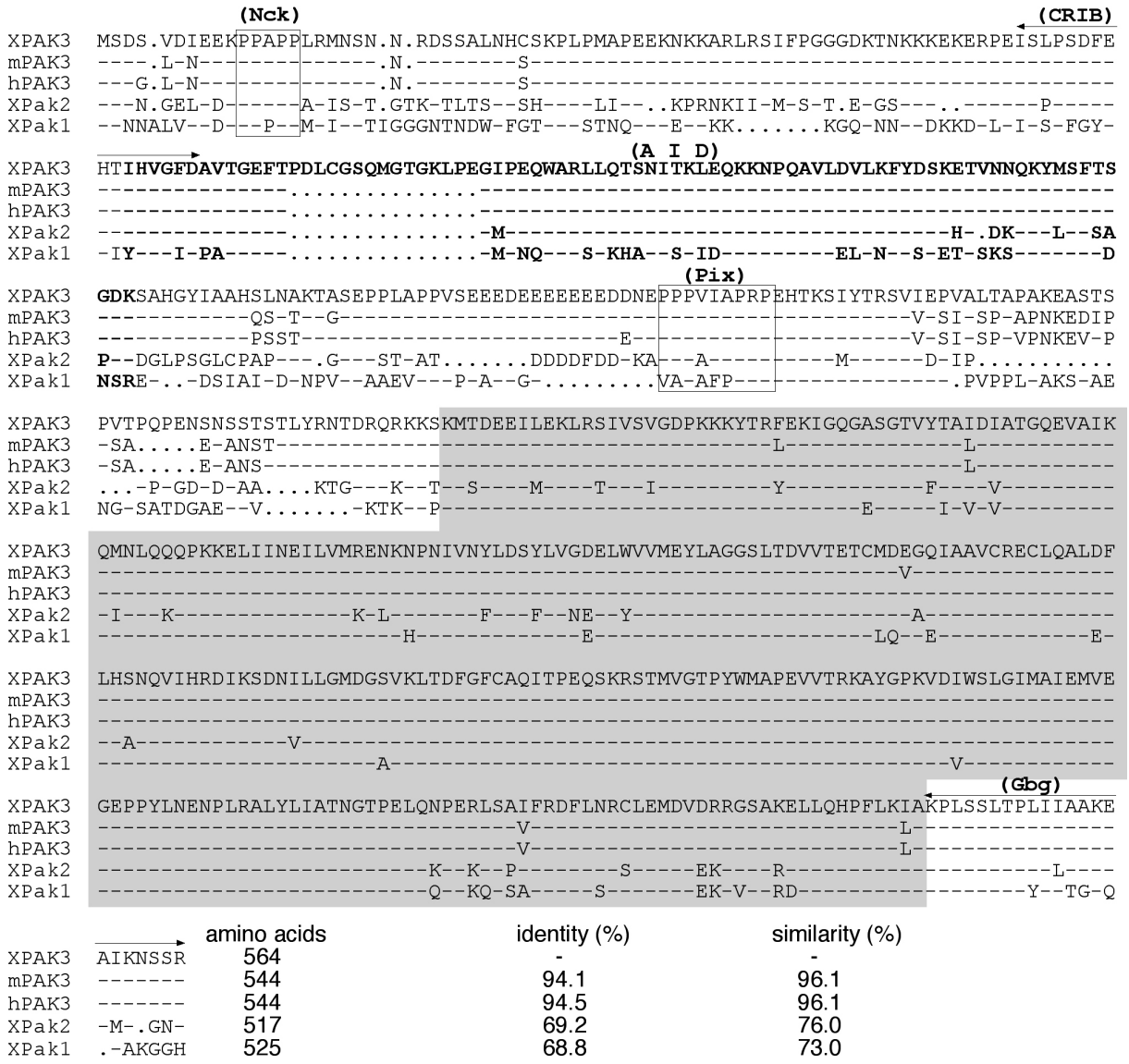


Figure 6: **Conserved structural features of Pak proteins in vertebrate species**

XPak3 protein sequence, predicted from the cDNA, was aligned with homologs from mouse and human, and with the two other *Xenopus* isoforms, namely XPak1 and XPak2. In this alignments dashes represent the identical amino acids while dots indicate the absence of the amino acids. The kinase domain is shaded in grey. The Cdc42/Rac1 interacting binding (CRIB) domain is indicated. CRIB partially overlaps with the autoinhibitory domain (AID) indicated in bold. The proline-rich motifs specific for Nck and Pix are boxed. The G-beta/gamma binding is conserved at the C-terminal edge. The percentage of homology is indicated as identity and as similarity.

and nature of amino acids (see grey shaded). Sequence elements specific for each individual Pak isoform are located in the regulatory domain and mostly in the hinge region, separating the regulatory from the kinase domains. This latter region shows the highest diversity in amino acid composition. Compared to other Pak proteins, XPak3 has a 15 amino acids insertion in the

regulatory domain. Alignments including the *Drosophila* homolog, DPak (not shown) reveal more such insertions in the regulatory domain but not in the kinase domain.

II. 1. 3 Sequence analysis of XSeb4 RNA binding protein

XSeb4 is a member of the RNA recognition motif (RRM) protein family. Its nucleotide sequence contains 1415 bp. The open reading frame encodes for a protein of 214 amino acids. The 83 amino acids RRM motif is located at the N-terminus and contains two putative ribonucleic protein motifs, RNP1 and RNP2 (fig. 7, 8). XSeb4 is one of the few RRM-like RNA binding proteins containing only one single RRM motif.

Database analysis reveals a significant homology with the corresponding sequences from mouse, human and *C. elegans* (fig. 8). Alignments of Seb4 proteins from theses species shows a strong homology in the RNA binding domain but not in the regulatory domain, spanning the C-terminal half of the putative protein. Overall, XSeb4 shares 71%, 70%, and 44% similarity with the sequence from the mouse, human, and *C. elegans*, respectively. However, the RRM motif from the *Xenopus* protein shows 94%, 95% and 87% identity with the corresponding motif from mouse, human, and *C. elegans*, respectively. These sequence homologies suggest that the Seb4 gene family might be conserved during evolution of vertebrate and invertebrate species.

Previous studies reported another Seb4 gene in *Xenopus,* but its expression was muscle and lens specific (Fetka et al., 2000). An alignment with this muscle/lens specific Seb4 isoform showed a highly conserved RRM sequence and three additional short conserved motifs in the C-terminal half of the protein sequence (not shown).

II. 1. 4 Sequence analysis of X-Mxi1

The other novel clone belonging to the to the N-tubulin synexpression group was clone JS97. In our expression pattern screen, three clones with similar expression pattern were picked. After sequencing, they turned out to represent the same mRNA and were found to be all 3'UTR, with JS97 being the longest clone (1165 bp). Using pBKCMV-forward primer and a specific JS97-reverse primer, located 300 bp downstream to the 5'end, an additional partial sequence, 1228 bp in size, was generated by PCR. Together with Kristine Henningfeld, this newly cloned fragment, namely JS40-97, was used as a probe to fish out a phage clone with

```
        AGC CAG CTT AGA CCC AGA GAG AGC GCT GGC CAG AAC AGA TGG TGC ATG GAG TTT  54
CTC TAG CAC CTT CCC ACC TGC TAC ACA GCT CTG CAT TAA CTA CCT CTT TAC CCA CTA CTG  114
CTG GCA CTC TTT AAC CGG CAC CCT TTT TAT TTT ATT ATA GAA GCA TTG CGA CAT TTT TAT  174
ATA AAA TAA GGA TTT AGC GAA AGA AAG AGA ATA TTT TTT TTG TAT CTG TTT TGT ATC TAT  234
TCC CTG GAG TTA GAG GGG CAG CCT GCG AAG ATT TGG ATG TTT TTT TTA TAA GGG CTG GTG  294

                    M   H   T   V   Q   K   D   T   T   F   T   K   I   F  V     15
AAT TTG CCT GTG ACC ATG CAC ACC GTG CAA AAA GAC ACA ACT TTC ACC AAG ATT TTT GTA  354

G   G   L   P   Y   H   T   T   D   A   S   L   R   K   Y   F   E   V   F   G    35
GGG GGT CTC CCG TAT CAC ACA ACG GAT GCC TCC CTC AGG AAA TAT TTT GAG GTT TTT GGG  414

D   I   D   E   A   V   V   I   T   D   R   Q   T   A   K   S   R   G   Y   G    55
GAT ATT GAC GAG GCG GTG GTC ATT ACG GAC AGA CAG ACG GCA AAG TCG AGG GGA TAC GGC  474

F   V   T   M   S   D   R   A   A   A   E   R   A   C   K   D   P   N   P   I     75
TTT GTG ACT ATG TCA GAC CGA GCT GCC GCA GAA AGA GCA TGC AAA GAC CCA AAC CCC ATT  534

I   D   G   R   K   A   N   V   N   L   A   Y   L   G   A   K   P   R   N   L    95
ATT GAT GGC CGC AAA GCT AAC GTC AAC CTT GCG TAC TTA GGA GCC AAG CCA AGG AAC CTG  594

Q   S   A   F   T   I   G   V   Q   Q   L   H   P   A   F   I   Q   R   P   F   115
CAG AGC GCA TTT ACT ATA GGA GTC CAG CAG TTA CAC CCA GCC TTC ATC CAG CGA CCA TTT  654

G   L   T   P   Q   Y   I   Y   P   P   A   I   V   Q   P   S   M   V   I   P   135
GGG CTG ACA CCA CAG TAT ATC TAC CCA CCT GCC ATC GTC CAG CCA AGC ATG GTC ATC CCA  714

T   P   I   P   S   L   Q   S   P   Y   I   D   Y   N   A   A   T   Q   A   Y   155
ACG CCG ATC CCT TCC CTG CAG TCT CCA TAT ATT GAC TAC AAT GCT GCT ACT CAA GCC TAC  774

T   H   Y   T   T   A   A   Y   E   Q   Y   P   Y   A   A   S   P   A   T   G   175
ACC CAT TAC ACC ACT GCG GCC TAT GAA CAG TAT CCC TAT GCA GCC TCG CCG GCC ACC GGA  834

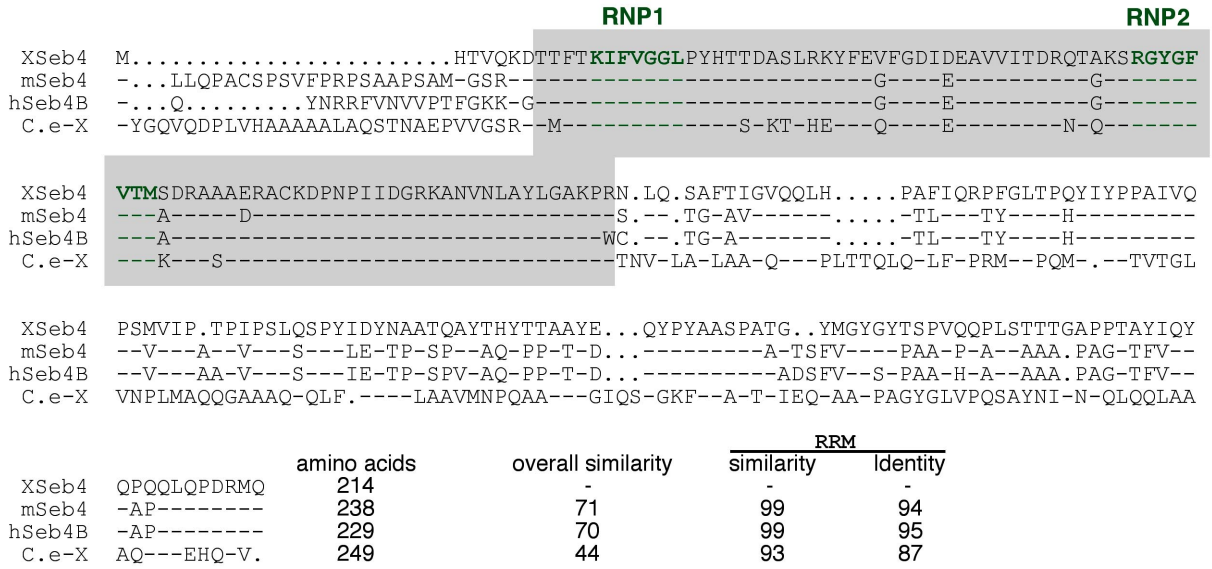
Y   M   G   Y   G   Y   T   S   P   V   Q   Q   P   L   S   T   T   T   G   A   195
TAC ATG GGC TAT GGA TAT ACT AGC CCA GTC CAA CAG CCA CTT TCC ACC ACC ACC GGA GCT  834

P   P   T   A   Y   I   Q   Y   Q   P   Q   Q   L   Q   P   D   R   M   Q       214
CCT CCC ACA GCG TAC ATC CAA TAC CAG CCT CAA CAG CTC CAG CCT GAC CGG ATG CAG TAA  894

AAA GCT CAG ACA CTA GAT TCC TCG TTG CCC CTA GCC ATG GAT GGG GAG AAA TGT AAC AAC  954
AAT AAT AAT ACC AGA TGG GAA AGT GTT GCT AAT AAC ACA GTA AGA AAA TGC TGC AGT CAA  1014
GTT CTG TGG ATC CCA GCT TTC AGT TCT GTT TCA GGC AAT ATC AAG GTC ATG GAT TTC TTT  1134
GGC GGA TTC CAG GCA AGA GGA GAA GCT TGT TGG CTG CAG AAG AAT AAA ACT GGG CGC CAC  1194
TAA CTC TTT GCC TCA ACT CTG CAA AAA ATT CAA TAA AAA CTT AAA TAA AAA CTT TCA AAA  1254
AAA AAC ACA AGG ATC TTT ATA AGG TAC TCT GAA CAC CGA AAT CAT CAA TAT TAT TAT TAT  1314
TTT GGG GAC CGG GAT AAA AAC TGA TAC TCA GCG ATG CCT TAA AGA AAT AAC TTC TTT TTT  1374
TTT TTT TTG AAT ATT TAT TTG CGA AAA AAA AAA AAA AAA AA                          1415
```

Figure 7: Prediction of XSeb4 protein sequence from the cDNA

The longest coding region of the XSeb4 cDNA sequence was translated into the corresponding amino acid sequence. The 5'UTR, upstream to the start ATG, shows an inframe TAA-stop codon before the start methionine. The 3'UTR contains 471 bp. Here, the poly(A) tail is bold. The amino acid sequence, numbered in bold characters contains the RRM domain marked at the N-terminal end. The two characteristic sequence motifs, RNP1 and RNP2, are underlined.



		amino acids	overall similarity	RRM similarity	RRM Identity
XSeb4	QPQQLQPDRMQ	214	-	-	-
mSeb4	-AP--------	238	71	99	94
hSeb4B	-AP--------	229	70	99	95
C.e-X	AQ---EHQ-V.	249	44	93	87

Figure 8: Conserved structural features of XSeb4 RNA-binding protein in vertebrate and invertebrate species.

The protein sequences of Seb4, as predicted from their corresponding cDNA were aligned. Dashes represent the identical amino acids, while dots indicate the missing ones. The RRM motifs, shaded in grey, contains the RNA interaction motif, RNP1 and RNP2, written in green. The homology is indicated as percentage of similarity between the *Xenopus* (xSeb4), the mouse (mSeb4), the human (hSeb4), and the *C. elegans* (C.e-X) homologs. The similarity and the identity within the RRM is indicated.

2777 bp in size. This clone contained an ORF encoding for a protein of 238 amino acids. The ORF is flanked upstream to the initiation codon by a very short 5'UTR (72 bp) which contains two in frame stop codons, TAG and TGA (fig. 9). Downstream of the ORF, a long 3'UTR of 2048 bp is flanked by a poly(A) tail. This structural composition of the DNA sequence indicates that this clone contains the complete ORF. Database analysis of the corresponding amino acid sequence revealed a strong homology with Max interactor1 (Mxi1), also known as mitotic arrest deficiency isoform 2 (Mad2). By analogy, we designated our clone X-Mxi1. Mad family proteins are related to Myc family of basic helix-loop-helix (bHLH) leucine zipper transcription factors which regulate cell proliferation. X-Mxi1, like other members of its family, has a protein sequence which includes the N-terminal Sin3-interacting domain (SID) necessary for the transcriptional silencing properties of Mad/Max heterodimers (Ayer et al., 1995, 1996; Schreiber-Agus et al., 1995). The delineation of this domain, as well as, the others was based on previous studies and on the alignments of X-Mxi1 with other related

```
         CGC ACA AAC TCC TCT CAG TGT CGG TAT TAG AGT CGG TGG AGC TGG TGG ATA TTG 54

                         M   E   L   Q   E   M   M   E   G   C   Q   E   P   P  14
 TGA GTC CTG GGG GGG GTG ATG GAG CTG CAG GAG ATG ATG GAG GGG TGT CAG GAG CCC CCA 114

  V   T   E   L   L   D   S   G   D   M   G   G   L   M   T   F   L   D   H   V  34
 GTG ACG GAG CTG TTG GAC TCG GGG GAC ATG GGG GGG CTG ATG ACG TTT CTG GAT CAT GTG 174

  Q   M   L   L   E   A   A   G   H   L   E   R   L   E   R   K   K   C   E   H  54
 CAG ATG TTG TTG GAG GCG GCG GGA CAT CTG GAG AGA CTG GAG AGG AAA AAG TGT GAA CAC 234

  G   Y   A   S   T   F   P   S   E   S   S   D   P   M   R   L   K   Q   K   R  74
 GGT TAC GCT TCG ACA TTC CCT TCA GAG AGT TCC GAC CCA ATG AGA TTA AAG CAG AAG AGG 294

  I   K   S   K   R   C   S   G   L   G   I   S   R   S   T   H   N   E   L   E  94
 ATA AAG TCA AAG AGA TGC AGT GGC CTC GGC ATT AGC AGA TCA ACC CAC AAT GAA CTG GAG 354

  K   N   R   R   A   N   L   R   L   C   L   E   R   L   K   D   L   I   P   L  114
 AAG AAC CGG AGA GCC AAC CTG CGC CTG TGT TTG GAG AGG TTA AAA GAC CTG ATC CCC CTG 414

  E   S   D   S   A   R   H   T   T   L   G   L   L   N   K   A   K   L   H   I  134
 GAG TCG GAC AGC GCC CGG CAC ACA ACT CTG GGG CTG CTG AAC AAG GCA AAG CTC CAC ATT 474

  K   K   L   E   E   N   S   R   R   G   Q   H   Q   L   E   V   L   E   R   E  154
 AAG AAA CTT GAG GAG AAT TCC CGG CGT GGG CAG CAC CAG CTG GAG GTT CTA GAG CGG GAG 534

  Q   R   F   L   K   R   R   L   E   Q   L   Q   G   G   P   E   L   E   R   V  174
 CAG AGG TTC CTG AAG AGA CGA CTG GAG CAG CTN CAG GGG GGC CCA GAA TTG GAG CGA GTG 594

  R   S   D   S   I   G   S   T   D   R   S   D   S   D   R   E   E   I   E   V  194
 CGC TCA GAC AGT ATT GGA TCC ACC GAC AGA TCC GAC TCT GAC AGA GAG GAG ATT GAA GTG 654

  D   V   E   S   T   E   L   L   Q   L   V   S   A   S   I   S   D   L   D   D  214
 GAC GTG GAG AGC ACA GAG TTG TTG CAA TTG GTC AGC GCC AGT ATC AGT GAT TTG GAT GAT 714

  Q   S   S   R   Q   S   L   A   S   D   E   G   Y   S   S   A   S   I   K   L  234
 CAG AGC AGC AGG CAG AGT TTG GCC AGT GAC GAG GGN TAC TCN AGC GCC AGC ATC AAG TTG 774

  S   F   S   S                                                                 238
 TCC TTC AGC TCG TAG AGT CCG ACA ACG GGG AGT CCA GTG GTG GGG GGC ACT TAC TGT CCG 834
 TGG ATC ACA GAC CAA CTG GGA CCA ACA GTT CTG ACT CAT GCA CCC CCC CCA CAC CCA AGC 894
 ACA GGC CGT TCA CTG AGG GGT CCC AGT GTC AGG GGG TTC TGA TGG GAC CCA GAC CAG GCC 954
 GGA CTC ACC TTG AAA CTG TCA GTT TGT TCT GCT GCA AAT CAC TGA CCC GCC CAC TGG TCT 1014
 CAA TGT GAA GAG TCT GGT TCT GCC TGT CGA CCA CCA GCA CTG ATC CCC ACT CAC ACT AGC 1074
 ACT GAT CCC CAC TCA CAC TCC AGC ACT AAT TCA CAC TCA CAC TCC AGC ACT GAT CCC CAC 1134
 TCA GCA GAC CCA GCC CAC AAG TGG CAG ATA AAC CAA GAT GGA CTC CTC CGT GGA CCA ATC 1194
 ACA AAC CTC TCT TGG ACT GTG TCT TTA GAA ATA GAT CTG CTG GTA AAA AAG TGG TTC CCT 1254
 GAG ATC CGA TTG GTT CCT GTT GAA CAT GGA GGG ACA ACT CCC ATC ATC CCA CAC ACT CGC 1314
 CGG ATG GAC TTC CAG AAC ACG GGG GGA CCT GGC GCG ACT ATT TCC GGG TCT CCC TGT ATC 1374
 GCT CCC TTA AAT TAT TTT TGC AGA TAA CAG AGA ATA TTT TTA AAG TGA GAA GAA TAA TAT 1434
 ATT ACT AGT GAC ATC ATG GTG ACA TCA CAG TGA CAA TTG GTG CCT TTA ATT CTC GTT TTT 1494
 TTT CTC CTT TTG TAT TTC TTT TTA ATT TAT TGT TAT ATT ATG TGT GTG ATA CAA TGT TTC 1554
 TCC CTT CCC AGA AGC CCC TGT CAT TGT CCT GTT GGA TGT GGG CGG GGC ATC TTG GGA CAC 1614
 AAA GCC CTA GAA TAT GAA AGG GAA CAT TTA GTT GCT ATG GCG ACT CCC CCC GCC CCT GAT 1674
 TAG GTG TAG TAT GTG GGT CAC ACC CGC AGG TGT ATA TAC CCG TCT TCT AGA CAA GGA AAT 1734
 GTT CAA CTT TGT AAG TTG TCA CAT GAT GGT GCA AGC TCC GCC TCC TAC AGT ACG AGG GCG 1794
 TTT TTA AAT GAC ACG CCC CCA GCT TGT ACC GCT AGA GCT CAG CCA CCC AAT AGC CTT TCT 1854
 GTG CCA AGA TGA TGC CGG TTG CAT GTG ACT CCG CCC AGG AAC TGT GAT TTT TGG TTA TTT 1914
 CAA TTG TCC TTC TGA TTC CCC ATT AAC CCT TTA TCA GTC CTC TCA GTC TCC TAC TGT GTG 1974
 GGA ATC AAA CCG AGG GGC AAT TAG AGC TCC TGC CCC TTT AAT TAA CCA CAT CCA ATC AGT 2034
 GAA GCT CAG GGG GAG AGT TGG GCA TCT CAT GGC TAA AGG GAA GAT CTA TAT ATA TAT AAT 2094
 TGC CTC TCT GTA TAT GGC CAG TAA TAG AGC ACC CTC TAG TGG CCA TGC CTG GAT TCT ACT 2154
 ATT CTG TTA CTA CCT AAG CTT ATC TAT ATG GCA GCT CCT GCT CAC ATT GTA AAG AAT GGG 2214
 CCC CAG TCA GGT ATA TGC CAT ATT TAT AGG GGC CCA TGT ACA ACA CTT TGT TAA AGG GCA 2274
 AAT CTA CTA AAA GGG ACA TTC CAC TTA AAA TGT ATT GCA CAG GGG GCG AGT CTG GCT GTT 2334
 AAG TAT AAA GGG GAA TTC TGC ATA AAC ATT GTG TTA TTG AAT ATT GAC AGT GAC TGT CTC 2394
 CCA CTG CAC ATT TAC TGC CCG GCT GCT GCT GCA GGT CAT TAT CAT TCT GCA ATC TGT GTT 2454
 TGG GTG GAC TTC CCC TTT AAT CTG TTA CAA ACC AGA AAT GAC ACA TCC CAG AAT CCT CAG 2514
 CTC CCC CTG GTA GAG CTG CTG AGA TGG TAT TTC CCC CTA TGG ATT NTG GGA AAT GAA GTN 2574
 TAA ATA AAG CTG CAG GGC ATC TCC GCA CCG TAA CCC AAT CCA CAG GCA TTT ATT GAC TCC 2634
 GCC TTT CCC AGA CCC GCC CCA TCC CCT TAA TTA TTA CAC TAA GAG TTA ATT TAA TTC ACT 2694
 ATT ATT ATT CAC TAT TTT ATT TGT TCC GTA GTT GTT GCT CTG ATG CGA ATA AAA ATT ATC 2754
 CAG AAA AAA AAA AAA AAA AAA AA                                                 2777
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<u>Figure 9</u>: **Prediction of X-Mxi1 (X-MAD2) amino acid sequence from cDNA.**

The longest coding region of the X-Mxi1 DNA sequence was translated into the corresponding amino acid sequence. The 5'UTR, upstream to the start codon contains two in frame stop codons indicated in bold-underline. The huge 3'UTR contains 2048 bp; here, the poly(A) signal is in bold and underlined. The poly(A) itself is also in bold. The amino acid sequence, numbered in bold characters contains the repressor domain marked in blue, the bHLH domain in green, and the leucine zipper domain in red.

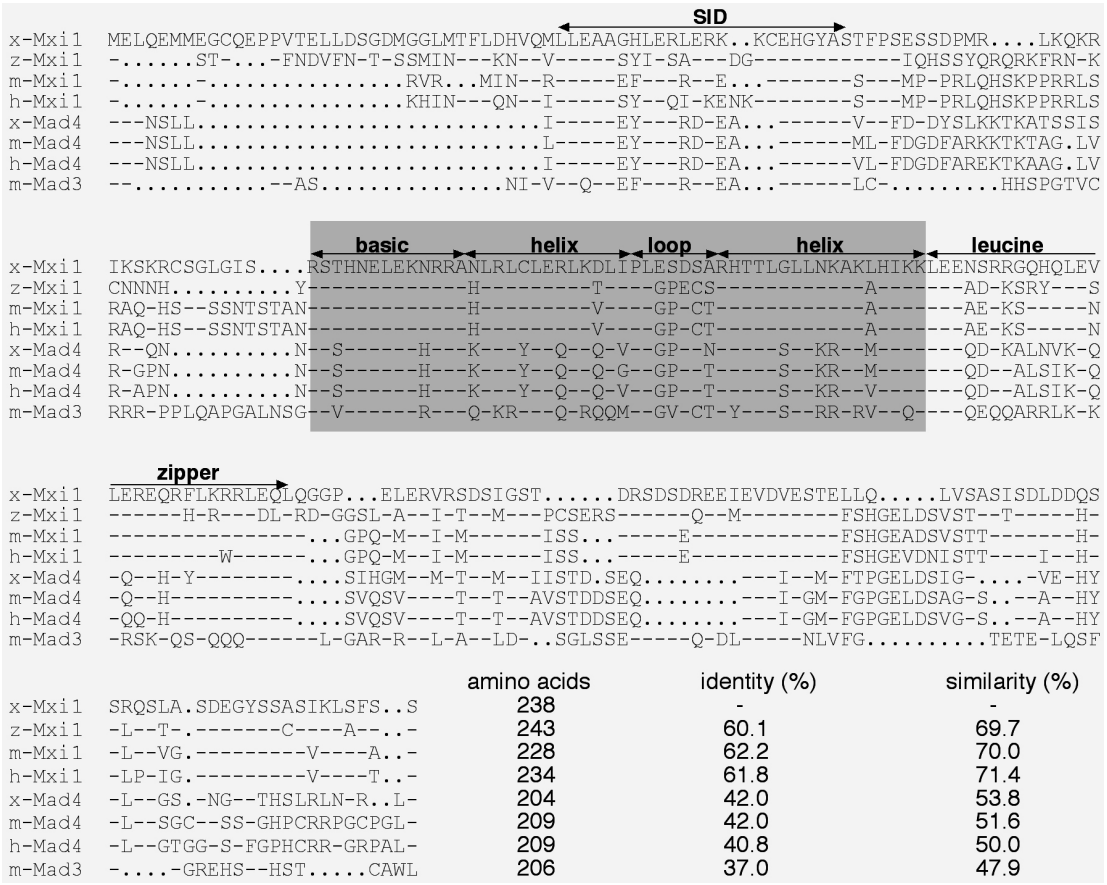

Figure 10: **Conserved structural features of Mad proteins in vertebrates.**

Different Mxi/Mad protein sequences, predicted from their corresponding cDNA, were aligned. This includes x-Mxi1and Mad4 for *Xenopus*, z-Mxi1 for Zebrafish, m-Mxi1 and Mad4-3 for mouse, and h-Mxi1 for the human proteins. The characteristic known functional domains are indicated: the bHLH domain is grey shaded. C-terminally adjacent to the bHLH domain, the leucine zipper domain is indicated. The repressor domain, (SID) in shown in the N-terminal half of the protein sequences. The homology is indicated in percentage (%) as similarity and identity. Identical amino acids are represented with dashes. The missing ones are represented with dots. The size of each protein sequence is indicated.

protein (fig. 10). In addition to the basic DNA binding domain, X-Mxi1 protein contains the helix-loop-helix and the leucine zipper domain required for dimerization with other bHLHZip proteins (fig. 9 and 10).

An alignment of X-Mxi1 protein with its homologs from Zebrafish, mouse, and human shows 69.7%, 70.0%, and 71.4% similarity, respectively. Furthermore, alignment with other isoforms, namely the *Xenopus* Mad4, the mouse Mad4, the human Mad4, and the mouse Mad3 shows a relatively low homology, comprising 53.8%, 51.6%, 50.0%, and 47.9% similarity, respectively (fig. 10), testifying that our novel gene is the Mxi1 (Mad2) orthologue. Some insertions, discriminating between different isoforms and different species, are

spanning the protein sequences. Interestingly, the characteristic functional domains are quite conserved (fig. 10).

II. 1. 5 Spatial- and temporal-expression of the selected genes

II. 1. 5. 1 XPak3 expression

To examine the role of XPak3 in neurogenesis, we analysed its early embryonic expression by use of RT-PCR and wholemount *in situ* hybridization. The first method revealed the presence of XPak3, but also XPak1-2 transcripts in the egg and in all embryonic stages (not shown). However, by wholemount *in situ* hybridization (fig. 11A1-14), XPak3 transcripts are first revealed at late gastrulation (stage 12) within the posterior neural ectoderm. At this stage two bilateral medial stripes are induced (not shown). As development proceeds, the cells expressing XPak3, are not evenly distributed throughout the neural plate, but fall into a pattern that is reminiscent of the neuron-specific N-tubulin gene, whose expression marks the territory of primary neurons (Oschwald et al., 1991). This correlation is even more evident at neurulation (stage 14) when XPak3 is clearly restricted to three groups of cells arranged in a bilaterally symmetrical pattern on either side in the dorsal midline of the posterior neural plate (fig. 11A2), thereby, providing evidence for the classification of this gene in the N-tubulin synexpression group. The same pattern of expression is maintained in later-stage neurula embryos, as the lateral stripes of XPak3-expressing cells extend along and converge towards the dorsal midline (fig. 11A5, 6).

The expression of XPak3 in the anterior neural plate starts at midneurulation (stage 14.5) in a lateral group of cells associated with the trigeminal placodes (fig. 11A8). At stage 16 (fig. 11A9), a second group of XPak3-expressing cells appears more centrally in a site corresponding to the future ventral forebrain/midbrain. As an exception compared to previously known proneural genes, XPak3 is expressed in the cement gland and its expression in this tissue starts at late neurula (stage 17) (fig. 11A10).

During secondary neurogenesis, XPak3 transcripts are revealed in the entire central nervous system (fig. 11A11-14). Remarkably, XPak3 expression in the neural tube (fig. 11B2) significantly precedes the expression of N-tubulin, localized specifically in the marginal zone (fig. 11B3), and follows that of X-Ngnr-1 located in the ventricular zone (fig.

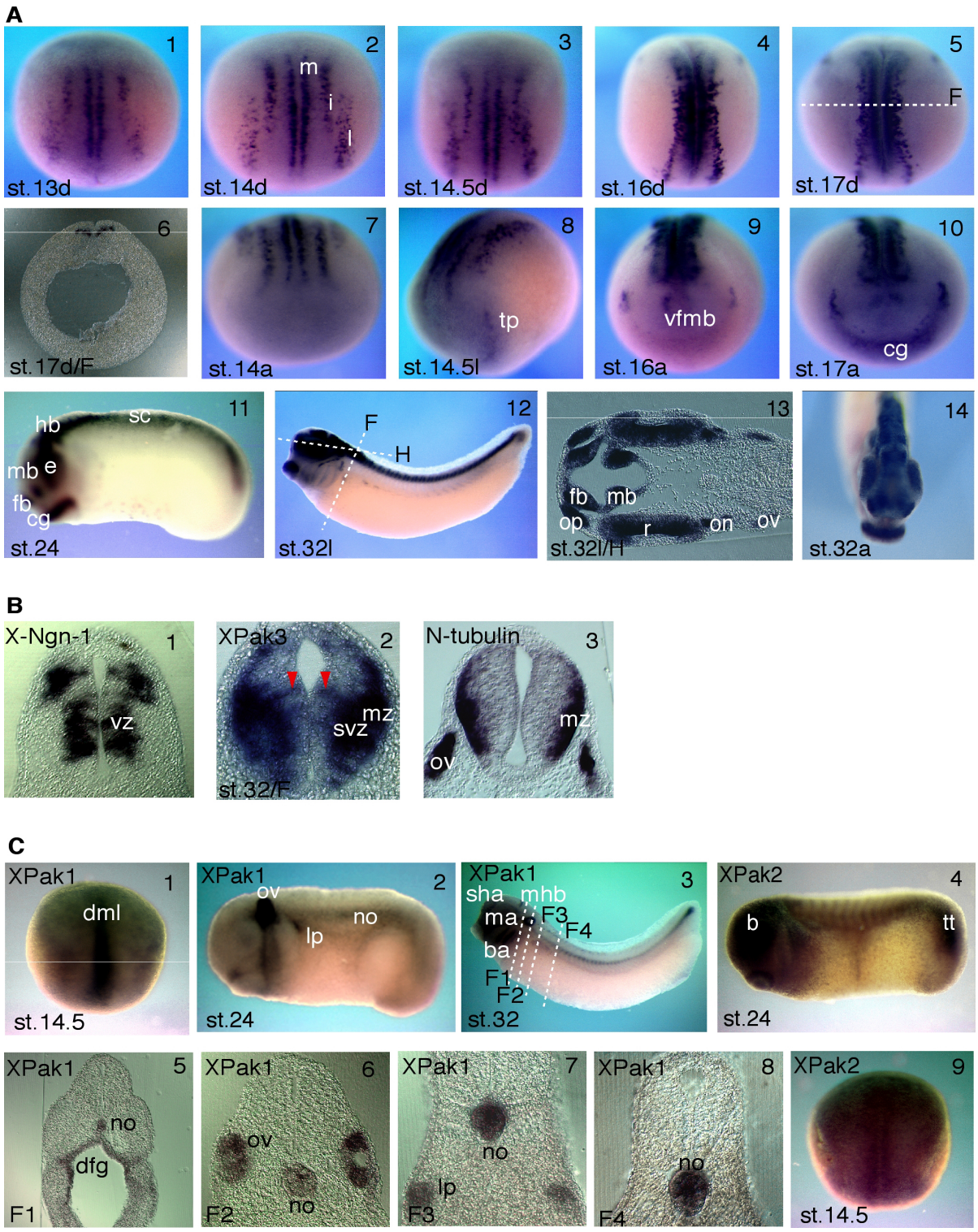

Figure 11: **Differential expression pattern of XPak genes during development**

(A) Temporal- and spatial-expression characteristics of XPak3 during primary (A1-10) and secondary (A11-14) neurogenesis. Transcripts are revealed by wholemount *in situ* hybridization. Embryos are shown in a dorsal (d) view with anterior up on panels A1-5. (A2), XPak3 transcripts are found in three bilateral stripes, lateral (l), intermediate (i), and medial (m) in the posterior neural plate. Panels A8-10 show the expression in the anterior neural plate; XPak3-expressing cells first appear (A8) in the trigeminal placode (tp), then (A9) in the area associated with the ventricular forebrain midbrain (vfmb), and (A10) in the cement gland (cg). During secondary neurogenesis (A12), horizontal section (A13) and frontal sections (B2) allow to monitor the expression in the brain (b) and spinal cord (sc); transcripts are revealed in the olfactory placode (op), retina (r),

optic nerve (on), otic vesicle (ov), forebrain (fb), midbrain (mb), and hindbrain (hb).
(B) Differential expression of neuronal marker genes in the neural tube. X-Ngnr-1 is expressed in the ventricular zone (vz), N-tubulin in the marginal zone (mz), and XPak3 in both the marginal and subventricular zone (svz). Some waves of XPak3 expressing-cells are revealed in the vz (read arrow heads).
(C) Expression pattern of XPak1 (C1-3, 5-8) and XPak2 (C4, 9). XPak1 transcripts are revealed (C1) in the dorsal midline (dml), and later in the otic vesicle, mandibular arch (ma), branchial arch, (ba), midbrain hindbrain boundary (mhb), stomodeal-hypophyseal anlage (sha), lateral placode (lp), notochord (no), and dorsal foregut (dfg). Early on, XPak2 is ubiquitously expressed in the neural plate (C9) and later (C4), the expression become stronger in the brain and tail tip (tt).

11B1). However, as indicated with red arrow heads, some waves of XPak3-expressing cells can be seen in the ventricular zone. According to the progression in the neuronal differentiation processes in the vertebrate neural tube or cortex, expression of XPak3 in the subventricular and marginal zones (fig. 11B2) suggests that this regulatory enzyme may have a function downstream of X-Ngnr-1 and upstream of N-tubulin.

Particularly striking, members of *Xenopus* Pak gene family are differentially expressed during development. Using specific probes from the regulatory domains, which are highly divergent among XPak genes, XPak1 is found to be expressed in a single stripe in the dorsal midline of the neural plate (fig. 11C1) while XPak2 is ubiquitously expressed in the neural plate (fig. 11C9). During tailbud/tadpole stages, XPak1 expression is restricted to the otic vesicle, lateral placode, notochord, and to the anterior foregut (fig. 11C2, 3, 5-8). Expression of XPak2 is stronger in the brain and tail tip (fig. 11C4). In summary, this analysis shows that XPak3 expression is predominently neural specific and XPak1 is strongly expressed in the notochord, while XPak2 is broadly expressed.

II. 1. 5. 2 X-Mxi1 expression

X-Mxi1 (Mad2) is the second Mad protein identified in *Xenopus*. Mad4 was previously described by Newman and Krieg (1999). Members of the Mad family proteins antagonize the oncogenic activity of Myc in cell-culture assays (reviewed in Zhou and Hurlin, 2001). Mice lacking Mxi1 show increased proliferation in several tissues, have an increased risk of certain spontaneous and carcingen-induced tumors, and show acceralerated tumor formation on a $p16^{JNK4a}$-null (cancer prone) genetic background (Shreiber-Agus et al., 1998). However, this Mxi1 activity cannot be directly correlated to cell differentiation.

To examine a potential role of X-Mxi1 in neurogenesis, we analysed its embryonic

expression pattern by wholemount *in situ* hybridization, using the 3'UTR sequence isolated in our expression screen. By this method, X-Mxi1 transcripts are detected at mid-late gastrula (stage 11.5) within the dorsal ectoderm which, at this stage, is just beginning to form the neural plate (fig. 12). As development proceeds, X-Mxi1-expressing cells are relatively broadly distributed thoughout the neural plate; however, they fall into a pattern that is reminiscent of the proneural marker genes, suggesting a classification of X-Mxi1 in the N-tubulin synexpression group. At the open neural plate stage 14 (fig. 12-2) the three characteristic stripes, in a radially symmetrical pattern on either side of the dorsal midline of the posterior neural plate, are almost completely fused to each other. Consitent with the early expression at gastrula stage, these broad expression patterns indicate that X-Mxi1 is likely to define an early marker of primary neurogenesis. Panel 3 of figure 12 shows that the expression of X-Mxi1 also appears to correlate with sites of neurogenesis in the anterior neural plate, including expression by a lateral group of cells associated with the trigeminal placode, by an extremely anterior group of cells associated with the olfactory placode, and by a central group of cells associated with the midbrain/forebrain. The same pattern of expression is maintained in later-stage neurula embryos, as the lateral stripes of X-Mxi1-expressing cells extend along and converge towards the dorsal midline (fig. 12-4). Thus, this analysis indicates that the expression of X-Mxi1 appears to be restricted to the developing central nervous system and, early on, correlates with sites of primary neurogenesis.

X-Mxi1 expression during secondary neurogenesis was analysed by use of tadpole-stage embryos. As shown in panel 8 of figure 12, cells expressing X-Mxi1 are restricted to the central nervous sytem. In the three transverse sections shown in panels 5, 6 and 7, X-Mxi1 appears to be strongly expressed in the ventricular and subventricular zones of the neural tube. The pituitary gland also appears to be a site of strong X-Mxi1 expression. In the retina, X-Mxi1 expression is strongest in the ciliary marginal zone (CMZ). Consistent with the broad expression of X-Mxi1 in the open neural plate stage embryos during primary neurogenesis, this strong expression in the ventriclar zone and in the CMZ further suggests that X-Mxi1 is an early marker of initiating events in neurogenesis.

II. 1. 5. 3 XSeb4 expression

To investigate if XSeb4 plays a role during neurogenesis, we examined the tissue distribution of its transcripts by use of wholemount *in situ* hybridisation. By this method,

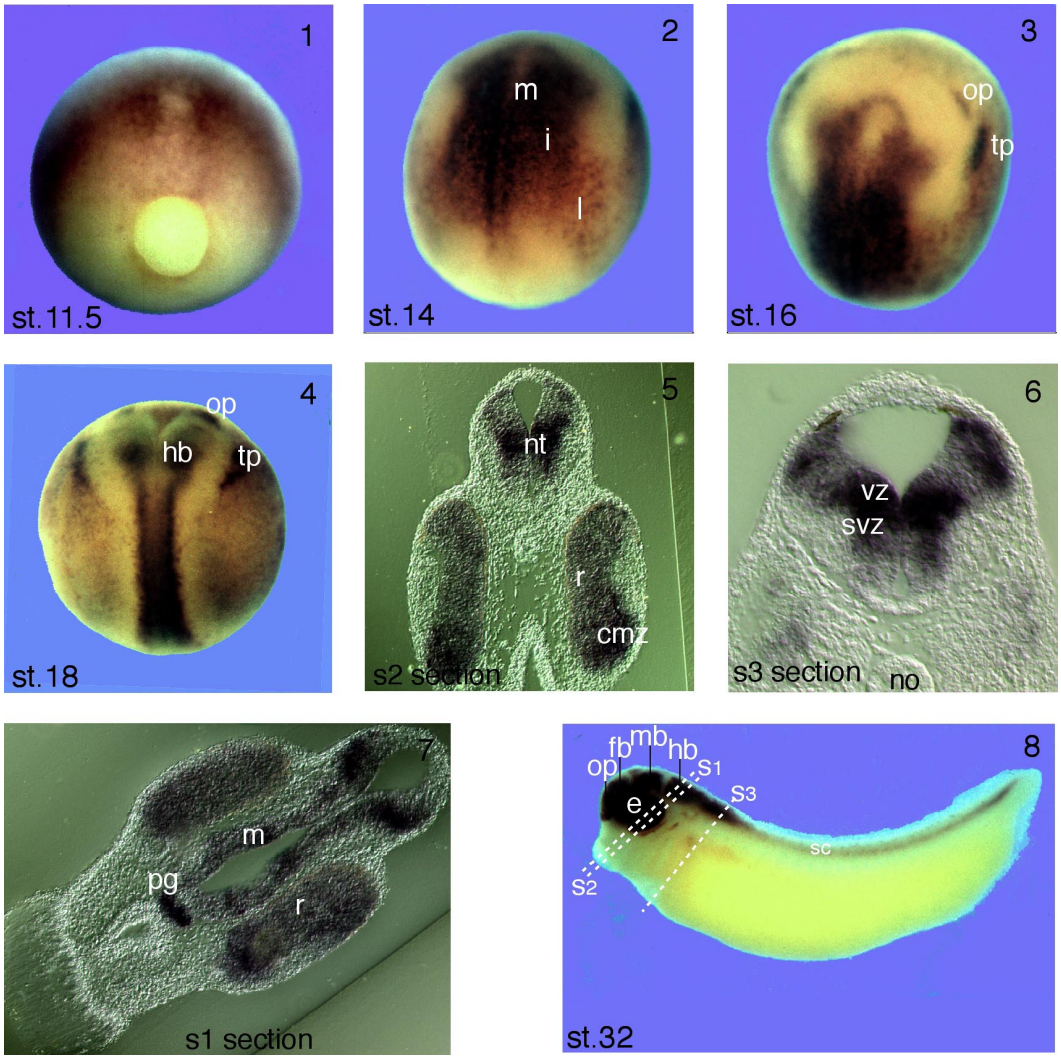

Figure 12: **Temporal- and spatial- expression characteristics of X-Mxi1**

X-Mxi1 expression during embryogenesis was analysed by wholemount in situ hybridization. Individual panels show the expression at different developmental stage: (1) X-Mxi1 expression starts during gastrulation in the forming neural ectoderm. (2) X-Mxi1-expressing cells are arranged in three broad stripes, medial (m), intermediate (i), and lateral (l). (3) Expression in the anterior neural plate starts with the trigeminal placodes (tp) and the olfactory placodes (op). (4) X-Mxi1 expression is restricted to sites of primary neurogenesis, with a strong signal in the cells associated with the hindbrain (hb). (5, 6, 7, 8) show the sites of X-Mxi1 expression during secondary neurogenesis. The following tissues are indicated: ventricular zone (vz), subventricular zone (svz), retina (r), ciliary marginal zone (CMZ), mesencephalon (m), pituitary gland (pg), eye (e), forebrain (fb), midbrain (mb), and hindbrain (hb). The transverse section are indicated by S1, S2, and S3.

XSeb4 transcripts are detected at early gastrula (stage 10.5) around the blastopore, in a region corresponding to the presumptive mesoderm (fig. 13, panel 1). A transversal section of an embryo at this stage shows clearly that XSeb4-expressing cells are located in the involuted mesoderm (fig. 13, panel 4). As the neural plate develops, the expression pattern of XSeb4 prefigures exactly, in shape and position, the expression of X-Delta-1 (fig. 13, panel 2), allowing for its classification in the Delta synexpression group. At this stage (st.14), XSeb4 is

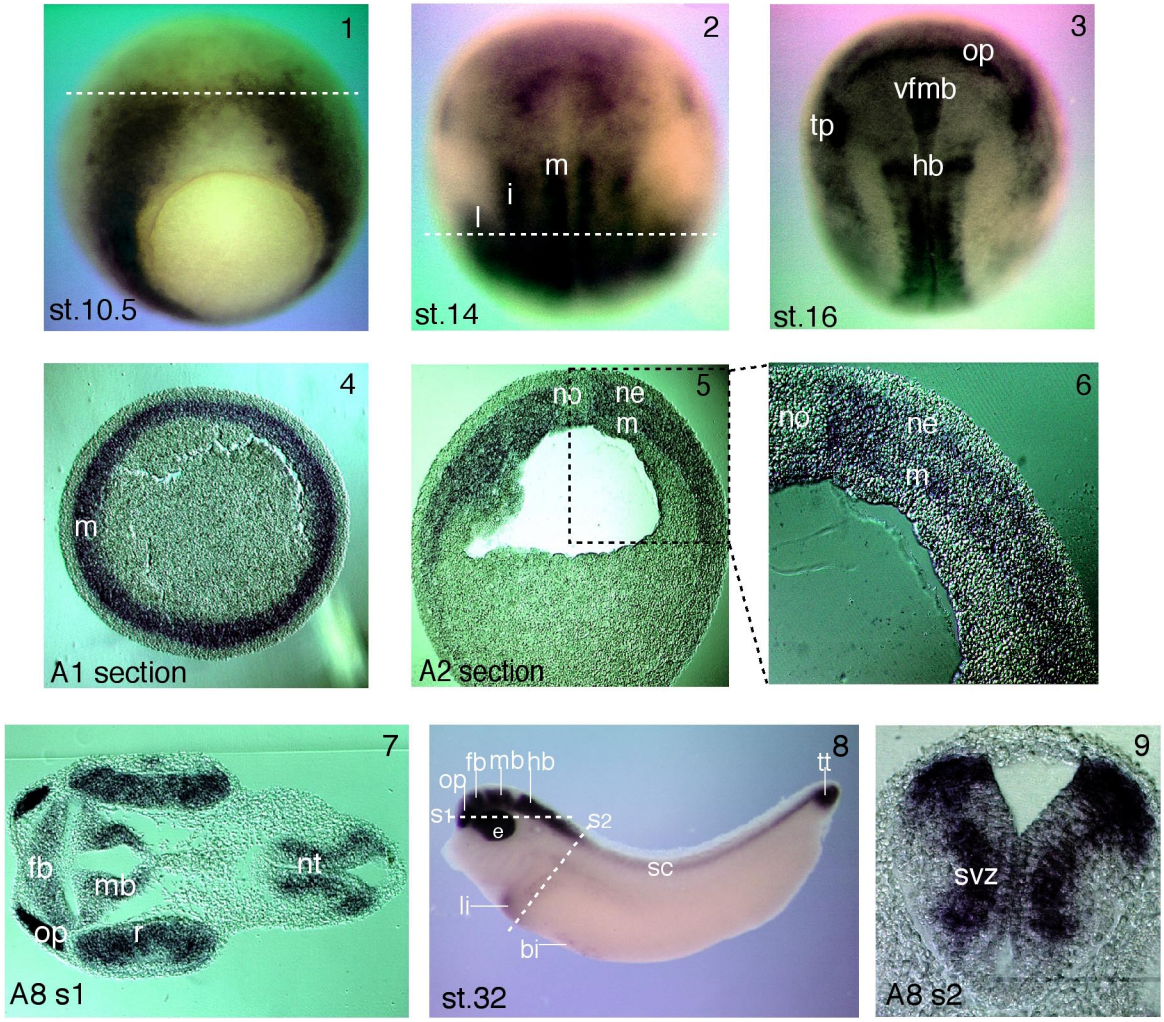

Figure 13: XSeb4 expression during primary and secondary neurogenesis in *Xenopus*

Wholemount *in situ* hybridization was used to analyse the expression pattern of XSeb4 during development. By this method, the first transcripts are revealed at early-mid gastrula stage in a region corresponding to the presumptive mesoderm (panel 1), as confirmed by the section shown panel 3. At neurula stage 14 (panel 2), three bilateral stripes of cells, including lateral (l), intermediate (i), and medial (m), are stained for XSeb4. Sections on panels 5 and 6 indicate that XSeb4-expressing cells are distributed in both the neural ectoderm and mesoderm. In the anterior neural plate, as shown on panel 3, XSeb4 is expressed in cells associated with the trigeminal placode (tp), the olfactory placode (op), and the central group of cells associated with the ventral forebrain midbrain (vfmb). In tadpole-stage embryos, as shown on panel 8, XSeb4 is expressed in the entire central nervous system, including the forebrain (fb), midbrain (mb), hindbrain (hb), eye (e), and the spinal cord (sc). In addition, XSeb4 transcripts are revealed in the liver (li), and tail tip (tt). The transverse sections, S1 and S2, show that XSeb4 is expressed in the retina (r), olfactory placode (op), forebrain (fb), midbrain (mb), and neural tube (nt). In this later, cells expressing XSeb4 are distributed in the subventricular zone (svz).

broadly expressed in three stripes in each half of the dorsal posterior neural plate. A transverse section of stage 14 embryo shows that XSeb4 expression is mostly restricted to the

dorsal portion of the embryo (fig. 13 panel 5). At high magnification, a distinct expression of XSeb4 in the neural ectoderm and in the mesoderm is observed (panel 6). In the neuroectoderm, the position of the lateral, intermediate, and medial stripes marks the wave of cells where the sensory neurons, interneurons and motor neurons differentiate during neurogenesis. The same pattern of expression is maintained in later-stage neurula (st.16), as the lateral stripes of XSeb4-expressing cells converge towards the dorsal midline during the folding of the neural tube (fig. 13, panel 3). At this stage, the expression of XSeb4 appears to correspond to the sites of neurogenesis in the anterior neural plate, including expression in the trigeminal placode precursors, in the olfactory placode progenitor cells, and in a central group of cells that corresponds to the territory of the future ventral forebrain/midbrain.

The expression of XSeb4 during secondary neurogenesis was carried out by analysing tadpole stage embryos. Overall, transcripts are revealed in the entire central nervous system, in the tail tip and in the liver anlage (fig. 13, panel 8). Transverse section through the hindbrain shows that XSeb4 is expressed in early postmitotic cells in the subventricular zone in the neural tube (panel 9). Another section across the brain (panel 7) shows that XSeb4 is strongly expressed in the olfactory placode, in the retina, forebrain, midbrain, and hindbrain.

II. 2 Regulation of expression of XPak3 and XSeb4 during neurogenesis

During primary neurogenesis in *Xenopus*, neuronal differentiation is regulated by the antagonistic effects of the proneural and the neurogenic pathways. Ectopic activation of the proneural gene network by overexpression of X-Ngnr-1 (Ma et al., 1996) induces ectopic expression of the downstream genes, including N-tubulin, whose expression marks neuronal differentiation. Conversely, ectopic activation of the Delta/Notch signaling, either with a constitutively active form of Notch, ICD-Notch, or by ectopic expression of X-Delta-1 suppresses the expression of X-Ngnr-1 and its downstream targets. Therefore, it is likely that new members of neuronal differentiation process will be regulated by these two pathways.

II. 2. 1 Regulation of XPak3 expression

If XPak3 is a marker of primary neuronal differentiation, its expression will likely be regulated by the proneural and the neurogenic genes during development. To address this question, embryos were injected at two-cell stage with 50 pg of X-Ngnr-1 capped-mRNA, along with LacZ mRNA as a tracer, and analysed at neural plate stage by wholemount *in situ*

hybridization staining for XPak3 expression, as well as with X-gal staining to reveal the distribution of the injected RNAs. Furthermore, similar analyses were carried out with several other proneural genes, functioning downstream of X-Ngnr-1, such as X-MyT1 (Bellefroid et al., 1996), X-NeuroD (Lee et al., 1995) and Xebf3 (Pozzoli et al., 2001). The results obtained show that overexpression of X-Ngnr-1 and X-NeuroD do indeed strongly activate ectopic expression of XPak3 (fig. 14-1,-2). The combination X-MyT1/Xash3 also induces strong ectopic XPak3 expression (not shown). However, overexpression of Xebf3 (kindly provided by Dr G. G. Consalez), only induces some scattered ectopic XPak3-expressing cells (fig. 14-3), similar to the ectopic expression of N-tubulin induced by the same protein as described earlier by Pozzoli et al., (2001). Based on these results, as well as, on the spatial correlation of X-Ngnr-1 and XPak3 expression described above, we conclude that XPak3 is likely to be transcriptionally activated by proneural regulators.

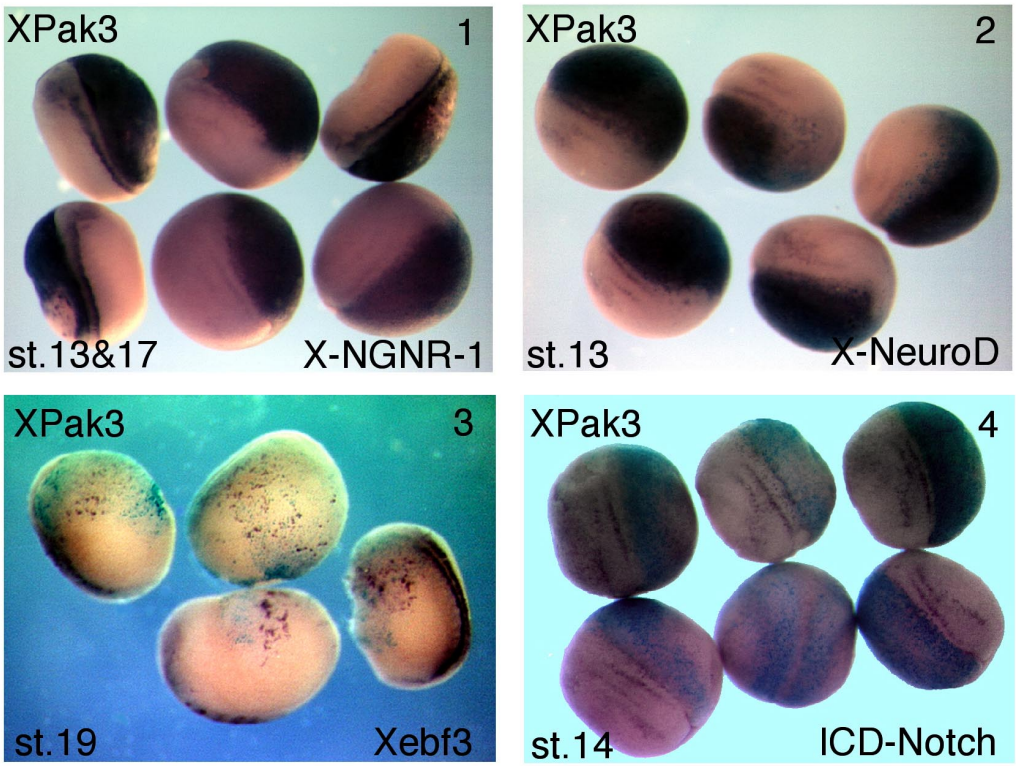

Figure 14: **XPak3 expression is positively regulated by X-Ngnr-1 and negatively regulated by lateral inhibition**

Xenopus albino embryos were injected into one cell at the two-cell stage with synthetic mRNA encoding X-Ngnr-1, X-NeuroD, Xebf3, and ICD-Notch, along with LacZ capped-RNA as a tracer. Embryos were grown and fixed at neurula stage, then stained with X-gal (blue) and analysed by wholemount *in situ* hybridization for XPak3 expression (purple). X-Ngnr-1 and X-NeuroD injections result in strong ectopic expression of XPak3 (A1 and A2: 100% and 96%, n = 76 and 64 respectively). Microinjections of Xebf3 are sufficient to turn on ectopically the expression of XPak3 but in a weak and scattered manner (A3; 62%, n = 48). The activated form, (ICD-Notch), of X-Notch-1 blocks XPak3 expression (A4; 100%, n = 56).

Expression of XPak3, like that of N-tubulin, occurs in scattered isolated cells. Previous studies indicated that the scattered pattern of neuronal differentiation is generated by an inhibitory cell-cell interaction, called lateral inhibition, mediated by Delta/Notch signaling (Chitnis et al., 1995). For instance, fewer N-tubulin-expressing cells form when Notch signaling is activated, and more N-tubulin-expressing cells form within the stripes when lateral inhibition is blocked (Chitnis and Kintner, 1996). As X-Ngnr-1 is regulated by the neurogenic pathway, its expression is suppressed by ectopic activation of Notch signaling (Ma et al., 1996). Because X-Ngnr-1 appears to regulate the expression of XPak3, we therefore asked if XPak3-expressing cells are also regulated by lateral inhibition. To test this, we examined XPak3 expression in ICD-Notch injected embryos. The results obtained reveal that ectopic activation of lateral inhibition, via ICD-Notch overexpression, is sufficient to repress XPak3 expression (fig. 14-4). Thus, this analysis reveals that XPak3 is trancriptionally repressed by lateral inhibition.

Neural induction involves anti-BMP4 signals from the mesoderm, therefore, making it possible to investigate, artificially, the regulation of a gene in animal cap assays. Together with Marion Soelter, we addressed the question as to how XPak3 and XSeb4 are regulated by use of this sytem. In these investigations, total RNAs were prepared from animal caps injected with X-Ngnr-1, Notch-ICD, and X-Ngnr-1 + Notch-ICD. Another set of injections included coinjections of Noggin. Consistent with the results in the embryos, RT-PCR results obtained, using XPaks specific primers, clearly show a transcriptional activation of XPak3, but not of XPak1, or XPak2 by X-Ngnr-1 overexpression. Similar results were obtained in Noggin-neuralized animal cap explants (fig. 15).

Strikingly, XPak3 expression is not sensitive to Notch-ICD upon coinjection of X-Ngnr-1 and Notch-ICD, indicating that XPak3 may be a direct target of X-Ngnr-1. An alternative explanation for this results is the involvement of an additional mediator, insentive to lateral inhibition. To discriminate between the two possibilities, we accessed our investigations at two levels. Firstly, we analysed the time course induction of XPak3 by means of X-Ngnr-1 injected caps. To achieve this, embryos were injected into two blastomeres of two-cell stage with low dose (50 pg) of X-Ngnr-1. Animal cap explants from these embryos were cultivated to stages 13, 14, and 15. By RT-PCR, XPak3 transcriptional activation was analysed in these latter caps; NeuroD, a known direct target of X-Ngnr-1 and N-tubulin, an indirect target of XNgnr-1 (Koyano-Nakagwa et al., 1999; Perron et al., 1999b) were used as controls. Results obtained reveal that XPak3 expression, like that of N-tubulin,

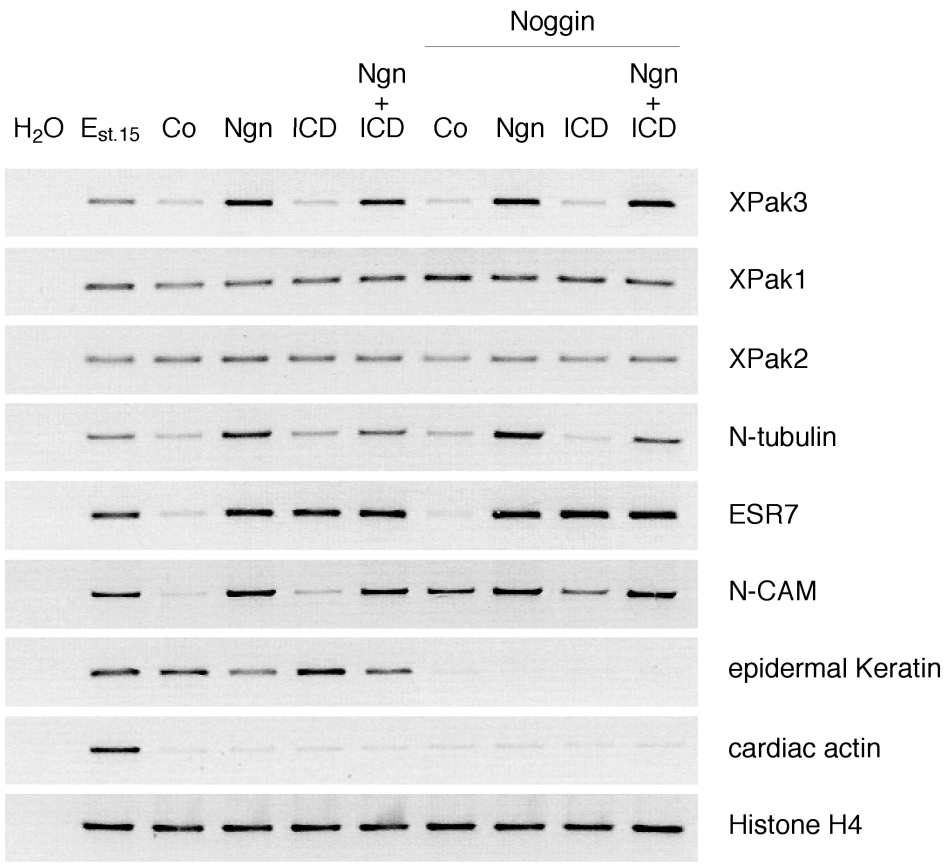

Figure 15: **Regulation of XPak genes in animal cap explants**

Pigmented *Xenopus* embryos were injected into two blastomeres of two-cell stage embryos with, first batch: 100 pg of X-Ngnr-1, 300 pg of ICD-Notch, 100 pg of X-Ngnr-1 + 300 pg of ICD-Notch; second batch: as the first but together with 500 pg of Noggin. Embryos were grown to stage 8-9. Using a gastromaster, animal caps were cut and cultivated to stage 15. Total RNAs were prepare from the caps and analysed by RT-PCR for XPak1-3 expression. From left to right, the columns represent respectively: water control (H_2O), control embryos stage 15 ($E_{st.15}$), Control caps (Co), X-Ngnr-1 injected caps (Ngn), ICD-Notch injected caps (ICD), coinjection (Ngn+ICD), and following with Noggin neuralized caps. Mesoderm contamination was controlled with cardiac actin, the neuralizing activity with epidermal keratin, neural induction with N-CAM, activation of Notch signalling with ESR7, and activation of the proneural signalling with N-tubulin. results of the first row show that XPak3 is transcriptionally activated in neuralized and non neuralized caps by X-Ngnr-1. ICD coinjection appears not to affect this activation of XPak3 in both cases. The second and third rows show that XPak1 and XPak2 are not regulated by X-Ngnr-1, both in the neuralized and non neuralized caps.

is increasingly induced in animal caps of stage between 13 and 15 (fig. 16A), while in the same conditions NeuroD is early strongly expressed already in stage 13 caps. Secondly, by used of hormone-inducible neurogenin, X-Ngnr-1-GR, construct (kindly provided by Dr. E. Bellefroid), our RT-PCR results reveal no XPak3 activation in caps treated with cycloheximide and dexamethasone, while NeuroD is found to be activated, though weakly (fig. 16B).

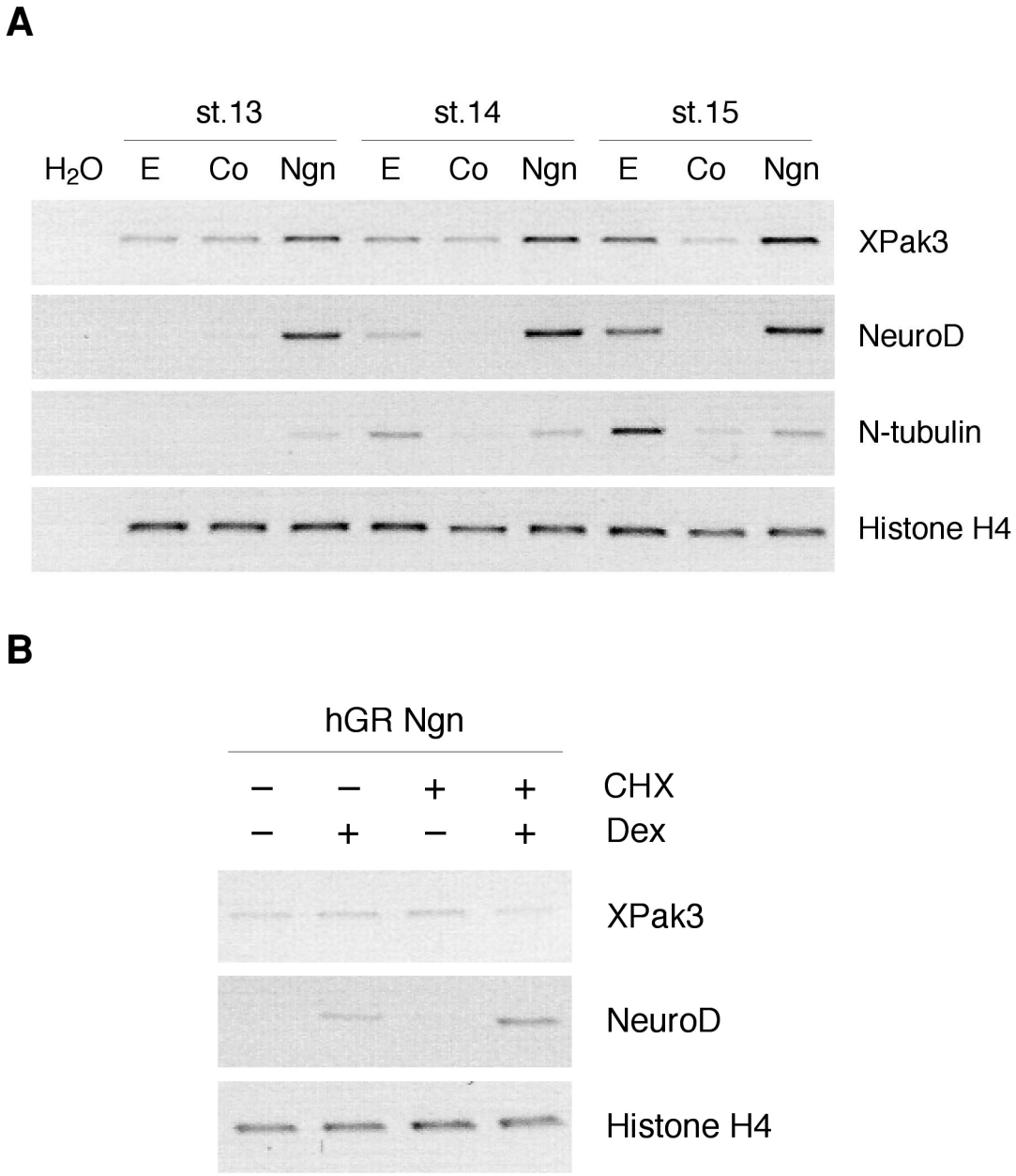

Figure 16: **XPak3 is not directly activated by X-Ngnr-1**

(A) XPak3 expression increases gradually in non neuralized caps from stage 13 to at 15. Embryos were injected as previously with 50 pg of X-Ngnr-1. Total RNAs were prepared from caps and related controlled embryos fixed at stage 13, 14, and 15. RT-PCR analysis shows that XPak3 expression, similar to that of N-tubulin, increased with during development, while NeuroD is early strongly induced already at stage 13.
(B) Embryos were injected into two blastomeres of two-cell stage with X-Ngnr-1-GR. Animal caps were treated for one hour as from stage 13.5 with or without cycloheximide (CHX) and followed by 2.5 hours treatment with CHX +/- dexamethasone (Dex). Total RNA were prepared from these caps and analysed by RT-PCR for XPak3 expression, controlled with NeuroD. Results show that XPak3 is not activated in caps treated with cycloheximide and dexamethasone while NeuroD is induced.

Consistent with the relatively late expression of XPak3 during development, these findings suggst strongly that XPak3 is not a direct target of X-Ngnr-1.

II. 2. 2 Regulation of XSeb4 expression

To investigate if XSeb4 is a member of the genetic cascade involved in the neuronal differentiation, we analysed the effect of X-Ngnr-1 overexpression on the transcription of XSeb4. Embryos were injected into one blastomere of two-cell stage with 50 pg of X-Ngnr-1 and 50 pg of ß-galactosidase capped sense RNAs, as a probe distribution control. At the open neural plate stage, these embryos were fixed, stained for X-gal and analysed by wholemount *in situ* hybridization for XSeb4 transcription (fig. 17). Our results show that X-Ngnr-1 injection induces ectopic XSeb4 expression (fig. 17, panel A1, white arrow head). Injection of NeuroD gave similar results (not shown). These findings further confirm that XSeb4 is upregulated by the proneural genes during primary neurogenesis, thereby, explaining the expression pattern of XSeb4 in the neuroectoderm at neurula stage 14, as described above.

X-Ngnr-1 is negatively regulated by the neurogenic genes. Its expression is suppressed by overexpression of X-Notch-1 (Ma et al., 1996) and other elements of the Delta/Notch pathway. Because X-Ngnr-1 appears to regulate the expression of XSeb4, we therefore asked if XSeb4-expressing cells are also regulated by lateral inhibition. To address this question, we examined XSeb4 expression in ICD-Notch injected embryos. Our results show that activation of lateral inhibition, via ICD-Notch overexpression, is sufficient to repress XSeb4 expression (fig. 17, panel A2, white arrow head), arguing that XSeb4 expression is likely to be regulated by lateral inhibition.

We further addressed the question if X-Ngnr-1 is sufficient to activate XSeb4. To achieve this, XSeb4 transcriptional activity was assayed in animal caps injected with X-Ngnr-1 alone or coinjected with Noggin. By RT-PCR, XSeb4 was found to be strongly activated in neuralized and non-neuralized caps injected with X-Ngnr-1 (fig. 17B). Surprisingly, XSeb4 is also activated by ICD-Notch; a combination of ICD-Notch and X-Ngnr-1 also results in strong activation of XSeb4. These findings define a regulatory situation in respect to transcriptional activation of XSeb4 in animal caps that cannot be reconciled with the current models of proneural/neurogenic gene network organisation. The muscle specific Seb4, MTG, though induced at a lesser extent by X-Ngnr-1, was not activated by ICD-Notch (fig. 17B).

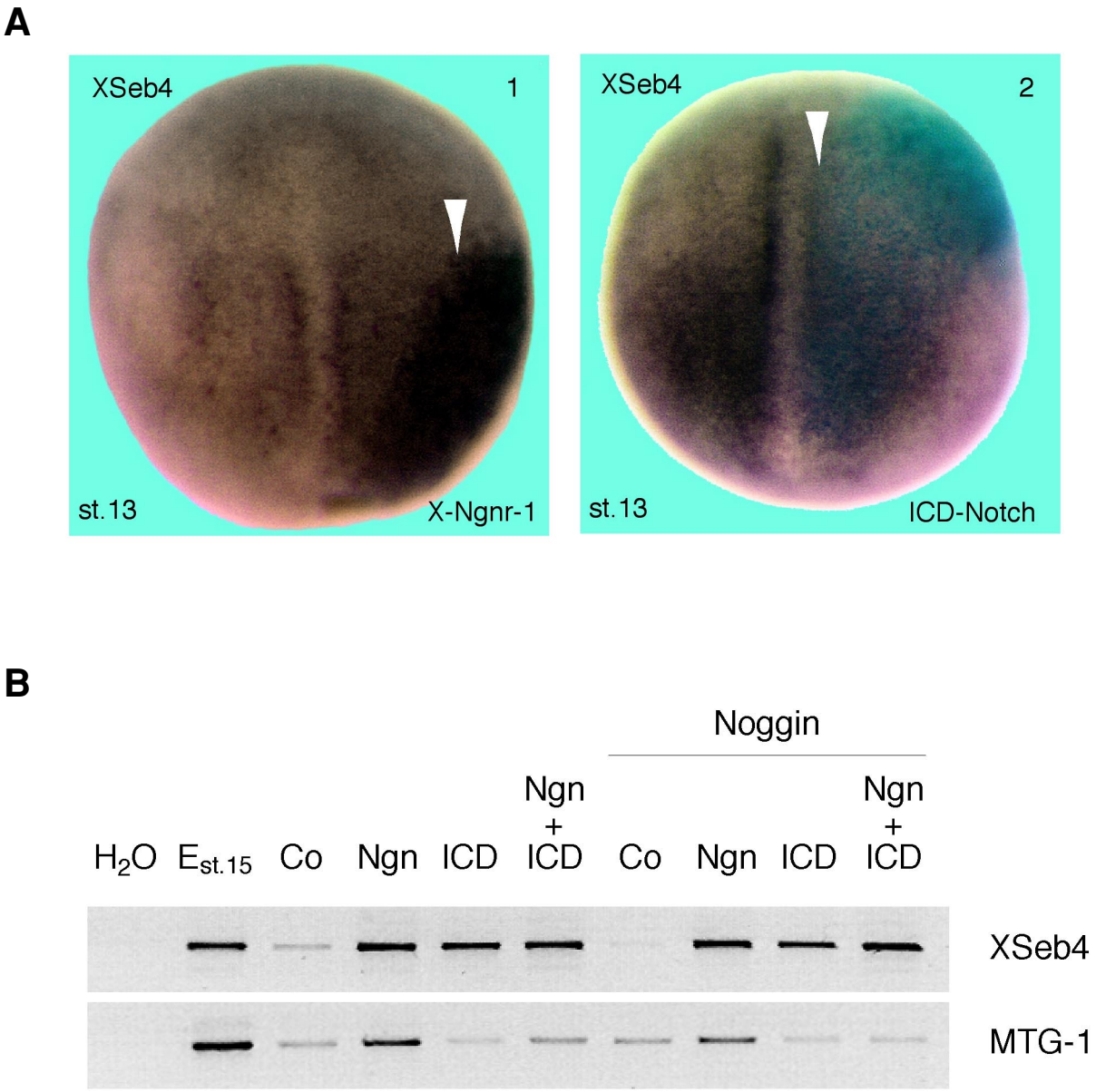

Figure 17: **XSeb4 is differently regulated in embryos and in animal cap assays**

(A) XSeb4 responds positively to X-Ngnr-1 and negatively to Notch signaling in embryos.
Embryos were injected into one blastomere of the two-cell stage with 50 pg of X-Ngnr-1 (panel 1) or 150 pg of ICD-Notch capped-RNA (panel 2) together with 50 pg of LacZ capped-RNA, as a tracer. Fixed at open neural plate stage, they were stained for X-gal and analysed by wholemount *in situ* hybridization for XSeb4 expression. X-Ngnr-1, as shown in panel 1, induces ectopic expression of XSeb4 (83 %, n = 56). As seen in panel 2, activation of Delta/Notch signaling downregulates XSeb4 expression (58 %, n = 48). Embryos are shown in the dorsal view with anterior up.
(B) Xeb4 responds positively to both X-Ngnr-1 and Notch signalling in animal caps.
As describe in figure 11, XSeb4 was analysed by RT-PCR using RNAs from caps injected as indicated. XSeb4 is transcriptionally activated upon activation of the proneural (X-Ngnr-1) and Delta/Notch (ICD) pathways.

II. 3 Functional characterization of XPak3 and XSeb4

The classification of a gene into a particular synexpression group (Niehrs, C. and Pollet, N., 1999) indicates not only a similarity in expression pattern, but also a functional link. We have shown from the expression pattern analysis that XPak3 is a member of the N-tubulin synexpression group and XSeb4 a member of the Delta synexpression group. If these classifications are correct, XPak3 and XSeb4 should also play a role in the proneural and in the lateral inhibition pathways, respectively. To address these questions, we analysed the effect of ectopic XPak3 and XSeb4 activation on the formation of the primary neurons, as marked by the expression of N-tubulin gene.

II. 3. 1 XPak3 functional characterization

If XPak3 plays a role during primary neurogenesis, its mis-expression could influence the pattern of N-tubulin expression. To address this question, we cloned the ORF of XPak3 into the pCS2+ expression vector. The recombinant vector was tested in the transcription and translation (TnT) assays for XPak3 protein synthesis. Results obtained reveal that full length XPak3 is synthesized in the TnT assays (not shown). To investigate the role of XPak3 in primary neurogenesis, capped-mRNA was prepared from pCS2+XPak3 (XPak3-wt) and injected into one blastomere of two-cell stage embryos, together with the capped-mRNA encoding LacZ for probe distribution control. Embryos were fixed at neurulation (stage 14), stained with X-gal, and analysed by wholemount *in situ* hybridization for N-tubulin expression. Results obtained reveal that ectopic expression of XPak3-wt has no effect on normal development of embryos and on the expression of N-tubulin gene (data not shown).

Previous studies had demonstrated that the sequestration of Pak proteins to the membrane leads to upregulation of its kinase activity (Lu et al., 1997; Manser et al., 1997). In *Drosophila*, recruitment of XPak3 homolog, DPak to the cell membrane by use of a myristylation signal was found to be sufficient to activate the kinase domain (Hing et al., 1999). Accordingly, we generated a membrane-anchored version of XPak3 by fusing a myristylation signal to the amino terminus of the wild type protein. This construct is designated XPak3-myr.

To test the effect of this construct on primary neurogenesis, capped-mRNA was prepared and injected at low concentration; a maximum of 10 pg/blastomere of two-cell stage,

together with 50 pg of LacZ mRNA as a tracer were optimal in this context. A batch of these embryos was fixed at the open neural plate stage (stage 14) and another at tadpole-stage.

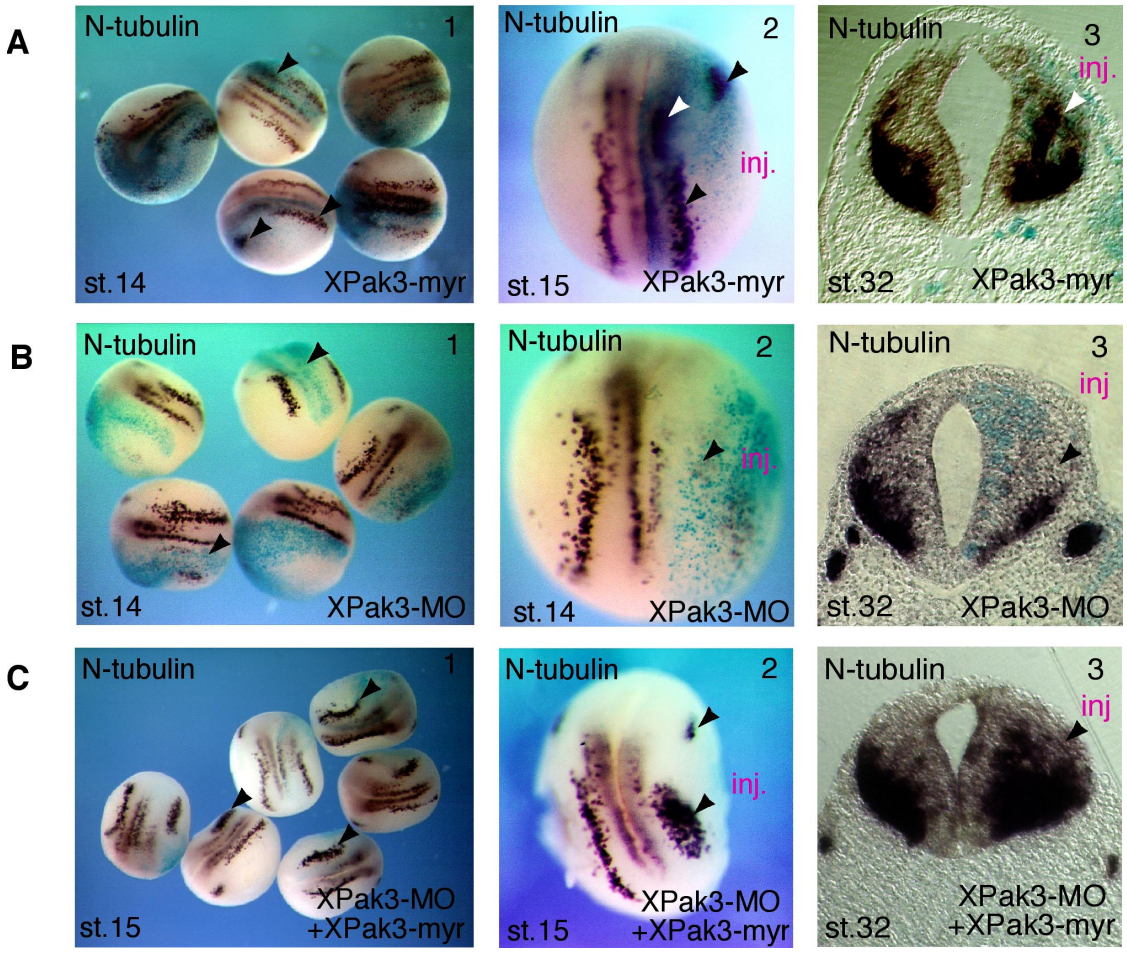

Figure 18: **XPak3 activation induces premature neuronal differentiation**

(A) *Xenopus* albino embryos were injected into one cell at the two-cell stage with 10 pg of XPak3-myr capped-mRNA along with LacZ mRNA as a probe distribution control. Embryos were fixed at neurula and tailbud stages, then stained with X-gal (blue). By wholemount *in situ* hybridization, these embryos were analysed for neuronal differentiation, as marked by N-tubulin expression. Panel A1-2 shows an increased (71%, n = 46) N-tubulin expression within the territory of primary neurons. In the neural tube, XPak3-myr injections shift the expression domain of N-tubulin from the marginal zone towards the ventricular zone (white arrow head).
(B) Embryos were injected as above with 2.5 pmoles of XPak3-MO, using LacZ mRNA as a tracer. They were allowed to grow to neurula and tailbud stages, were fixed and stained with X-gal. As judged by wholemount *in situ* hybridization, XPak3-MO injections strongly block neuronal differentiation (100%, n = 92).
(C) Coinjections of XPak3-MO and XPak3-myr capped-mRNA (2.5 pmoles/12.5 pg) effectively (84%, n = 73) rescue N-tubulin exppression.

These embryos were stained for X-gal activity (blue) and analysed by wholemount *in situ* hybridization for N-tubulin expression. As shown in figure 18A1-2, ectopic expression of XPak3-myr leads to increased N-tululin expression within its normal expression domain

(black arrow heads). Such phenotype was described earlier for genes confering resistance to lateral inhibition (Bellefroid et al., 1996; Lamar, et al., 2001b). Another explanation of these results is premature cell cycle exit, resulting in premature neuronal differentiation. To discriminate between these two possibilities, we examined the effect of XPak3-myr on N-tubulin expression during secondary neurogenesis because previous studies had shown that neuronal differentiation progresses from the ventricular towards the marginal zone in the neural tube in *Xenopus* (Bellefroid et al. 1996). Therefore, an activity leading to early cell cycle exit could induce premature neuronal differentiation and thereby expand/shift the pattern of N-tubulin-expressing cells towards the ventricular zone. As shown in figure 18A3, microinjections of XPak3-myr capped-mRNA do indeed result in markedly reduced N-tubulin expression in the marginal zone and in an expansion/shift of N-tubulin-expressing cells domain in the subventricular zone (white arrow head). These findings argue strongly that XPak3 activation induces premature neuronal differentiation.

These results from ectopic XPak3 activation using its constitutively active form, XPak3-myr, thus suggest that XPak3 activity regulates cell cycle exit during primary neurogenesis. If this is right, XPak3 loss-of-function should result in defects concerning the formation of primary neurons; in this case neuronally programmed cell will be inhibited in respect to their exit from the mitotic cell cycle. To address this question, we produced a XPak3 loss-of-function situation, using a morpholino antisense oligo (XPak3-MO). Results showed in figure 18B reveal that microinjection of XPak3-MO strongly inhibits N-tubulin expression (black arrow heads). As the XPak3 expression spans to neural tissues, including the cement gland, we addressed the question if XPak3 suppression impairs cement gland formation. This was done by analysing the effect of XPak3-MO injection on XAG expression and on cement gland formation. Our results clearly show that inhibition of XPak3 activities leads to suppression of XAG expression and inhibition of the formation of cement gland cells, as marked by the absence of pigmented cells (fig. 19).

The morpholino antisense oligo was directed at the 5' UTR of the endogenous transcripts such that it could not bind to XPak3-myr capped-mRNA. Therefore, rescue experiments could be carried out just by coinjection of XPak3-MO and XPak3-myr. Hence, to investigate if N-tubulin suppression phenotype could be rescued, coinjection experiments were achieved as summarized in table 2. As above, normal looking embryos resulting from these injections were fixed at open neural plate and tailbud stages, then judged by wholemount *in situ* hybridization. Results obtained reveal that inhition of neurogenesis by 2,5

pmoles of XPak3-MO is effectively rescued by coinjection of 12,5 pg of XPak3-myr (fig. 18C).

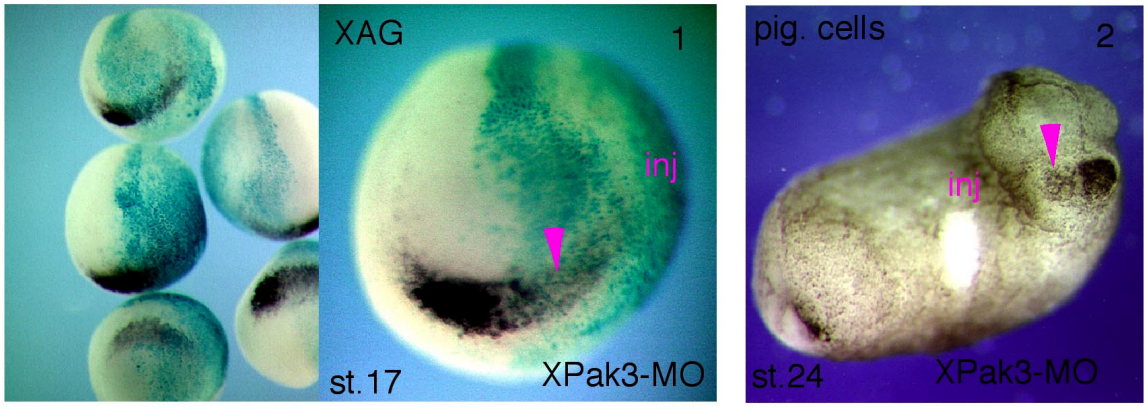

Figure 19: **XPak3 is required for cement gland cells differentiation**

Embryos were injected into one blastomere of two-cell stage embryos with 2.5 pmoles of XPak3-MO and 50 pg of LacZ RNA as a tracer. A batch of these embryos was fixed at st.17, stained for X-gal activity (green-blue) and bleached. The other part was fixed at stage 24. By wholemount in situ hybridization, XAG is found to be suppressed (1). In stage 24 embryos, pigmented cement gland cells are missing on the injected side (2).

Table 2: Summarized data of the rescue experiments.

XPak3-MO (pmoles)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
XPak3-myr (pg)	0	1.5	3.125	6.25	12.5	25	50	100	200
number of embryos	80	88	70	92	73	104	110	96	120
Neural plate expansion	67%	45%	0%	0%	0%	0%	0%	0%	0%
Normal looking	33%	55%	100%	93%	82%	0%	0%	0%	0%
cell cycle arrest (CCA)	0%	0%	0%	7%	18%	97%	100%	100%	100%

RT-PCR analysis, revealed the presence of XPak3 transcripts in eggs and in all embryonic stages. In addition XPak1 was reported to induce cleavage arrest of early frog embryos (Rooney et al., 1996). Thus, the presence of XPak3 transcripts in all embryonic stages may correlate with its role in early cell cycle regulation. To address this question, we injected a series of different concentrations of XPak3-myr into one blastomere of two-cell

stage embryos, as summarized in table 3. Our results show that ectopic expression of XPak3-myr induces cell cycle arrest (CCA) in a dose dependent manner (table 3), associated in some extreme cases with a loss of pigmentation (fig. 20A1, 2). Because embryo microinjection of high dose (1 ng) of the wild type Pak3 capped-RNA or other myristylated constructs, such as ED/Pix (fig. 21) is found to have no CCA effects (data not shown), we conclude that the cleavage arrest activity induced by XPak3-myr is not a result of side effects coming from the toxicity of XPak3 protein or the myristylation signal.

Table 3: Summarized data of the cell cycle arrest (CCA) activities induced by XPak3-myr.

XPak3-myr mRNA (pg)	500	250	125	62.5	31.25	15.62	7.81	0
Number of embryos	162	116	134	102	96	107	86	80
developmental arrest	100%	100%	100%	100%	98%	100%	6%	0%
	before midblastula transition (MBT)			after MBT				
Normal looking	0%	0%	0%	0%	2%	0%	94%	100%

If the above cell arrest phenotype reflects the right function of XPak3 during embryonic development, its suppression may produce the opposite effect. Interestingly, microinjection of morpholino antisense oligonucleotide results in an expansion of the neural plate (fig. 20A3-4), indicating increased proliferation, as indeed suggested by increased/expanded PCNA staining (fig. 20A5-6). The number of apoptotic cells seems to increase in proportion with the increased cell number (fig. 20A8). Thus, modulation of XPak3 activity correlates with proliferation control but with no significant effect on apoptosis.

The question as if the endogenous XPak3 protein and XPak3-myr synergize in this CCA phenotype was addressed. Results obtained have shown that injection of 12.5 pg of XPak3-myr capped RNA induces developmental arrest at around gastrula stage, while coinjection of 2.5 pmoles of XPak3-MO rescues effectively this phenotype (fig. 20B1-2); these findings reveal that endogenous XPak3 contribute to the CCA activity upon XPak3-myr injection.

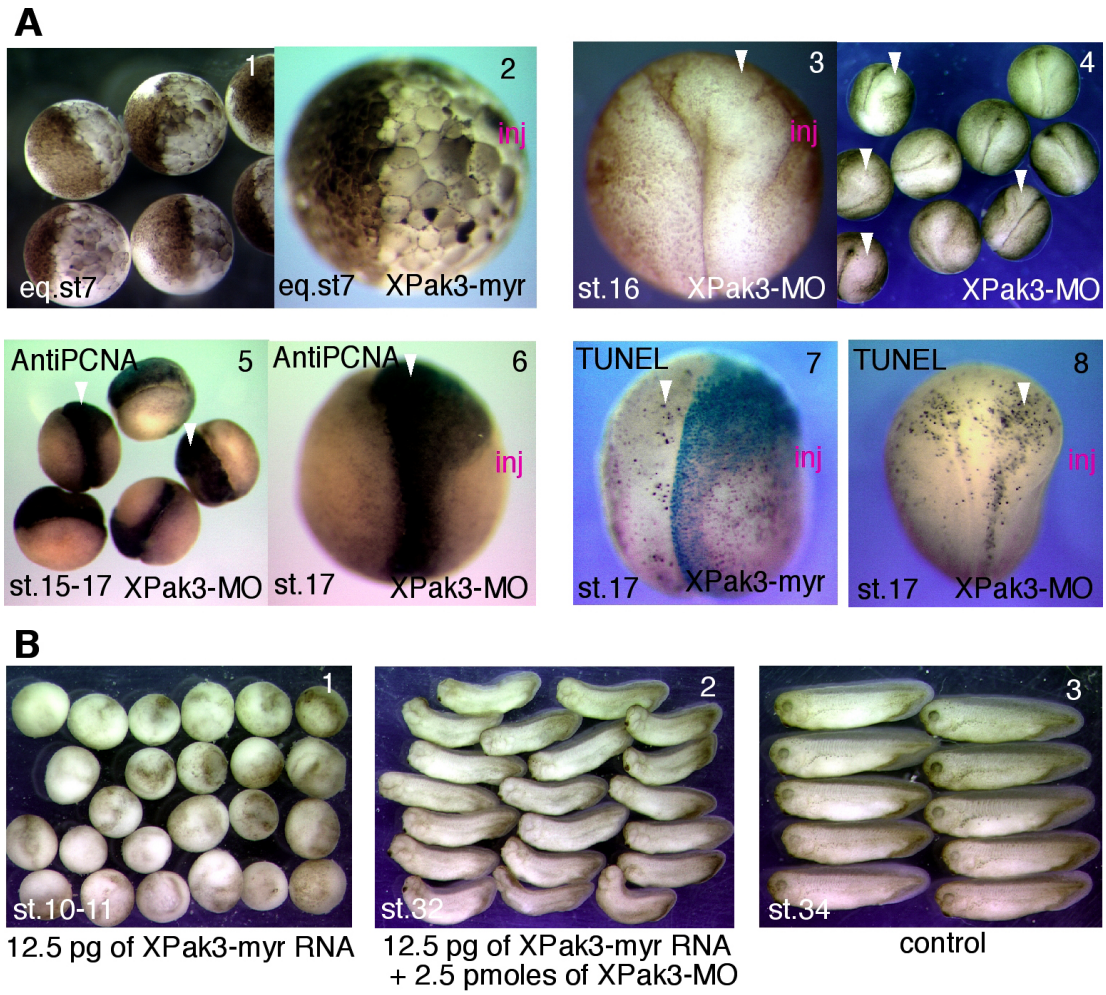

Figure 20: **XPak3 activities are involved in cell cycle regulation.**

(A) Embryos were injected with XPak3-myr capped mRNA into one blastomere of two-cell stage with 200 pg (1-2) and 7.5 pg (7). The high concentration induces early developmental arrest while the lower shows no morphological change. Apoptotic cell death was analysed by the TUNEL assay; no increased apoptosis was detected (panel 7). Another batch of embryos was injected with XPak3-MO at a concentration of 5 pmoles/blastomere at the two-cell stage. By completion of primary neurogenesis, a significant number (67 %, n = 116) of injected embryos exhibits neural plate expansion (A3-4). Antibody staining reveals increased PCNA positive cells in the injected side (A5-6). Investigation of programmed cell death by TUNEL staining shows increased apoptosis in the injected side, proportional to the increased cell number (A8).
(B) Rescue of the cell cycle arrest phenotype. A batch of embryos was injected into one blastomere of the two-cell stage with XPak3-myr capped-RNA, a nother batch was coinjected with XPak3-myr and XPak3-MO. Embryos were incubated at 18 °C for 48 hours. Panel B1 shows embryos whose development was arrested just after MBT after injection of 12.5 pg of XPak3-myr. Panel B2 shows the rescue of this CCA phenotype by coinjection of 2.5 pmoles of morpholino antisense oligo along with 12.5 pg of XPak3-myr (79 %, n = 95). Panel B3 shows the uninjected control embryos.

Previous studies showed that the kinase activity of the *Drosophila* Pak3 orthologue, is essential for R cell axon guidance (Hing et al., 1999). To investigate which domains are involved in the cell cycle arrest described above, we generated a series of XPak3 deletion constructs, containing a myristylation signal at their amino terminus (fig. 21). Overexpression

of these constructs reveal that misexpression of the N-terminal deletion constructs induces cell cycle arrest, suggesting a requirement for the kinase domain. However, kinase domain deleted constructs induce cell cycle arrest as well (fig. 21). Strikingly, an internal fragment, (ED/Pix), containing the Pix binding site, part of the autoinhibitory domain and the acidic domain (ED), was found to induce neural plate expansion, therefore, appears to function as a dominant negative form of XPak3. Furthermore and in contrast to XPak3-MO, this construct was found to impair the anterior-posterior axis formation in about 40% of the affected embryos (not shown), indicating a cross-reaction with XPak1 whose expression is strong in the notochord.

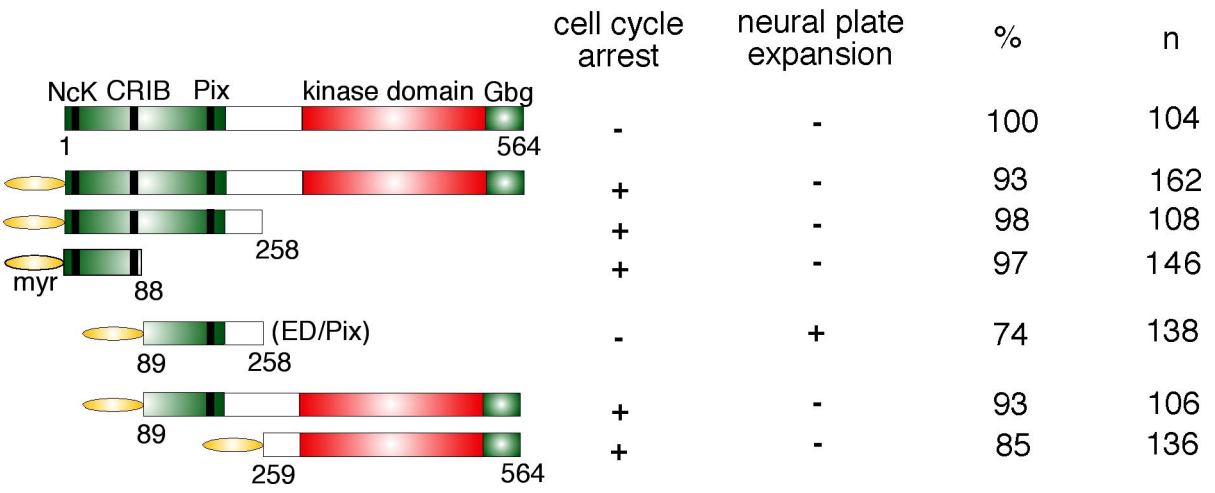

Figure 21: XPak3 functional domains involved in proliferation control
A series of constructs, as indicated, was generated by use of PCR cloning techniques. The construct corresponding to the wild type (wt) protein comprises the main regulatory domain in green, the hinge (white portion), the kinase domain, and the G-beta/gamma subunit binding motif, respectively. The myristylation signal (yellow) is indicated at the N-teminus. Capped-RNAs were prepared from these constructs and used in microinjection experiments. As indicated, myristylated kinase deletion and N-terminal deletion constructs both induce CCA. The construct containing part of the autoinhibitory domain, the guinine exchange factor, Pix, binding site (ED/Pix) induces neural plate expansion. (%) percentage of respective phenotypes, n number of injected embryos.

Taken together , based on the expression pattern of XPak3 in form of the stripes in the neural plate, the regulation of XPak3 expression by the proneural and neurogenic genes, the premature neuronal differentiation effects, and the cell cycle arrest phenotype induced upon XPak3 activation, as described above, we would like to define XPak3 as a member of the proneural pathway which belongs to the N-tubulin synexpression group. Furthermore, we would like to suggest that activation of XPak3 is required to drive neuronally specified cells

into terminal differentiation. Otherwise, these cells remain in the mitotically active state and are inhibited from entering neuronal differentiation as supported by our gain- and loss-of-function experiments.

II. 3. 2 XSeb4 functional characterization

The expression of XSeb4 mimics in shape and tissue distribution the expression of X-Delta-1 and other members of the Delta/Notch signaling cascade. This expression pattern is modified in embryos with altered X-Ngnr-1 activities; overexpression of X-Ngnr-1 in growing *Xenopus* embryos reveals ectopic expression of XSeb4. Mis-expression of X-Notch-1 by injecting its constitutively active form, ICD-Notch, suppresses XSeb4 expression in whole embryos. This regulation of XSeb4 expression by proneural and neurogenic genes suggests that XSeb4 may have a role during primary neurogenesis. To address this question, we injected a range of concentrations varying from 25 pg to 500 pg of XSeb4 capped-mRNA into one blastomere of the two-cell stage embryos, together with 50 pg of ß-galactosidase capped-mRNA as a tracer. Embryos were grown to the open neural plate stage (stage 14), fixed, stained for X-gal activity (blue), and analysed by wholemount *in situ* hybridization for the expression of neuronal marker genes, including X-Ngnr-1, X-MyT1, X-Delta-1, and N-tubulin. Our results reveal that microinjection of concentrations less than 150 pg had no effect on the expression of N-tubulin (not shown). However, ectopic expression of concentrations above 200 pg were found to downregulate X-Ngnr-1 and its downstream targets, indicating that XSeb4 is involved in the cascade of neurogenic genes that govern lateral inhibition (fig. 22).

Previous studies show that ectopic expression of elements of the Delta/Notch signal transduction pathway results in etopic expression of Enhancer of split-related (ESR) genes, which in turn repress the expression of X-Ngnr-1. As XSeb4 appears to inhibit the expression of X-Ngnr-1, its ectopic expression could also induce ectopic expression of the ESRs genes. To address this question, we analysed by microinjection and wholemount *in situ* hybridization effects of XSeb4 overexpression on the expression of ESRs genes. Our results show that XSeb4 also suppresses these genes (fig. 23), suggesting a different mechanism in X-Ngnr-1 repression.

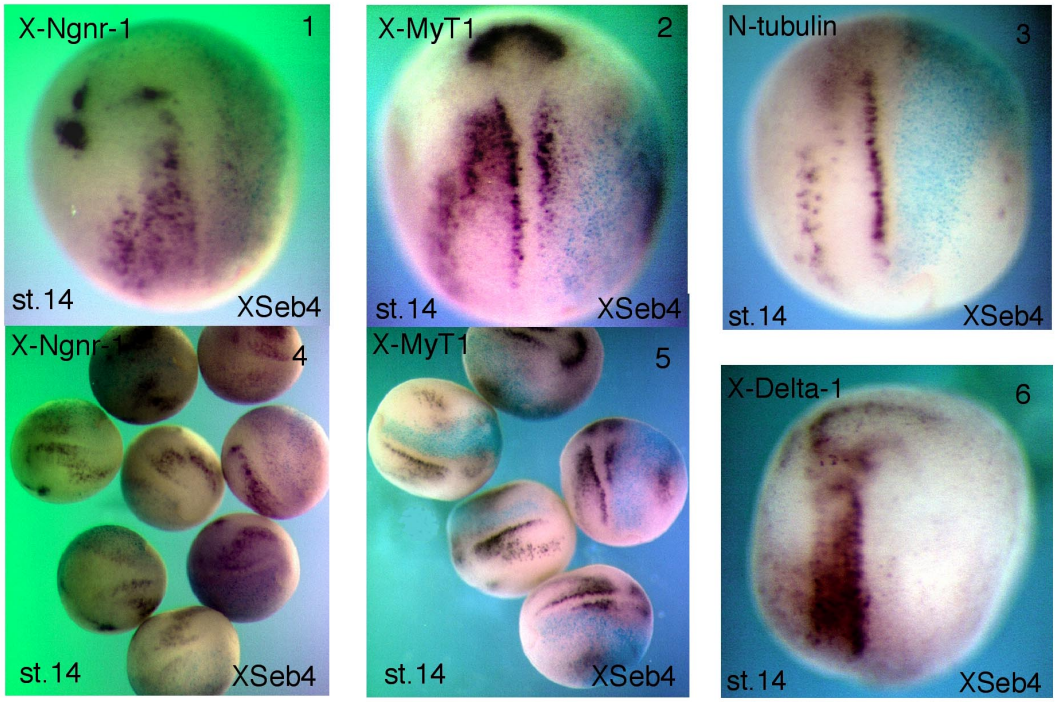

Figure 22: **Overexpression of XSeb4 suppresses proneural and neurogenic genes**

Embryos were injected with 250 pg of XSeb4 capped-mRNA into one blastomere of the two-cell stage, along with LacZ capped-mRNA, for probe distribution control. They were grown to the open neural plate stage (stage 14), fixed and stained for X-gal activity (blue). By wholemount *in situ* hybridization techniques, these injected embryos were analysed for neuronal marker gene expression. Our results show that microinjection of XSeb4 is sufficient to suppress the expression of X-Ngnr-1 (83%, n = 56) and its downstream targets, including X-MyT1 (89%, 48), X-Delta-1 (71%, n = 54), and N-tubulin (100%, n = 85).

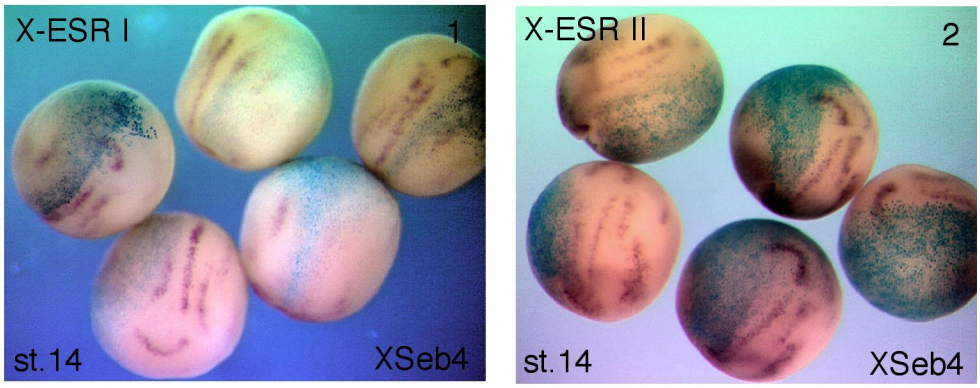

Figure 23: **Activation of XSeb4 suppresses the expression of members the ESR gene family.**

Embryos were injected with 400 pg of XSeb4 capped-RNA into one blastomere of the two-cell stage, along with LacZ capped-RNA, as tracer. They were grown to the open neural plate stage (stage 14), fixed and stained for X-gal activity (blue). By wholemount *in situ* hybridization techniques, these injected embryos were analysed for ESRs marker gene expression. Our results show that microinjection of XSeb4 efficiently represses the expression of ESR I (89 %, n = 52) and ESR II (98 %, n = 64).

Previous studies have shown that some RRM-like RNA binding protein may act as transcription factors (Brzostowski et al., 2000). If this is the case also with XSeb4, XSeb4 should be nuclearly localised. To address this question, we generated a Flag-tagged version of XSeb4 and analysed the protein localisation in growing embryos. Our results show that XSeb4 protein accumulates in the nucleus (fig. 24), though lacking nuclear localization signal.

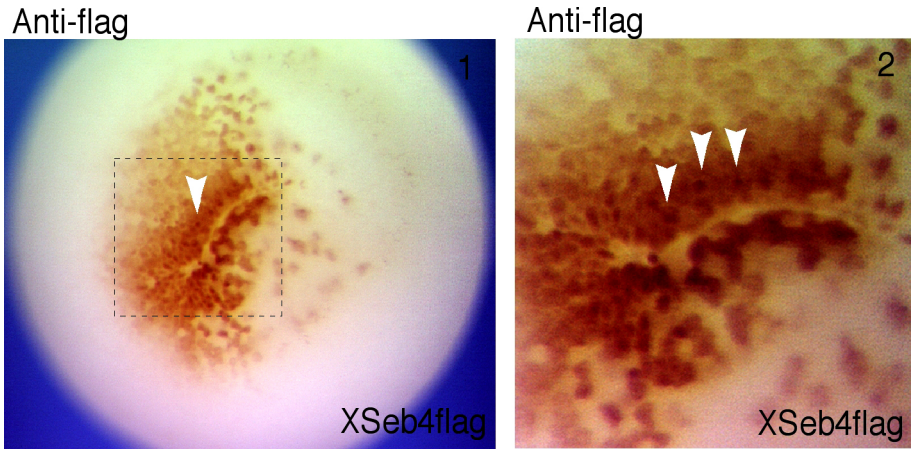

Figure 24: **XSeb4 is a nuclear localised protein**

Capped-RNA encoding XSeb4Flag-tagged was injected into one blastomere of the two-cell stage embryos and embryos were allowed to grow up to stage 9. They were fixed and analysed by immunostaining for XSeb4 translation and cellular localisation, using antiflag antibody. By this method, XSeb4 protein is detected in the nucleus. (see white arrow head), as shown in panel 1 (entire embryo) and panel 2 (high magnification).

Previous studies had demonstrated that inhibition of Delta/Notch signaling by mis-expression of the antimorphic form of X-Delta-1, X-Delta-1^{Stu} (Coffman et al., 1993; Chitnis et al., 1995), or by reducing the amount of the intracellular domain (ICD) of Notch (Lamar et al., 2001a) increase N-tubulin-expression within the territory of primary neurogenesis. As mis-expression of XSeb4 appears to mimic lateral inhibition, its suppression could induce an increased density of N-tubulin-expressing cells within the territory of primary neurogenesis. We addressed this question by producing a loss-of-function situation, targeting a specific morpholino antisense oligo (XSeb4-MO) to the endogenous XSeb4 transcripts. Our results, unexpectedly, reveal a suppression of N-tubulin expression, an inhibition of neuron formation (fig. 25). This suppression was found to be more severe at the neural plate stage (fig. 25, panel 1-2) than at tailbud stage (fig. 25, panel 3-4), indicating a reduction of the total amount of morpholino antisense oligo as development proceeds.

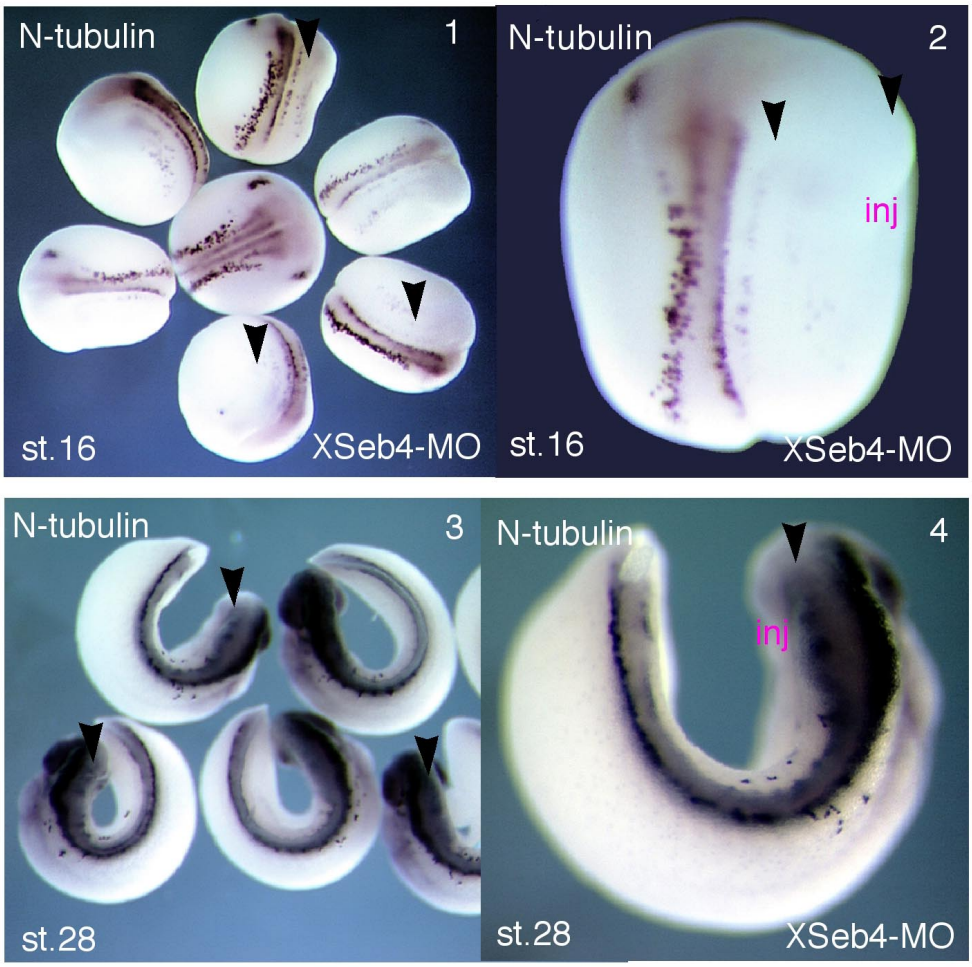

Figure 25: **XSeb4 loss-of-function inhibits neurogenesis**

Xenopus albino embryos were injected into one blastomere of the two-cell stage with 2 pmoles of XSeb4-MO, along with LacZ capped-mRNA as a tracer. They were allowed to grow to the open neural plate stage for one batch (panel 1-2) and to tailbud stage (panel 3-4) for the other. Fixed and stained for X-gal activity, embryos were analysed by wholemount *in situ* hybridization for N-tubulin expression. As shown in all the panels, microinjection of XSeb4-MO inhibits N-tubulin expression. This inhibition appears more severe at neural plate stage (panel1-2) than at tadpole stage (panel 3-4).

Taken together, these reproducible but contradictory gain- and loss-of-function experiments obtained with XSeb4, suggest that only investigations using a different approach may clairify the real function of XSeb4 during primary neurogenesis in *Xenopus*.

III DISCUSSION

III. 1 Identification of novel members of the N-tubulin and Delta synexpression groups

The amphibian frog, *Xenopus laevis*, is a suitable model system to study neurogenesis in vertebrates. At early stages of embryogenesis, the territory of primary neurons is nicely defined by a set of neural genes expressed in a typical pattern in the form of stripes within the open neural plate, which is defined by three bilateral groups of cells giving rise to motor neurons, interneurons, and sensory neurons, respectively (Chitnis et al., 1995). Niehrs and Pollet (1999) defined "synexpression groups" in eukaryotes as sets of genes that share a complex spatial expression pattern and that define a functional network. Accordingly, one subset of neural genes comprises genes whose expression pattern mimics the spatial-expression of N-tubulin. Members of this group are involved in the determination/differentiation of primary neurons. We propose the term N-tubulin synexpression group to define this subset. Another subset, designated Delta synexpression group, includes genes whose expression pattern is similar to that of X-Delta-1. Members of this group regulate the local cell-cell interactions in a process known as lateral inhibition. Overall, such patterns of expression have been a valuable criterion to screen for novel genes involved in primary neurogenesis.

In this thesis, we report the identification of XPak3, X-Mxi1, and XSeb4, three novel neuronal marker genes. The *Xenopus* homolog of the p21-activated kinase isoform 3 (XPak3) is expressed in the form of stripes in the open neural plate, similar to the N-tubulin expression pattern. The spatial expression of XPak3 correlates with sites of primary neurogenesis, as reflected by timing (late gastrula) and position in the anterior and posterior neural plate. Consistent with this, XPak3 is expressed during secondary neurogenesis in the subventricular and marginal zones, territories where one finds postmitotic neuronal cells within the neural tube.

The *Xenopus* homolog of the MAX interactor 1 (X-Mxi1) also shows, in time and space, an expression pattern similar to N-tubulin. However, X-Mxi1 expression starts very early at mid-late gastrula stage, and arises in three broad stripes, almost completely fused to each other in the open neural plate at stage 14. Moreover, X-Mxi1 is later restricted to the ventricular and subventricular zones within the neural tube. Bellefroid et al. (1996) showed, in a timing of expression analysis of neuronal markers, a correlation between the width (cell

number) of gene expression and the timing of expression. Overall, the earlier a neuronal regulator is expressed, the broader are the stripes, which means the higher is the number of cells expressing that gene. The reduction of cell number later on has been suggested to result from lateral inhibition (Chitnis and Kintner, 1996). Furthermore, the earlier a gene participates in neuronal differentiation, the nearer is its expression territory to the ventricular zone in the neural tube. Based on this analytical approach, we propose that X-Mxi1 is an early marker of primary neurogenesis belonging to the N-tubulin synexpression group

Different from the previous two genes, XSeb4, although expressed in stripes, exhibits a pattern of expression similar to X-Delta-1. Early during gastrulation, XSeb4 is expressed in the involuting mesoderm. With the particularity that the pattern is broader, this expression in the presumptive mesoderm is characteristic of Delta and other elements of lateral inhibition, including X-Notch-1 (Coffman et al., 1993), ESR (Koyano-Nakagawa et al., 2000), and Nrarp (Lamar et al., 2001a). As the neural plate forms, the expression of XSeb4 arises in three bilateral stripes in the neuroectoderm. At neurula stage 14 and later in the neural tube, cells expressing XSeb4 define a pattern that is closely related to genes involved in the Delta/Notch signaling cascade. We propose therefore that XSeb4 is a marker of primary neurogenesis belonging to the Delta synexpression group.

III. 2 XPak3 is a novel element in the proneural gene network

We have shown that XPak3 is expressed in scattered cells in areas of the neural plate where primary neurons are being born, as marked by the expression of N-tubulin. Temporally, XPak3 expression anticipates that of N-tubulin, as it starts at late gastrulation (stage 12) and is later found to be restricted to the subventricular and marginal zones of the neural tube. Conversely, XPak3 is expressed after X-Ngnr-1, whose expression starts by mid-gastrulation (stage 10.5) and is later detected only in the ventricular zone of the neural tube (Ma et al., 1996). Embryo microinjection experiments suggest that the same factors which control N-tubulin expression regulate XPak3 expression. Both genes are activated by X-Ngnr-1 and some of its downstream genes, including X-NeuroD, X-MyT1 and Xebf3. However, compared to X-Ngnr-1 or X-NeuroD, X-MyT1 alone increases XPak3-expressing cells density only within the stripes, while its combination to Xash3 promotes a strong ectopic XPak3 expression; these findings correlate with the regulation of N-tubulin expression by X-MyT1 (Bellefroid et al., 1996). Moreover, Xebf3 only weakly induces XPak3 in scattered

cells, suggesting that additional factor may also be required to result in a strong ectopic expression. Xebf3 is proposed to be the immediate upstream transcription factor of N-tubulin (Pozzoli et al., 2001). Both, XPak3 and N-tubulin encoding genes, also appear to be negatively regulated by lateral inhibition; animal cap experiments reveal that XPak3 expression is mediated via a regulatory transcription control region that is not directly responsive to lateral inhibition. Taken together, our data place XPak3 downstream of Xebf3 and upstream of N-tubulin (fig. 26).

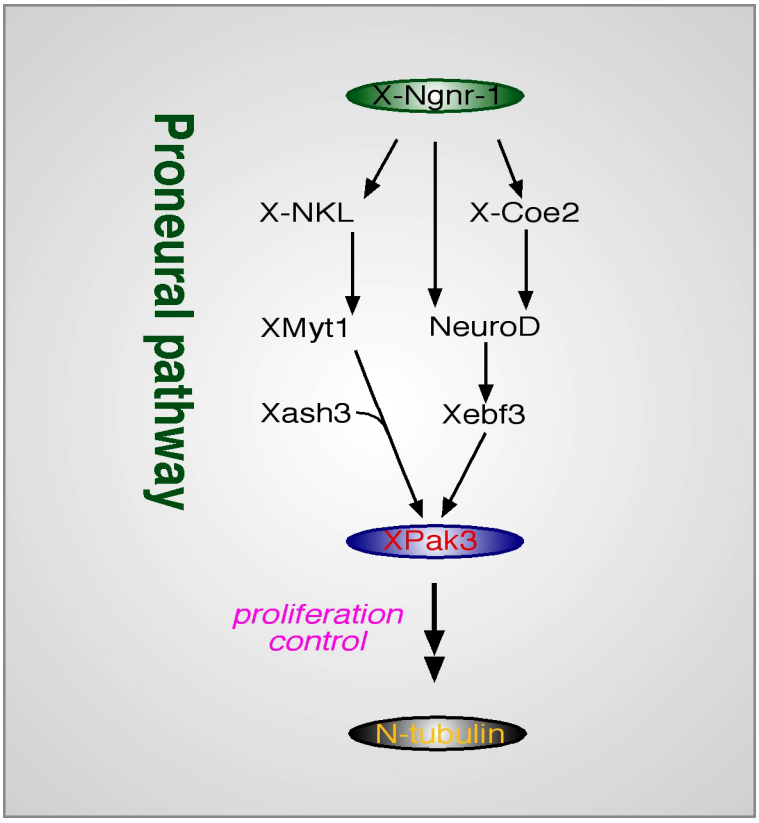

Figure 26: **XPak3 links the proneural regulators and proliferation control during primary neurogenesis in *Xenopus***

Based on embryo microinjection experiments described above, XPak3 is transcriptionally activated by X-Ngnr-1 and some of its downstream target genes. In turn, ectopic expression of the constitutively active form of XPak3 induces premature neuronal differention, a phenotype correlating with the induction of cell cycle arrest.

Results obtained upon ectopic activation of XPak3, as well as upon inhibition of XPak3 activity in *Xenopus* embryos, provide further evidence for a function of this protein in the context of primary neurogenesis. Misexpression of a constitutively active variant of XPak3 enhances and accelerates neurogenesis, whereas suppression of XPak3 reduces neurogenesis, as reflected by the effects on the expression of N-tubulin as a neuronal

differentiation marker. Interestingly, Pak3 is mutated in particular form of X-linked mental-retardations, a mutation which produces premature termination, disrupting kinase function (Allen et al., 1998), or a mutation which substitutes Arg67 for Cys, interefring with G-protein binding (Bienvenu et al., 2000). In *Drosophila*, the kinase activity of DPak, the homolog of XPak3, was found to be essential in photoreceptor axon guidance (Hing et al., 1999). In addition, a Pak-related protein from *Drosophila*, mushroom bodies tiny (MBT), was identified in a screen for viable mutations affecting adult central brain structures and is believed to play a role in the generation of neuronal cells involved in learning and memory (Melzig et al., 1998).

III. 3 XPak3 acts as cell cycle regulator in the context of primary neurogenesis

The first two *Xenopus* Pak protein isoforms, XPak1 and XPak2, were previously reported to be involved in oogenesis, regulating the G2/M phase transition during the process of oocyte maturation. Microinjection of a catalytically inactive form of XPak1, with a K/R substitution in the ATP-binding site, or of a N-terminally deleted construct, greatly facilitates oocyte release from G2/prophase arrest by progesterone and insulin (Faure et al., 1997); functionally, XPak1 appears to negatively regulate the maturation-promoting factor (MPF) (Faure et al., 1999). Similarly, XPak2 phosphorylation results in its inactivation and allows maturation to proceed to completion. Consistently, activation of mitogen-activated protein kinase and cyclin B-p34 (Cdc 2) is coincident with XPak2 inactivation, and purified active MPF inhibits XPak2 (Cau et al., 2000). Interestingly, microinjection of an activated form of Pak1 was found earlier to induce cleavage arrest of early frog embryos (Rooney et al., 1996). We report that ectopic expression of XPak3-myr induces cell cycle arrest (CCA) in a dose dependent manner, without affecting apoptosis. Importantly, this CCA phenotype results from a synergistic action of the endogenous XPak3 and the injected, constitutively active XPak3-myr. Conversely, reduction of XPak3 activity induces hyperproliferation. These findings support the notion of a general role of Pak proteins in cell cycle regulation.

In the context of neurogenesis in the retina of *Xenopus* embryos, p27Xic1, a cdk inhibitor whose ectopic expression also inhibits cell proliferation in growing embryos, promotes Müller glial cell formation from retinoblasts. Inhibition of expression of this protein by use of antisense constructs reduces the population of Müller glial cells at the expense of other neuronal cell types (Ohnuma et al., 1999). Although the regulation of p27Xic1

expression was recently shown to be dependent on XBF-1, a specific anterior neural plate winged-helix transcription factor (Hardcastle and Papalopulu, 2000), the pattern of expression of these genes doesn't correlate with primary neurogenesis. Thus, XPak3 appears to be the prime candidate that regulates cell cycle exit during the generation of primary neurons, thereby establishing a link between the proneural/neurogenic genes and proliferation control.

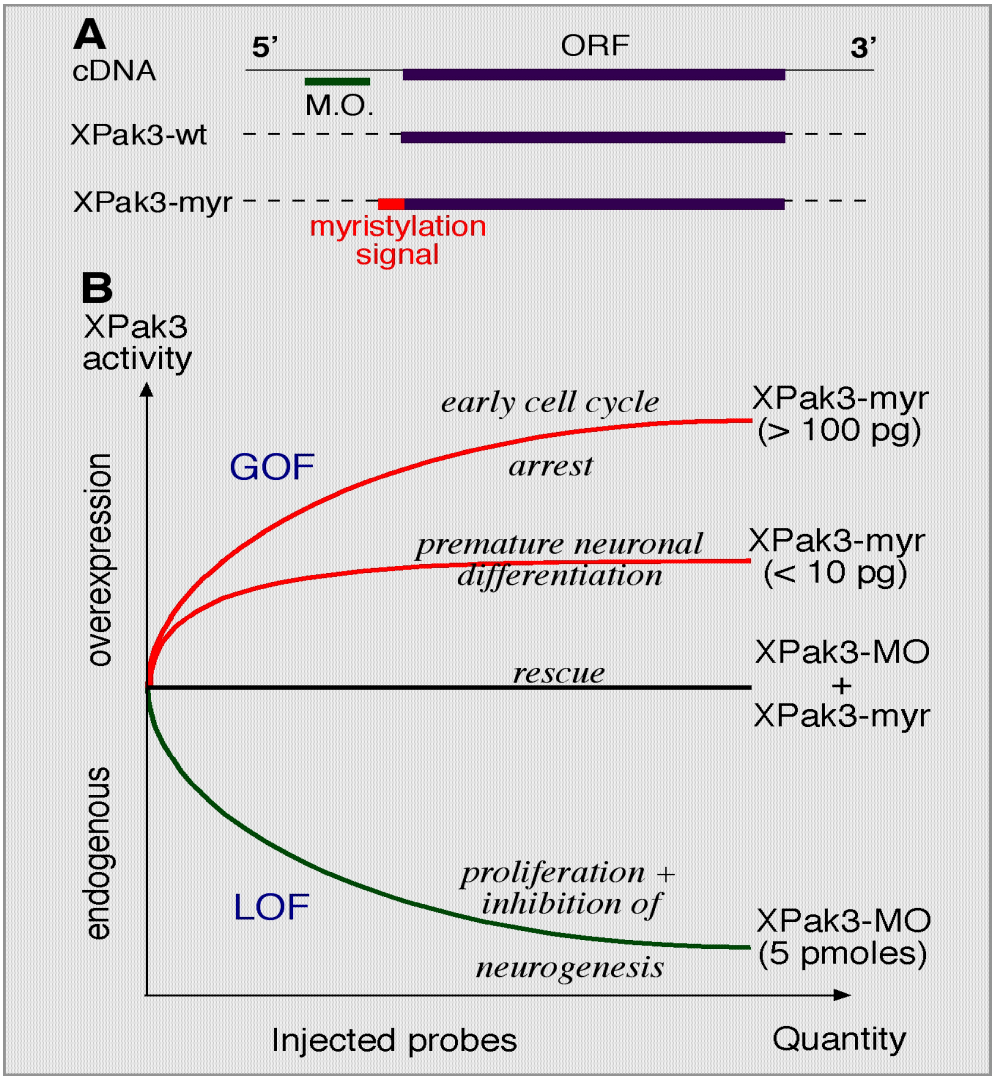

Figure 27: **Summary of XPak3 functional characteristics**

(A) Constructs used in this analysis. The constitutively active form, XPak3-myr, was generated by fusing a myristylation signal to the N-terminus of the wild type (XPak3-wt) protein. The morpholino antisense oligo (M.O.) was targeted the 5'UTR of the mRNA. The wild type and the activated XPak3 coding regions were cloned into the pCS2+ expression vector.
(B) Functional assays. Ectopic expression of XPak3-wt does not produce a phenotype. By fusing a myristylation signal to the N-terminus (XPak3-myr) XPak3 becomes constitutively activated. Its ectopic expression promotes cell cycle withdrawal and induces premature neuronal differentiation. Conversely, XPak3 loss-of-function by use of morpholino antisense oligo inhibits neurogenesis and increases cell proliferation. This inhibition of neurogenesis was rescued by XPak3-myr coinjection.

III. 4 Molecular mechanism of XPak3 activation

The enzymatic activity of Pak proteins is modulated by homomeric interactions between N- and C-terminal protein domains, as well as by other specifically interacting regulatory proteins (reviewed by Knaus and Bokoch, 1998; Lei et al., 2000; Parrini et al., 2002). The recruitment of the the Pak/Pix complex to the cell membrane by Nck (Dock) or other, as yet unidentified adaptors in response to specific receptor activation, is thought to promote GTP binding and activation of Cdc42/Rac1. Activated Cdc42/Rac1 can, in turn, bind to the CRIB motif in Pak and induce a conformational change. This displaces the autoinhibitory domain, thereby, activating Pak kinase activity (Galisteo et al., 1996; Bokoch et al., 1996; Lu et al., 1997; Manser et al., 1998; Hing et al., 1999). This complex situation seems to be reflected in our observation that ectopic expression of the wild type version of XPak3 by itself does not produce a phenotypic effect.

We also addressed the question if the kinase domain of XPak3 is required for the induction of cell cycle arrest in *Xenopus* embryos. Our experimental data reveal that both N-terminal and C-terminal deletion constructs did interfere with CCA activity. Mechanistically, it has recently been demonstrated that Pak1 forms trans-inhibited homodimers in vivo that dissociate upon GTPase binding (Parrini et al., 2002). In case of N-terminal truncations that maintain the kinase domain, it seems likely that these fragments are constitutively active because they lack the dimerisation ability. In case of C-terminal truncations that lack the kinase domain, a different mechanism of activation must apply. Such fragments might interact with the endogenous XPak3, thereby disrupting the inactive homodimers and allowing for the formation of active heterodimers that contain one copy of the endogenous wild type protein and one copy of the respective N-terminal fragment.

G-proteins are regulators of the cell cycle through the reorganisation of the cytoskeleton (reviewed by Ridley, 1995; Philips et al., 2000; Welsh et al., 2001). As an effector of G-proteins, XPak3 may mediate CCA via its G-protein binding domains. Interestingly, an internal fragment appears to act in a dominant negative fashion, as it exerts the same effects as the morpholino antisense oligonucleotide. It may either titrate out regulators of Pak kinase activity that are members of the PIX family (Manser et al., 1998) or bind and inhibit endogenous Paks, as it contains part of the autoinhibitory domain (Frost et al., 1998; Zhao et al., 1998; Zenke et al., 1999; Parrini et al., 2002). The same fragment also impairs anterior-posterior axis formation, suggesting that XPak1, which is expressed exclusively in the notochord during gastrulation, may be inhibited via the same mechanism.

III. 5 XSeb4 responds differently to activated Notch signaling in embryos and animal cap explants

When ectopically activated, X-Ngnr-1 induces the expression of a set of genes required for neuronal differentiation. At the same time, however, X-Ngnr-1 induces the expression of genes that antagonize neuronal differentiation by lateral inhibition. In this process, investigations at the molecular level have defined a genetic network in which ESRs are activated by X-Notch-1 and in turn suppress X-Ngnr-1 (Wettstein et al., 1997; Koyano-Nakagawa et al., 1999). Members of this pathway have been shown to inhibit neurogenesis through this mechanism. On the basis of its expression pattern, we classify XSeb4 as a novel member in this pathway. Consistently, XSeb4 is found to be activated by X-Ngnr-1. However, ectopic expression of ICD-Notch results in the repression of XSeb4 expression in *Xenopus* embryos. Conversely, investigations making use of the animal cap system reveal that XSeb4 behaves like other members of the Delta synexpression group; XSeb4 is transcriptionally activated by X-Ngnr-1 and also by ICD-Notch. Taken together, these two different situations in caps and embryos suggest alternative mechanisms of XSeb4 regulation.

III. 6 Ectopic expresion of XSeb4 affects neurogenesis in *Xenopus* embryos

XSeb4 expression and regulation suggest its implication in the process of lateral inhibition. To address this question experimentally, we ectopically expressed XSeb4 in developing *Xenopus* embryos. Microinjection of low amounts (<100 pg) of XSeb4 mRNA showed no effect on the transcriptional activity of N-tubulin, a neuronal specific marker. However, microinjection of high concentrations (>150 pg) downregulates the expression of N-tubulin, indicating that XSeb4 possesses a neurogenic activity. Attempts to investigate if XSeb4 functions in the neurogenic gene cascade revealed, surprisingly, that XSeb4 cannot activate known downstream targets of X-Notch-1; instead, ectopic expression of high amounts of XSeb4 suppressed both, members of the proneural and of the neurogenic pathway.

We further induced XSeb4 loss-of-function by microinjection of antisense morpholino oligonucleotides specific to the endogenous XSeb4 transcripts. Reproducible data obtained in this analysis revealed an inhibition of neurogenesis. This results again indicate that XSeb4 is involved in neuronal differentiation. But further investigations are required to resolve the apparent contradictions in respect to the true function of XSeb4 in the context of neurogenesis in *Xenopus laevis*.

IV SUMMARY

The South African claw-toed frog, *Xenopus laevis*, is a good model to study neurogenesis in vertebrates. Early on, at the open neural plate stage, the territory of primary neurons is nicely defined by a set of neural genes expressed in a typical pattern in the form of stripes which define three bilateral groups of cells giving rise to motor neurons, interneurons, and sensory neurons, respectively. One subset comprises genes whose expression pattern mimics that of N-tubulin, a neuronal differentiation marker. This subset has been refered to as N-tubulin synexpression group, thanks to the observation that members of this group are involved in the differentiation of primary neurons. Another subset, designated Delta synexpression group, includes genes whose expression pattern is similar to that of X-Delta-1. Members of this group regulate the local cell-cell interactions in the context of primary neurogenesis known as lateral inhibition. Overall, these patterns of expression are a valuable criterion to search for novel genes involved in primary neurogenesis.

We have analysed 500 clones from a *Xenopus* tailbud stage head cDNA library by use of a systematic expression pattern screen. From the panel of neurally expressed genes, five were expressed in form of stripes. Three of these genes have not been described in *Xenopus*. These genes are XPak3, a serine/threonine protein kinase, and X-Mxi1, a basic helix-loop-helix leucine zipper protein, both belonging to the N-tubulin synexpression group, and XSeb4, a RRM-type RNA binding protein, belonging to the X-Delta-1 synexpression group. Based on the fact that XPak3 and XSeb4 define regulatory molecules with unknown function in neurogenesis, their functional characterization was carried out. By embryo microinjection experiments, XPak3 and XSeb4 were found to be transcriptionally activated by the proneural factor X-Neurogenin related-1 (X-Ngnr-1) and repressed by lateral inhibition.

Comparative expression pattern analysis showed that XPak3 but not XPak1 or XPak2 is a neuronally expressed XPak isoform. Interestingly, overexpression of a constitutively active form of XPak3, XPak3-myr, induced premature neuronal differentiation, a phenotype correlating with the induction of cell cycle arrest. Conversely, XPak3 loss-of-function, generated by using a morpholino antisense oligonucleotide, blocked the formation of primary neurons and induced increased cell proliferation. This inhibition of neurogenesis was rescued by coinjection of XPak3-myr. In conclusion, we propose that XPak3 is induced by neurogenin to inhibit the mitosis of neuronally programmed cells and thereby allowing for their differentiation.

Ectopic expression of high concentrations (>150 pg) of XSeb4 inhibits the formation of primary neurons. Unexpectedly, the suppression of XSeb4 expression, using morpholino antisense oligonucleotide, also led to inhibition of neurogenesis. Microinjection of low concentrations of XSeb4 mRNA showed no effect on the expression of N-tubulin. These contradictory findings require further systematic investigations.

V ZUSAMMENFASSUNG

Der Südafrikanische Krallenfrosch *Xenopus laevis* ist ein gutes System für das Studium der Neurogenese in Vertebraten. Bereits früh, im offenen Neuralplattenstadium, wird das Gebiet der primären Neuronen durch eine Gruppe von neuralen Genen definiert, die in einem typischen Muster in Form von Streifen exprimiert werden und die drei bilaterale Zellgruppen definieren, aus denen jeweils Motorneuronen, Interneuronen und sensorische Neuronen hervorgehen. Eine Untergruppe enthält Gene, deren Expressionsmuster dem von N-tubulin, einem neuronalen Differenzierungsmarker, entspricht. Diese Untergruppe wird aufgrund der Beobachtung, dass Mitglieder dieser Gruppe bei der Differenzierung von primären Neuronen eine Funktion besitzen, als N-tubulin Synexpressionsgruppe bezeichnet. Eine andere Untergruppe, die als Delta Synexpressionsgruppe bezeichnet wird, beinhaltet Gene, deren Expressionsmuster dem von X-Delta-1 ähnelt. Mitglieder dieser Gruppe regulieren die lokalen Zell-Zell-Interaktionen, im Zusammenhang mit der primären Neurogenese bekannt als laterale Hemmung. Insgesamt sind solche Expressionsmuster ein wertvolles Kriterium für die Suche nach neuen Genen, die bei der primären Neurogenese eine Funktion besitzen.

Wir haben 500 Klone aus einer Xenopus Schwanzknospenstadium Kopf cDNA Bank durch Verwendung einer systematischen Expressionsmusteranalyse untersucht. Aus der Fülle der neural exprimierten Gene werden fünf in Form von Streifen exprimiert. Drei dieser Gene sind in Xenopus bisher nicht beschrieben worden. Diese beeinhalten XPak3, eine Serin/Threonin Proteinkinase, und X-Mxi1, ein basisches Helix-Loop-Helix Leucin Zipper Protein, die beide zur N-tubulin Synexpressionsgruppe gehören, sowie XSeb4, ein RRM-Typ RNA bindendes Protein, das zur Delta Synexpressionsgruppe gehört. Basierend auf der Tatsache, dass XPak3 und XSeb4 regulatorische Moleküle mit unbekannter Funktion bei der Neurogenese definieren, wurde ihre funktionelle Charakterisierung durchgeführt. Durch Mikroinjektionsexperimente in Embryonen wurde gefunden, dass XPak3 und XSeb4 auf Transkriptionsebene durch den proneuralen Faktor X-Neurogenin related-1 (X-Ngnr-1) aktiviert und durch laterale Hemmung reprimiert wurden.

Eine vergleichende Expressionsmusteranalyse zeigte, dass XPak3, aber nicht XPak1 und XPak2, eine neuronal exprimierte XPak Isoform ist. Interessanterweise induzierte die Überexpression einer konstitutiv aktiven Form von XPak3, XPak3-myr, vorzeitige neuronale Differenzierung, ein Phänotyp, der mit der Induktion von Zellzyklusarretierung korreliert. Umgekehrt blockierte der XPak3 Funktionsverlust, der durch Verwendung eines Morpholino-antisense-Oligonukleotids erzeugt wurde, die Bildung von primären Neuronen und induzierte eine gesteigerte Zellproliferation. Diese Hemmung der Neurogenese wurde durch Koinjektion von XPak3-myr wieder aufgehoben. Schlussfolgernd schlagen wir vor, dass XPak3 von Neurogenin induziert wird, um die Mitose neuronal programmierter Zellen zu inhibieren und so deren Differenzierung zu erlauben.

Ektopische Expression hoher Konzentrationen (>150 pg) von XSeb4 hemmt die Bildung primärer Neuronen. Unerwarteterweise führte die Unterdrückung der XSeb4 Expression durch die Verwendung eines Morpholino-antisense-Oligonukleotids ebenfalls zur Hemmung der Neurogenese. Die Mikroinjektion niedriger Konzentrationen von XSeb4 mRNA zeigte keinen Effekt auf die Expression von N-tubulin. Diese widersprüchlichen Befunde erfordern weitere systematische Untersuchungen.

VI. MATERIALS AND METHODS

VI. 1 Materials

VI. 1. 1 Chemicals

Acetic anhydride	(Sigma)
10 X PCR buffer	(Perkin – Elmer)
Acetic acid	(Merck)
Acetone	(Merck)
Agar	(Difco)
Agarose	(Gibco - BRL)
Ammonium Persulfate	(Serva)
Ampicillin	(Biomol)
Bacto Trypton	(Difco)
Bacto Yeast Extract	(Difco)
BCIP	(Boehringer Mannheim)
Benzyl alcohol	(Sigma)
Boehringer block	(Sigma)
Boric acid	(Merck)
Bovine Albumin	(Sigma)
BSA	(Sigma)
$Ca(NO_3)_2$	(Merck)
$CaCl_2$	(Merck)
CHAPS	(Sigma)
Chloroform	(Merck)
DAB	(Pharmacia)
Dextran sulfate	(Merck)
DMSO	(Merck)
DTT	(Gibco)
EDTA	(Paesel & Lorei)
EGTA	(Sigma)
Entelan	(Merck)
Ethanol	(Merck)
Ethidium Bromide	(Roth)
Ficoll	(Sigma)
Ficoll 400	(Pharmacia)
Formaldehyde	(Merck)
Formamid	(Merck)
Gelatin	(Sigma)
Glutaraldehyde	(Sigma)
Glycerol	(Merck)
Glycogen, Molecular Biology grade	(Boehringer Mannherim)
Goat Serum	Gibco – BRL
H_2O_2	(Baker), (ROTH, N^r 2014)
HCl	(Merck)
Heparin	(Sigma)
HEPES	(Sigma)
Human Chorionic gonadotropin (HCG)	(Sigma)
Isopropanol	(Merck)
$K_3Fe(CN)_6$	(Sigma)

$K_4Fe(CN)_6$	(Sigma)
KCl	(Merck)
L-Cysteinhydrochloride	(Fluka)
LiCl	(Sigma)
Methanol	(Merck)
$MgSO_4$	(Merck)
MOPS	(Sigma)
Mowiol	(Calbiochem)
Na acetate	(Roth)
Na citrate	(Fluka)
Na_2HPO_4	(Merck)
NaCl	(Merck)
NaH_2PO_4	(Merck)
$NaHCO_3$	(Merck)
NaN_3	(Sigma)
NaOH	(Merck)
NBT	(Boehringer Mannherim)
NH_4 acetate	(Merck)
Nile blue chloride	(Sigma)
Penicillin	(Sigma)
Phenol	(Merck)
SDS	(Biomol)
(^{35}S)Met	(Amersham)
ß-mercaptoethanol	(Sigma)
Streptomycin	(Sigma)
Sucrose	(BRL)
Technovit 7100	(HeraeusKulzer)
TEMED	(Serva)
Triethanolamine	(Sigma)
Tris	Paesel & Lorei)
Triton-X-100	(Sigma)
Tween-20	(Sigma)
Urea	(Pharmacia)

VI. 1. 2 Solutions, buffers and media

VI. 1. 2. 1 Embryos and explants

L-Cystein hydrochloride 2 %

L-Cystein hydrochloride 1 X H_2O	**:** 10 g
dH_2O	**:** to 500 ml
pH	**:** 7.8 – 8.0

Ficoll 10 %

Ficoll	**:** 10 g
ddH_2O	**:** to 100 ml

Filter through 45µl filter and store at 4 °C.

Human chorionic gonadotropin (HCG) 10.000 U

Resuspend in 5 ml dH_2O to make a stock of 2000 U/ml, aliquote in fractions of 1 ml each, and store at – 20 °C.

Injection buffer

5 X MBS	: 100ml (1 X)
Ficoll 10 %	: 50 ml (1 %)
ddH_2O	: to 500 ml

MBS (Modified Barth Solution) 1 X

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

MBS (Modified Barth Solution) 5 X

NaCl, l5 M	: 88 ml (440 mM)
$NaHCO_3$ 1 M	: 12 ml (12 mM)
KCl, 1M	: 5 ml (5 mM)
HEPES, 1 M, pH 7.0	: 50 ml (50 mM)
$MgSO_4$, 1 M	: 4.1 ml (4.1 mM)
$CaCl_2$, 1 M	: 2.05 ml (2.05 mM)
$Ca(NO_3)_2$, 1 M	: 1.65 ml (1.65 mM)
dH_2O	: to 1 l
pH	: 7.4

Penicillin / Streptomycin

Penicillin	: 100 U / ml
Streptomycin	: 10 mg / ml

Working concentration : Dilute 1 / 1000

Postinjection buffer

5 X MBS	: 10 ml (0.1 X)
Ficoll 10 %	: 25 ml (0.5 %)
dH_2O	: to 500 ml

VI. 1. 2. 2 Wholemount *in situ* hybridization

Alkaline Phosphatase Buffer (APB)

Tris-HCl 1 M, pH 9.5	: 50 ml (100 mM)
$MgCl_2$ 1 M	: 25 ml (50 mM)
NaCl 5 M	: 10 ml (100 mM)
Tween-20, 20 %	: 2.5 ml (0.1 %)
dH_2O	: to 500 ml

Bleaching solution

H_2O_2 (ROTH)	**:** 1.67 ml (1 %)
Formamid	**:** 25 ml (50 %)
5X SSC	**:** to 50 ml

Boehringer Block (BMB) 10 % (autoclaved)

5 X MAB	**:** 20 ml (1 X)
BMB*	**:** 10 g (10 %)
dH_2O	**:** to 100 ml

Takes about 1 h at 60 °C to dissolve.
Autoclave and store at – 20 °C.

Denhart´s 100 X solution

BSA	**:** 2 g (2 %)
PVP	**:** 2 g (2 %)
Ficoll 400	**:** 2 g (2 %)
dH_2O	**:** to 100 ml

Store at – 20 °C.

Hybridization solution

Deionized formamid*	**:** 50 ml (50 %)
20 X SSC	**:** 5 ml (5 X)
Torula RNA 50 mg / ml	**:** 2 ml (1 mg / ml)
Heparin 10 mg / ml	**:** 1 ml (100µg / ml)
Denhart´s 100 X	**:** 1 ml (1 X)
Tween-20, 20 %	**:** 0.5 ml (0.1 %)
CHAPS 10 %	**:** 1 ml (0.1 %)
EDTA 0.5 M	**:** 2 ml (10 mM)
DEPC dH_2O	**:** to 100 ml

Store at – 20 °C.
***** To deionize formamid: Add 50 g of mixed bead resin (BioRad) to 500 ml formamid, mix on magnetic stirrer for 2 h and filter on Whatman paper. Reuse reusin.

Heparin 10 mg / ml

Heparin	**:** 100 mg (10 mg / ml)
DEPC dH_2O	**:** to 10 ml

Store at – 20 °C.

HEPES buffer pH 7.5 (autoclaved)

HEPES	**:** 238.3 g (1M)
dH_2O	**:** to 1 l
pH	**:** 7.5

Autoclave.

5 X MAB solution (autoclaved)

Maleic acid	:	29 g (500 mM)
NaCl	:	21 g (750mM)
dH_2O	:	to 500 ml
pH	:	7.5

Autoclave.

$MgCl_2$, 1 M

$MgCl_2$, 6 H2O	:	203.3 g (1 M)
dH_2O	:	to 1 l

5 X NaCl (autoclaved)

NaCl	:	292.2 g (5 M)
dH_2O	:	to 1liter

Autoclave.

1 X PTw

10 X PBS	:	50 ml (1 X)
Tween-20, 20 %	:	2.5 ml (0.1 %)
dH_2O	:	to 500 ml

PTw / Proteinase K solution

PTw	:	20 ml
Proteinase K 20 mg / ml	:	20 µl

2 X SSC / RNase A and RNase T1 solution

20 X SSC	:	1 ml (2 X)
RNase A 10 mg / ml	:	20 µl (20 µg / ml)
RNase T1 20,000 U / ml	:	5 µl (10 U / ml)
dH_2O	:	to 10 ml

20 X SSC (autoclaved)

NaCl	:	175.3 g (0.3 M)
Sodium citrate	:	88.2 g (0.3 M)
dH_2O	:	to 1 l
pH	:	7.0

Staining solution : NBT / BCIP

APB	:	1 ml
NBT 75 mg / ml in 70 % dimethylformamide	:	2 µl
BCIP 50 mg / ml in 100 % dimethylformamide	:	3,5 µl

Torula RNA 10 mg / ml

Torula RNA	:	100 mg (10 mg / ml)
DEPC - dH_2O	:	to 10 ml

Store at – 20 °C.

Tris buffer, pH 9.5 (autoclaved)

Tris-HCl	**:** 121.1 g (1 M)
dH_2O	**:** to 1 l
pH	**:** to 9.5

Tween – 20, 20 %

Tween – 20	**:** 20 ml (20 %)
dH_2O	**:** to 100 ml

Store at – 20 °C.

VI. 1. 2. 3 Histology and immune whole mount staining

DAB 10 mg / ml solution

DAB	**:** 100 mg (10 mg / ml)
dH_2O	**:** to 10 ml

Store at – 20 °C.

Dent´s fixative

DMSO	**:** 20 ml (20 %)
Methanol	**:** 80 ml (80 %)

Gelatin – albumin medium

10 X PBS	**:** 45 ml (1X)
Gelatin	**:** 2.2 g
dH_2O	**:** to 450 ml

Stir under heating at 60 °C, cool down to room temperature and add:

Albumin	**:** 135 g
Sucrose	**:** 90 g

Filter through 45 µm filter and store at – 20 °C.
For embedding, add 105 µl of 25 % Glutaraldehyde to 1.5 ml of Gelatin-albumin medium.

$K_3Fe(CN)_6$ solution

$K_3Fe(CN)_6$	**:** 8.23 g (500 mM)
dH_2O	**:** to 50 ml

$K_4Fe(CN)_6$ solution

$K_4Fe(CN)_6$, 3 H_2O	**:** 10.56 g (500 mM)
dH_2O	**:** to 50 ml

10 X MEM (autoclaved)

MOPS	**:** 209.3 g (1 M)
EGTA 0.5 M	**:** 20 ml (20 mM)
$MgSO_4$ 1 M	**:** 5 ml (10 mM)
dH_2O	**:** to 500 ml

Autoclave

1 X MEMFA

10 X MEM	: 10 ml (1 X)
Formaldehyde 37 %	: 10 ml (3.7 %)
dH_2O	: to 100 ml

Mowiol solution

Mowiol	: 9.6 g
Glycerine	: 19.35 ml
dH_2O	: 24 ml
Tris 0.2 M, pH 8.5	: 48 ml

Stir under heating at 50 °C, centrifuge at 5000 rpm for 15 min and store at – 20 °C.

PBT solution

10 X PBS	: 10 ml (1 X)
BSA	: 200 mg (2 mg / ml
Triton-X-100, 20 %	: 0.5 ml (0.1 %)
dH_2O	: to 100 ml

X-Gal solution

X-Gal	: 1 g (40 mg / ml)
DMSO	: to 25 ml

Store at – 20 °C

X-Gal staining solution

10 X PBS	: 46.750 ml
X-Gal 40 mg / ml in DMSO	: 1.250 ml (1 mg / ml)
$K_3Fe(CN)_6$ 500 mM	: 0.5 ml (5 mM)
$K_4Fe(CN)_6$ 500 mM	: 0.5 ml (5 mM)
$MgCl_2$ 100 mM	: 1 ml (2 mM)

VI. 1. 2. 4 Molecular Biology

VI. 1. 2. 4. 1 SDS – PAGE

30 % Acrylamide stock solution

Acrylamide	: 29.2 % (w / v)
Bis-acrylamide	: 0.8 % (w / v)

10 % Ammonium persulfate (APS) solution

Ammonium persulfate	: 10 g (10 %)
dH_2O	: to 100 ml

2 X Laemmli loading buffer

Tris-HCl	: 0.1 M, pH 6.8
SDS	: 2 % (w / v)
Glycerol	: 20 % (w / v)
Bromphenol blue	: 0.002 % (W / v)

VI. 1. 2. 4. 2 Agarose gel electrophoresis of DNA

Ficoll loading buffer

Tris-HCl 1 M, pH 7.5	:	0.5 ml (10 mM)
EDTA 0.5 M	:	0.1 ml (1 mM)
Bromphenol blue	:	0.025 %
Xylencyanol	:	0.025 %
Ficoll 400	:	5 g (10 %)
dH_2O	:	to 50 ml

Glycerol loading buffer

Tris-HCl 1 M, pH 7.5	:	0.5 ml (10 mM)
EDTA 0.5 M, pH 8	:	1 ml (10 mM)
Bromphenol blue	:	0.025 %
Xylencyanol	:	0.025 %
Glycerol 99 %	:	15.15 ml (30 %)
dH_2O	:	to 50 ml

Formamid loading buffer

EDTA 0.5 M, pH 8	:	0.2 ml (10 mM)
Bromphenol blue	:	0.025 %
Xylene cyanol FF	:	0.025 %
Formamid 99 %	:	to 10 ml

10 X TBE buffer

Tris	:	108 g (0.89 M)
Boric acid	:	55 g (0.89 M)
EDTA 0.5 M, pH 8	:	40 ml (20 mM)
dH_2O	:	to 1 l

VI. 1. 2. 4. 3 Plasmid DNA preparation

TELT buffer

Tris-HCl 1 M, pH 7.5	:	2.5 ml (50 mM)
EDTA 0.5 M, pH 8	:	0.1 ml (1 mM)
LiCl, 10 M	:	16 ml (3.2 M)
Triton-X-100, 20 %	:	1.25 ml (0.5 %)
dH_2O	:	to 50 ml

TE buffer

Tris-HCl 1 M, pH 7.5	:	1 ml (10 mM)
EDTA 0.5 M	:	200 µl (1 mM)
dH_2O	:	to 100 ml
pH	:	8

Lysozym solution

Lysozym	:	100 mg (10 mg / ml)
dH_2O	:	to 10 ml

Store at – 20 °C

Tris buffer (autoclaved)

Tris-HCl	: 121.1 g (1 M)
dH2O	: to 1 l
pH	: to 7.5

Triton-X-100, 20 % solution

Triton-X-100	: 20 ml (20 %)
dH_2O	: to 100 ml

VI. 1. 2. 4. 4 Frequently used buffers and solutions

EDTA solution

$Na_2EDTA, 2H_2O$	: 186.12 g (0.5 M)
dH_2O	: to 1 l
pH	: 8

EGTA solution

EGTA	: 190 g (0.5 M)
dH_2O	: to 1 l
pH	: 8

MgSO4 1 M (autoclaved)

$MgSO_4, 7\ H_2O$	: 246.48 g (1 M)
dH_2O	: to 1 l
Autoclave.	

NaPhosphate 1 M

$NaH_2PO_4, 1\ H_2O$	: 137.9 g (1 M)
dH_2O	: to 1 l

Na2Phosphate 1 M

$Na_2HPO_4, 12\ H_2O$	: 358.14 g (1 M)
dH_2O	: to 1 l

Sodium Phosphate buffer

NaH_2PO_4, 1 M	: 15 ml
Na_2HPO_4, 1 M	: 84.5 ml
pH	: 7.5

10 X Phosphate buffered saline buffer

$Na_2HPO_4, 12\ H_2O$	: 36.3 g (0.1 M)
KH_2PO_4	: 2.4 g (20 mM)
NaCl	: 80 g (1.4 M)
KCl	: 2 g (28 mM)
dH_2O	: to 1 l

Sodium acetate 3 M

Sodium acetate, 3 H_2O	: 40.81 g (3 M)
dH_2O	: to 100 ml
pH	: 5.2

SDS 10 %

SDS	: 10 g (10 % w / v)
dH_2O	: to 100 ml

SM buffer

Tris-HCl 1 M, pH 7.5	: 50 ml (10 mM)
$MgSO_4$ 1 M	: 8 ml (8 mM)
NaCl 5 M	: 20 ml (100 mM)
Gelatin 2 %	: 5 ml (0,01 %)
dH_2O	: to 1 l

20 X SSPE (autoclaved)

NaCl	: 174 g (3.6 M)
NaH_2PO_4, 1 H_2O	: 27.6 g (0.2 M)
EDTA	: 7.4 g (20 mM)
dH_2O	: to 1 l
pH	: to 7.4

X-Gal solution

X-Gal	: 0.5 g (20 mg / ml)
Dimethylformamide	: to 25 ml

Isopropylthiogalactoside (IPTG) solution

IPTG	: 238.3 mg (100 mM)
dH_2O	: to 10 ml

Store at – 20 °C

VI. 1 . 2. 4. 5 Media and antibiotics

All the media were autoclaved for at least 15 min at 120 °C, under 1.5 bar.

LB – medium

Bacto Trypton	: 10 g (1 %)
Bacto Yeast Extract	: 5 g (0.5 %)
NaCl	: 10 g (17.1 mM)
dH_2O	: to 1 l
pH	: to 7.5

LB –Agar medium

Bacto Trypton	: 10 g (1 %)
Bacto Yeast Extract	: 5 g (0.5 %)
NaCl	: 10 g (17.1 mM)
Agar	: 15 g (1.5 %)
dH_2O	: to 1 l
pH	: to 7.5

Tetracyclin stock solution

Tetracyclin	: 250 mg (25 mg / ml)
Ethanol 100 %	: to 10 ml
Store at – 20 °C	
Working concentration	: 12.5 µg / ml

Ampicillin stock solution

Ampicillin	: 1000 mg (100 mg / ml)
dH_2O	: to 10 ml
Store at – 20 °C	
Working concentration	: 75 µg / ml

VI. 1. 2. 4. 6 RNA isolation

Diethylpyrocarbonat (DEPC) – dH_2O (autoclaved)

Diethylpyrocarbonat	: 0.5 ml (0.1 %)
dH_2O	: 500 ml

Incubate 2 h at 37 °C and autoclave.

2 X lysis buffer

SDS 20 %	: 5 ml (1 %)
EDTA 0.5 M, pH 8.0	: 2 ml (10 mM)
Tris – HCl 1 M, pH 7.5	: 10 ml (100 mM)
NaCl 5 M	: 2 ml (100 mM)

Lysis buffer

2 X lysis buffer	: 5 ml
DEPC – dH_2O	: 4.5 ml
Proteinase K* (20 µg / µl)	: 0.5 ml

*Added immediately before use.

10 X DNase buffer

Tris – HCl	: 400 mM, pH 7.9
NaCl	: 100 mM
$MgCl_2$	: 60 mM
$CaCl_2$	: 1 mM

DNase I digestion mix

10 X DNase buffer	: 5 µl
DTT 20 mM	: 2.5 µl
RNasin (40 U / µl)	: 1 µl
DNase I (10 U / µl)	: 1.5 µl
RNA sample	: 40 µl

Phenol – Chloroform mix

Ratio	: 1 : 1 (v / v)
pH	: 8 – 8.3

Ammonium acetate (NH_4Ac) 7.5 M

NH_4Ac	: 57.8 g (7.5 M)
dH_2O	: to 100 ml

VI. 1. 3 Bacterial strains and vectors

Escherichia coli Xl1-Blue (Stratagene); *Escherichia coli* TG1 (Stratagene); *Escherichia coli* XLOLR (Stratagene); were used in this study. The following vectors were used: *pBluescriptKS* (Stratagene); *pGEM-T* (Promega); *pBSRN3* (Lemaire et *al*., 1995); *pCS2; pCS2+Flag, MycTag* (Turner and Weintraub, 1994); *pCS2+Myr; pCS2+Myr/Flag*.

VI. 1. 4 Plasmid constructs

Below are listed the constructs used for RNA synthesis in this study. The restriction enzymes, used for DNA linearization, and the RNA polymerases used for *in vitro* transcription are indicated:

For the Wholemount *in situ* hybridization

Constructs	Restriction enzyme	RNA polymerase
XSeb4 (in CMV, clone JS124)	EcoRI	T7
XPak3 (in CMV, clone JS464)	EcoRI	T7
X-Mxi1 (in CMV, clone JS097)	EcoRI	T7
RBP erlC (in CMV, clone JS4.3)	EcoRI	T7
Tau protein (In CMV, clone JS5.22)	EcoRI	T7
N-tubulin (in CMV, clone JS5.42)	EcoRI	T7
X-Delta-1 (1F6)	XhoI	T7
X-Ngnr-1	BamHI	T3
X-MyT1 (1F5)	ClaI	T7
ESRI (Ac AF146088)	EcoRI	T7
ESRII (clone 8C9)	Sal I	T7
XPak1-RD (in pGEM T)	NotI	T7
XPak2-RD (in pGEM T)	NotI	T7
XAG (in CMV, clone JS003)	EcoRI	T7

For embryo microinjection

Constructs	Restriction enzyme	RNA polymerase
ß-galactosidase (1D1, pCS2+)	NotI	Sp6 (Ambion kit)
X-Ngnr-1 (1F2)	NotI	Sp6 (Ambion kit)
ICD-Notch (1E1)	NotI	Sp6 (Ambion kit)
NeuroD	NotI	Sp6 (Ambion kit)
XMyT1	NotI	Sp6 (Ambion kit)
Xash3	NotI	Sp6 (Ambion kit)
Xebf3	NotI	Sp6 (Ambion kit)
Noggin	NotI	Sp6 (Ambion kit)
XPak3 in pCS2+	NotI	Sp6 (Ambion kit)
XPak3 in pCS2+myr	NotI	Sp6 (Ambion kit)
XPak3 in pCS2+myr-Flag	NotI	Sp6 (Ambion kit)
XPak3-RD in pCS2+myr	NotI	Sp6 (Ambion kit)
XPak3-KD in pCS2+myr	NotI	Sp6 (Ambion kit)
XPak3-ED/Pix in pCS2+myr	NotI	Sp6 (Ambion kit)
XPak3-Ntle in pCS2+myr	NotI	Sp6 (Ambion kit)
XPak3-Ntle deleted in pCS2+myr	NotI	Sp6 (Ambion kit)
XPak3-KD/Ctle deleted in pCS2+myr	NotI	Sp6 (Ambion kit)
XSeb4 in pCS2+	NotI	Sp6 (Ambion kit)
XSeb4 in pCS2+Flag	NotI	Sp6 (Ambion kit)
XSeb4 in pCS2+Myc	NotI	Sp6 (Ambion kit)

VI. 1. 5 Animals

Wild-type and albino frogs, *Xenopus laevis.*

VI. 1. 6 Antibodies

Anti-digoxigenin-alkaline phosphatase conjugated (Roche Biochemicals)
Anti-flureseeine-alkaline phosphatase conjugated (Roche Biochemicals)
Anti-PCNA monoclonal antibody; PCNA (C10) sc-56 (Santa Cruz Biotechnology)
Anti-mouse alkaline phosphatase conjugated (Sigma)
Antiflag monoclonal antibody (Sigma)

VI. 1. 7 Enzymes

Restriction enzymes (with supplied buffers)	(NEB, Promega)
Proteinase K	(Merck)
RNase A	(Worthington)
RNase T1	(Sigma)
RNasin (Ribonuclease Inhibitor)	(Promega)
RNase – free DNase I (2000 U / ml)	(Ambion)
Taq polymerase	(Perkin Elmer)
T4 DNA ligase	(Promega)
SP6, T3 and T7 RNA polymerases	(Stratagene)
T4 Polynucleotide kinase	(Boehringer Mannheim)
Terminal Deoxynucleotidyl Transferase (TdT)	(GibcoBRL)

VI. 1. 8 Others

Salmon sperm DNA	(Sigma)
RNA molecular weight markers	(Gibco BRL)
1 kb molecular weight marker mix	(Gibco BRL)

VI. 1. 9 Computers and software

- **Computer** : Apple Macintosh
- **Computer progams** :
  - Adope Photoshop 5.0;
  - DNA-Star software;
  - Free Hand 9.0;
  - Image Quant 2.0;
  - MS Win Word 6.0.
  - OW 98 PowerPoint

VI. 1. 10 Nucleic acids

VI. 1. 10. 1 Nucleotides

(γ-^{32}P)dATP 10 mCi / ml	(Amersham)
dNTPs	(Biomol)
Fluorescein – 12 – UTP	(Boerhinger)
Digoxigenin – 11 - UTP	(Boerhinger)
rNTP solutions (10 mM)	Stratagene
RNA cap structure m7G(5´)ppp(5´)G, sodium salt	(New EnglandBiolabs)
Digoxigenin – 11 - dUTP	(Roche Biochemicals)

VI. 1. 10. 2 Oligonucleotides

For the sequencing: pBRCMV-F 5'-CGCGCCTGCAGGTCGACACTA-3'
pBRCMV-R 5'-GCAAGGCGATTAAGTTGGGTA-3'

XPak3:	JS464-F1	5'-CCTCCACTCCCTACAGAAGA-3'
	JS464-F2	5'-GGTGGTTCTCTGACAGATGT-3'
	JS464-F3	5'-CGACTGTCAACACCAACTAC-3'
	JS464-F4	5'-CGCTGAATGCAAAGACTGCA-3'
	JS464-F5	5'-CCTGAACTTCAGAACCCAGA-3'
	JS464-F6	5'-ACGAGATCTTGGTCATGAGG-3'
	JS464-F7	5'-TGAACACACCATCCACGTTG-3'
	JS464-R1	5'-CCTGTGCTGAAACAGACAAC-3'
	JS464-R2	5'-GCATGCGTTCTGCTGGAATC-3'
	JS464-R3	5'-CCTGTCCACATCCATCTCTA-3'
	JS464-R4	5'-CATCTGTCAGAGAACCACCA-3'
	JS464-R5	5'-CTGTATTGGTCCAGAGAGCC-3'
XSeb4:	JS124-F1	5'-GTGACTATGTCAGACCGAGC-3'
	JS124-F2	5'-GGATTCCAGGCAAGAGGAGA-3'
	JS124-R1	5'-GCAGAGTTGAGGCAAAGAGT-3'

	JS124-R2	5'-GTGTAACTGCTGGACTCCTA-3'
XMxi1:	JS097-F1	5'-GGATGATCAGAGCAGGCAGAG-3'
	JS097-F2	5'-GTCCTCTCAGTCTCCTACTG-3'
	JS097-R1	5'-CGCATCAGAGCAACAACTACGGAAC-3'
	JS097-R2	5'-CAGTGGGAGACAGTCACTGT-3'
	JS097-R3	5'-GAGCTTGCACCATGATGTGA-3'
	JS40.97-F1	5'-CCACTGGTCTCAATGTGAAC-3'
	JS40.97-R1	5'-GTCACTGTGATGTCACCATG-3'

For the cloning:

To engineer the pCS2+myr and pCS2+myr/Flag, the following primers were used:

Myr-F 5'-GAC**CAATTG**CCATGGGTTCATCGAAGTCCAAGCCAAAGGACCCATC-3'
Myr-R 5'-CAA**GAATTC**CGTCGTCGTTGCGATGGGTCCTTTGGCTTGGACTTCG-3'
Flag-F 5'-**AATTG**TATGGACTACAAGGACGACGATGACAAGAATTC-3'
Flag-R 5'-TCGA**GAATTC**TTGTCATCGTCGTCCTTGTAGTCCATAC-3'

To subclone the ORF of XPak3 and deleted fragments, and XSeb4, the following primers were used:
XPak3-MfeI 5'-CGC**CAATTG**CAAGATGTCCGATAGTGTGGATATTG-3'
XPak3-XhoI 5'-ATGT**CTCGAG**CTAGCGGCTGCTATTCTTAATGGC-3'
KD-MfeI 5'-CTC**CAATTG**CCAGCCAGAAAACTCAAACTCAAGC-3'
RD-XhoI 5'-ATGT**CTCGAG**CTAGTCGGTGTTCCTATACAAAG-3'
Other XPak3 deletion constructs were generated by restriction digestions using EcoRI.

XSeb4-EcoRI 5-ACA**GAATTC**GGCCATGCACACCGTGCAAAAAGAC-3'
XSeb4-XhoI 5'-AAGTT**CTCGAG**TTTTACTGCATCCGGTCAGGCTG-3'

For the loss-of-function:

XPak3 antisense morpholino oligo. 5'-CAGCACTCCTCACAGCCTCCTGGC-3'
XSeb4 antisense morpholino oligo. 5'-GTGCATGGTCACAGGCAAATTCACC-3'

For the RT-PCR:

N-tubulin-F	5'-ACACGGCATTGATCCTACAG-3'
N-tubulin-R	5'-AGCTCCTTCGGTGTAATGAC-3'
ESR7-F	5'-GAAATCCCTCATAACAATGACATC-3'
ESR7-R	5'-CTACCAATGCCATTTTCAGGTTTG-3'
Histone H4-F	5'-CGGGATAACATTCAGGGTATCACT-3'
Histone H4-R	5'-ATCCATGGCGGTAACTGTCTTCCT-3'
Cardiac actin-F	5'-TCCCTGTACGCTTCTGGTCG-3'
Cardiac actin-R	5'-TCTCAAAGTCCAAAGCCACA-3'
epikeratinXK81F	5'-CACCAGAACACAGAGTAC-3'
epikeratinXK81R	5'-CAACCTTCCCATCAACCA-3'
N-CAM-F	5'-GCCCCTCTTGTGGATCTTAGTGA-3'
N-CAM-R	5'-ACAGCGGCAGGAGTAGCAGTTC-3'
XPak1-F	5'-GTGCTGAAGCAGCAGAGGTCCCT-3'

XPak1-R	5'-GTCTACGCTGCTTTCTGCTCCATC-3'
XPak2-F	5'-GTCTGTGCCCTGCACCTAATGCAA-3'
XPak2-R	5'-CTTTGCAGCACTGTCTGAATCTCC-3'
XPak3-F	5'-TGAATGCAAAGACTGCATCTGAGC-3'
XPak3-R	5'-GCTCGTGCTTGAGTTTGAGTTTTC-3'
XSeb4-F	5'-GGAACCTGCAGAGCGCATTTACTA-3'
XSeb4-R	5'-GTCAGGCTGGAGCTGTTGAGGCTG-3'
XSeb4/AJ271404-F	5'-GGATAATGCAGCCAGGTTTTGCA-3'
XSeb4/AJ271404-R	5'-GTAAGTGGTTGCGGGACTGCATA-3'
NeuroD-F	5'-GTGAAATCCCAATAGACACC-3'
NeuroD-R	5'-TTCCCCATATCTAAAGGCAG-3'

VI. 1. 11 Kits

Big dye terminator Cycle sequencing kit	(PE Applied Biosystems)
SP6 Message Mashine *in vitro* transcription	(Ambion)
Qiagen Plasmid Midi kit	(Qiagen)
Qiagen PCR Purification kit	(Qiagen)
RNA PCR Core kit	(Perkin Elmer)
RNeasy mini kit	(Qiagen)
In vitro transcription/translation kit	(Promega)
BrdU-labelling and Detection kit II	(ROCHE)

VI. 2 Methods

VI. 2. 1 Handling and manipulation of embryos

Pigmented and albino X*enopus laevis* were obtained by hormone-induced egg-laying and and *in vitro* fertilization. Eggs were collected from *X. laevis* females, which had been injected with 400 – 800 U of human chorionic gonadotrophin approximately 10 hours prior to eggs collection. Eggs were fertilized with minced testis in 0.1 X MBS, dejellied with 2 % cystein hydrochloride, pH 7.8 – 8.0 and cultured in 0.1 X MBS. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For further in situ hybridisation analysis, albino embryos were injected with synthetic RNA along with 30 pg of NLS ß – Galactosidase RNA. The RNA was injected into two cells at the four-cell stage in a volume of 10 nl, in 1 X MBS solution containing 10 % Ficoll. After injection, embryos were transferred in 0,1 X MBS containing 0.5 % Ficoll, and finally cultured into 0,1 X MBS until the desired stage. For phenotypic analysis, pigmented embryos were injected into two dorsal or ventral blastomers at four-cell stage. The dorsal side was identified by cell size and pigment difference compared with ventral blastomeres.

VI. 2. 2 Preparation of animal cap explants

Embryos were injected into two blastomer of two-cell stage with the specific probes. They were grown to blastula stage 9 and the animal cap was excised by use of a Gastromaster. In 0.5X MBS solution, explants were cultivated to the appropriete stage. They were subsequently frozen in liquid nitrogen and store at -80 °C. Total RNAs were prepared by use

of Qiagen kit. The following data indicate the probes, their injected amounts, and the developmental stage of caps.

Construct (capped-mRNA)	***injected amount*** (pg)	***stage of caps***
X-Ngnr-1	50 or 100	13, 14, 15
ICD-Notch	300	14
X-Ngnr-1/ ICD-Notch	100/300	14
Noggin	500	14
Noggin/ X-Ngnr-1	500/100	14
Noggin/ ICD-Notch	500/300	14
Noggin/X-Ngnr-1/ICD-Notch	500/100/300	14

VI. 2. 3 Fixation and histological procedures

VI. 2. 3. 1 MEMFA fixation of *Xenopus* embryos

Embryos were transferred in 5 ml glass vials and fixed in 1 X MEMFA for 1 h on the rocking platform, washed twice or more for 5 min in 100 % ethanol and kept at – 20 °C for further analysis by *in situ* hybridization. For sectioning or X-Gal staining, embryos were rinsed in 1 X MEM, 2 – 3 times washed with 1 X PBS and stored in 1 X PBS at 4 °C.

VI. 2. 3. 2 Gelatine-medium embedding and vibratome sectioning of embryos

Embryos stained by *in situ* hybridization were fixed for 1 h in 1 X MEMFA, washed once with MEM, and transferred into 1 X PBS. Vibratome sectioning was performed essentially as previously described (Hollemann et *al.*, 1996). After 20 min equilibration in gelatin-albumin medium, embryos were mounted addition of glutaraldehyde. Thirty micrometers sections were cut with a vibratome 1000 (Technical Products International Inc.) and mounted in Mowiol solution.

VI. 2. 4 Wholemount *in situ* hybridization

Whole mount *in situ* hybridization of albino embryos was carried out essentially as described (Harland, 1991) and improved by Hollemann et al., (1999) using digoxigenin-11-UTP- (Dig) or fluorescein-12-UTP (Flu)-labelled antisense RNA probes.

For double staining *in situ* hybridization, Fast Red and NBT/BCIP were used as alkaline phosphatase substrates. After first staining reaction with Fast Red, alkaline phosphatase activity was stopped by heating the embryos for 15 min in 1 X MAB + 0.1 M EDTA at 65 °C. the second staining reaction was done using NBT/BCIP.

DAY 1:

1. *Rehydratation of embryos*

Reagent(s)	**Temperature**	**Time**
100 % ethanol	RT	5 min
75 % ethanol in water	RT	5 min
50 % ethanol in water	RT	5 min

25 % ethanol in PTw	RT	5 min
100 % PTw	RT	5 min, 4 times

2. *Proteinase K treatment*

Embryos in each vial were treated with 2 ml PTw containing 10 µg/ml proteinase K for 15 – 30 min at RT. The treatment time depended on the embryos developmental stage and the value of the real RT. Reasonable time for gastrula stage embryos was 15 – 20 min and longer time (20 – 30 min) for tailbud stage embryos.

3. Acetylation

After proteinase K treatment, embryos were immediately subjected to the following treatments.

Reagent(s)	**Temperature**	**Time**
0.1 M triethanolamine, pH 7.5	RT	5 min, 2 times
5 ml 0.1 M triethanolamine, pH 7.5 + 12.5 µl acetanhydride	RT	5 min
Above mixture + 12.5 µl acetanhydride	RT	5 min
100 % PTw	RT	5 min, 2 times

4. *Refixation*

Reagent(s)	**Temperature**	**Time**
PTw + 4 % formaldehyde	RT	20 min
100 % PTw	RT	5 min, 4 times

* If dealing with pigmented embryos, bleach by the following treatment: 5X SSC + 50 % formamid + 1 % H_2O_2 (ROTH, N^r 2014), 15 – 60 min at room temperature. Wash 4X with 100 % PTw.

5. *Hybridization*

Reagent(s)	**Temperature**	**Time**
250 µl PTw + 250 µl hybridization mix	RT	Mix and discard
500 µl hybridization mix	65 °C	10 min
500 µl hybridization mix	60 °C	6 hr
+ probe (1 µg / ml in hybridization solution)	60 °C	Overnight

DAY 2:

6. Washing

The antisense RNA probes (which can be repeatedly used for several probing cycles), were collected and stored at – 20 °C. the embryos were then wash as follow:

Reagent(s)	**Temperature**	**Time**
500 µl hybridization mix	60 °C	10 min
2 X SSC	60 °C	20 min, 3 times
2 X SSC + 20 µg/ml RNase A and 10 U RNase T1	37 °C	30 min, 2 times
2 X SSC	RT	10 min
0.2 X SSC	60 °C	30 min, 2 times

7. *Antibodies incubation*

Reagent(s)	**Temperature**	**Time**
1 X MAB	RT	15 min, 2 times
1 X MAB + 2 % BMB	RT	15 – 60 min
1 X MAB + 2 % BMB + 20 % serum	RT	60 min
1 X MAB + 2 % BMB + 20 % serum + antidigoxigenin (antifluorescein) antibody (1 : 5000)	RT	4 – 6 hours
1 X MAB	RT	30 min, 2 times
1 X MAB in 50 ml corning tubes	4 °C	Overnight

DAY 3:

8. Colour reaction

Reagent(s)	**Temperature**	**Time**
1 X MAB	RT	60 min
APB	RT	5 min, 2 times
APB + NBT / BCIP	4 °C	5 min – 1 week

NBT / BCIP staining solution

APB	:	1 ml
75 mg / ml NBT in 75 % dimethylformamide	:	2 µl
50 mg / ml BCIP in 100 % dimethylformamide	:	3.5

After staining, the embryos were clarified in some cases by the following treatment :

Reagent(s)	**Temperature**	**Time**
H_2O	RT	30 sec
100 % MeOH	RT	1 min
75 % MeOH	RT	1 min
50 % MeOH	RT	1 min
25 % MeOH	RT	1 min
H_2O	RT	1 min
MEMFA	4 °C	Store

VI. 2. 5 Immune wholemount staining

Immune wholemount staining was done as described in (Dent *et al*., 1989) with modifications.

Fixation:

1. Fix the embryos by incubating in Dent´s fixative for 2 hrs at room temperature after ß-gal staining.
2. Permeabilize in Dent´s fixative overnight at – 20 °C.
3. Transfer the embryos in 100 % methanol and store at – 20 °C.

Day 1:

Embryo rehydration, washes and primary antibody incubation

1. Rehydrate embryos in 90 % methanol/H_2O_2, 70 % methanol/H_2O_2, 50 % methanol/1X PBS, 30 % methanol/1X PBS
2. Wash embryos 2 – 3 times for 15 min with PTw.
3. Block embryos in 20 % goat serum in PTw for two hours at room temperature.
4. Dilute the primary antibody (antiflag antibody, dilution 1 : 500; antiPCNA dilution 1:50) in 20 % horse serum + 5 % DMSO/PBS) and incubate the embryos overnight at 4° C with gentle shaking.

Day 2:

Second antibody incubation

1. Wash embryos with 3 – 5 changes, 30 min to 1 hour each, in PBS-TB at RT. The longest the wash the better the background. Washes longer than 10 hours should be done at 4° C.
2. Dissolve secondary antibody (anti-mouse IgG-HRP, 1 : 500 dilution; ant-mouse IgG-fluores.conj., 1 : 200 dilution; anti-mouse IgG-AP, dilution 1: 500 in 20 % goat serum + 5 % DMSO/PBS and incubate embryos overnight at 4° C, with gentle shaking (or 4 – 6 hours at RT).

Day 3:

Immunodetection, AP, and HRP staining

1. Wash embryos with 4 – 5 changes, 30 min – 1 hour each, in PBS-TB at RT.
2. Wash embryos with one or two changes PBS-TBN, 30 min.
3. Wash embryos with one or two changes PBS-TB, 2 – 4 hour at RT or ON at 4° C. Ready for microscope detection for fluoresceine conjugated. Sections are some time required.
4. Preincubate (HRP conjugated) 30 to 45 min 0.3 mg/ml DAB in PBS (600 µl).
5. Incubate embryos in 300 µl of 0.3 mg/ml DAB + 0.06 % H_2O_2 (PIERCE) for 10 min or several hours.
6. Stop with PBS or MeOH.
7. Destroy DAB solutions with Clorix or with saturated permanganate).
8. For the AP staining, proceed as described in the wholemount *in situ* hybridization.

VI. 2. 6 BrdU-labelling Whole mount staining

This was done in principle according to Hardcastle and Papalopulu (2000) with modifications. After injection of BrdU, embryos were fixed in MEMFA at RT for one hour. The vitellin membrane was remove by proteinase K treatment as described earlier in whole mount *in situ* hybridisation. Embryos were then washed with 5 changes PTw and dehydrated ON in ethanol.

Day 1:
1. Rehydration

Ethanol 100 %		5 min	1x
Ethanol 75 %	1X PBS 25 %	5 min	1x
Ethanol 50 %	1X PBS 50 %	5 min	1x
Ethanol 25 %	1X PBS 75 %	5 min	1x
	1X PBS 100 %	5 min	5x

2. HCl-treatment

2 N HCl	1 hour	1x
1X PBS	5 min	3 – 5x

3. Antibody incubation
Rinse embryos in 1X PBS + 0.3 Triton-X-100, 30 min 2times at RT.
Block 1 hour in 1X PBS + 3 % horse serum
Wash 15 min in incubation buffer (Boehringer kit 1299964)
Replace with anti-BrdU solution (anti-Brdu 1 : 10 dilution in incubation buffer), incubate at 37° C for 3 – 4 hours (shake once a while).
Wash embryos with 3 changes 1X PBS, 15 – 30 min each, then ON at 4° C in 1X PBS.

Day 2:
Secondary antibody incubation.

1X PBS + 0.1% Tween-20	5 min	5x
1X PBS + 20 % horse serum	1 hour	1x
1X PBS + 20 % horse serum + anti-mouse-IgG-AP	5 – 6 hours (RT)	1x
1X PBS + 0.1% Tween-20	15 – 30 min	3x
1X PBS + 0.1% Tween-20	ON at 4° C	

Day 3:
BrdU positive cells Staining

1X PBS + 0.1% Tween-20	5 min (RT)	2x
Substrate buffer	5 min (RT)	2x
Substrate buffer + NBT + X-phosphate	5 – 30 min (4° C)	
Stop the reaction with 1X PBS and fix embryos in MEMFA.		

VI. 2. 7 TUNEL Wholemount staining

This was done according to Hensey and Gautier (1997) with modifications. Embryos were fixed in MEMFA at RT for one hour. The vitellin membrane was remove by proteinase K treatment as described earlier in whole mount *in situ* hybridization. Embryos were then washed with 5 changes PTw and dehydrated ON in ethanol.

Day 1:
1. Rehydration and end labeling
The rehydration was done as described above.
2. End labeling
Wash embryos 1 hour in TdT buffer at RT. (dilute 5X buffer in 1X PBS, not H_2O).

Incubate embryos in TdT buffer containing 0.5 µM digoxigenin-dUTP and 150 U/ml TdT ON at RT. Use 5 ml glass vials, with flat bottom, Fisher 3 338 AA (minimal volume 200 µl, use 1X PBS to ajust the volume).

Day 2:
Wash embryos with two changes 1X PBS/EDTA at 65° C (water bath or hybridization oven) to inactivate TdT. Further wash with four changes 1X PBS, one hour each and proceed for detection as described by Harland, R. M., 1991.

VI. 2. 8 Molecular biology methods

VI. 2. 8. 1 Preparation of probes for the expression pattern screen

PCR cloning:

In a 96-well PCR-plate, 25ul of PCR mix, containing both forward and reverse CMV primers, were distributed on ice. One ul of individual phase or plasmid clone was added per well. The following programme was designed to amplify the inserts of interest, using the Biometra "Uno" PCR machine:

Pre-denaturation	:	94 °C	3 min
Denaturation	:	94 °C	1 min
Annealing	:	56 °C	45 sec
Elongation	:	72 °C	2.5 min
Number of cycles	:	38	
Final extension	:	72 °C	10 min
Soak	:	12 °C	

On a 1 % agarose gel, the PCR products were analysed by electrophoresis. Clones with at least 1 Kb in size were selected for futher analysis.

Antisense RNA synthesis:

In a 96-well PCR-plate, 20 ul of transcription mix, containing the T7 RNA polymerase and Flu-UTP, were distributed on ice. Five microliters of PCR product were added and mixed. The final mixture was incubated at 37 °C for 2.5 hours. Using the G-50 gel, the synthesized RNAs were purified and used in wholemount in situ hybridization as described above.

VI. 2. 8. 2 RT-PCR analysis

Total RNAs, prepared from embryos or from the caps as described by the RANsin kit protocole, were analysed for specific genes expression by use of the following conditions:

Gene	**GB Accession**	**Product size** (bp)	**Annealing** (°C)	**Nb. Cycles**
XPak3	AF485330	242	60	34
XPak1	AF169794	198	60	30
XPak2	AJ242726	190	60	31
XSeb4	not submitted	356	60	33
XSeb4 (muscle)	AJ271404	310	60	28

ESR7	AF146088	212	56	38
N-tubulin	X15798	250	57	32
NeuroD	U28067	238	57	32
Cardiac actin	X03469	253	57	26
epiKeratinXK81	M11940	217	57	27
N-CAM	M76710	413	57	32
Histone H4	X03017	189	57	25

VI. 2. 8. 3 Molecular cloning of the constructs used in embryo microinjection

The enoding regions of interest of each clone was amplified by PCR from the corresponding pBKCMV recombinant plasmid, using the appropriate primers described above. Analysed by agarose gel electrophoresis, the amplified DNA fragments were purified from the gel with the Qiagen kit, as described in the Handbook. The DNAs, including our clones and different version of pCS2+ expression vector (Turner and Weintraub, 1994) were digested overnight with the corresponding restriction enzymes. The digested products were purified and ligated. The resulting recombinant plasmids were amplified in vivo, using XL1-Blue bacteria (Stratagene). Each clone was further sequenced and analysed by TnT *in vitro* transcription/translation system (Promega). ^{35}S-methionine was used for labelling and the translated products were analysed by SDS PAGE and phosphoimaging.

To generate the pCS2+myr expression plasmid, the primers corresponding to the myr signal (Resh, 1994) were assembled, endfilled, digested with MunI/EcoRI, purified and ligated into pCS2+. All the myristylated versions of clones could then be cloned appropriately using EcoRI and other downstream restriction enzymes.

VI. 2. 8. 4 Preparation and analysis of plasmid DNA

VI. 2. 8. 4. 1 Preparation of electrocompetent bacteria

Electrocompetent cells used in this study was prepared according to the BioRAD protocole as followed:

1. Inoculate a single colony of *E. coli* XL blue in 200 ml of LB broth with tetracycline and culture at 37 °C overnight. Simultaneously, incubate 2 liters of LB medium at the same condition without bacteria.
2. Put the 200 ml bacteria culture to the 2 liters LB broth (1:100 dilution) and culture for additional 3-3.5 hours at 37 °C. After culturing for 2.5 hours check OD_{600} every 20-30 min until OD_{600} reaches to 0.8 and store the culture on ice.
3. Harvest the cells by centrifuging at 4000 rpm for 10 min at 4 °C. It is better to do all the following steps in the cold room. Decant the liquid from the cell pellets completely.
4. Wash the cells by resuspending the pellets in 1.2 liters of 10% glycerol or pure water and centrifuge at 4000 rpm for 10 min at 4 °C.
5. Wash again with 600 ml of 10% gycerol or pure water and centrifuge at 4000 rpm for 10 min at 4 °C.
6. Suspend the cells in 4 ml 10% glycerol, make 40 µl aliquots, freeze with liquid nitrogen and store at -80 °C.

VI. 2. 8. 4. 2 Transformation of bacteria and plating

1. Put on ice the electrocopetent bacteria (for tawing), an aliquot of LB medium, the probe (ligation mix or others, in a low ionic strength buffer), and the cuvettes.
2. Set Gene Pulser at 25 µF, 2.5 kV when using the 0.2 cm cuvettes.
3. Mix 40 ul bacteria and 1 µl probe then transfer into a pre-cooled cuvette. Cover with the lid and insert in the Gene Pulser.
4. Press the corresponding two butons on the Gene Pulser until the sound comes (time constant should be 4-5 msec).
5. Put 500 µl cooled LB-medium in the cuvette and resuspend the cells (steps 4 and 5 should be very face to improve the recovery of the transformants).
6. Plate 20 µl and 200 µl to LB-Amp (Kan or Tet) plates, incubate overnight at 37 °C.

VI. 2. 8. 4.3 Plasmid DNA preparation from bacteria

Mini-preparation with TELT
1. Collect cells from 1.5 ml bacteria culture by centrifuging at 6000 rpm, RT, 5 min.
2. Remove supernatant completely with a vacuum system and resuspend the cells in 150 µl TELT solution.
3. add 15 µl of 10 mg/ml lysozyme to the cells, vortex and incubate at RT for 5 min.
4. Keep the cells in boiling water for 2 min and thenc transfer on ice for 5 min.
5. Centrifuge with full speed (14 000 rpm, microcentrifuge), RT, 10 min and remove the pellet of bacteria debrits with a sterilized toothpick.
6. Add 100 µl of isopropanol to the sample and vortex.
7. Centrifuge with full speed at RT for 15 min. Remove the supernatant with pipette and wash the pellet with 200 µl of 70 % ethanol.
8. Centrifuge with full speed at RT for 5 min. Suck off the supernatant with pipette and dry the pellet under speed vacuum for 5 min or at 37 °C for about 15-30 min.
9. Dissolve the pellet in 30 µl TE with 10 ng/µl RNase A and incubate first at RT for 2-5 min and then 65 °C for 5 min. Store the preparation at -20 °C.

Large scale plasmid DNA preparation

For the preparation of the large amounts of highly pure plasmid to be use for in-vitro RNA synthesis, sequencing and TnT, Qiagen or Nucleobond Plasmid Midi kit was used according to manufacturer's instructions.

VI. 2. 8. 4. 4 Agarose gel electrophoresis of DNA

0.8 to 2.5 % agarose gels were used for analysis of DNA fragments 0.2-5 kb. The agarose gels were prepared using 1X TBE buffer with 0.5 µg/ml ethidium bromide; 1X TBE was used as running buffer. Low or high size molecular marker (Gibco BRL) was used. The DNA band were visualised at the UV-transillumination; The picture of the gels were made using the IMAGER computer. In case of cloning, the bands of interest were purified from the agarose gels according to Qiagen Handbook.

VI. 2. 8. 5 Preparation and analyis of RNA

VI. 2. 8. 5. 1 Synthesis and purification of mRNA for injections

The constructs and the restriction enzymes used for the linearization are listed above. Approximately 10 ug of each clone were digested and purified using PCR Purification kit (Qiagen). Eluted in TE buffer, linearized constructs were diluted to 200 ng/ul. The mRNA synthesis was done according to the instruction manual of the mMESSAGE mMACHINE (Ambion). Capped-RNAs were purified using RNeasy kit (Qiagen). Eluted in RNAase-free water, mRNA were analysed on a 1% agarose gel and diluted to 500 ng/ul and store at -80 °C.

VI. 2. 8. 5. 2 Synthesis and purification of Dig(Flu-)-labelled antisense RNA for *in situ* hybridization

The plasmid probes of interest were linearized using the appropriate restriction enzyme, as indicated above. Analysed on a 1 % agarose gel, the linearized DNAs were purified and diluted to 200 ng/ul in TE buffer. For 25 ul of *in vitro* transcription reaction, the following components were mixed in a 1.5 ml eppendorf tube:

5X transcription buffer (Stratagene)	5 ul
ATP (10 mM)	1 ul
GTP (10 mM)	1 ul
CTP (10 mM)	1 ul
UTP (10 mM)	0.64 ul
Dig-or Flu-UTP (10 mM)	0.36 ul
DTT (0.75 M)	1 ul
RNAsin (40 U/ul)	1 ul
RNA polymerase (1:5 diluted)	0.5 ul
DNA template (200 ng/ul)	5 ul
RNAase-free H2O	up to 25 ul

Mixed and centrifuged briefly, the reaction mix was incubated at 37 °C for 2.5-3 hours. The template DNA was next digested by treatment with 10 U of DNAaseI RNAase-free for 20-30 min. The Digoxigenin (Fluorescein)-labelled RNA probes were purified using the RNeasy kit (Qiagen). Eluted in 35 ul of RNAase-free water, equivalent volum of formamid was immediately added to stabilize the antisense probes. The quality of the purified RNAs was analysed by electrophoresis on 1 % agarose gel. In hybridization mix, these probes are stable for years at -20 °C.

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VIII ACKNOWLEDGEMENTS

The accomplishment of this thesis has been made possible thanks to support of divers nature from many people, all whom cannot be cited but may find here the expression of my heart-felt gratitude.

I am so grateful to Prof. Dr. Tomas Pieler for offering me a position in the Graduate program, "Molekulare Genetik der Entwicklung" which he is the Director and accepting me in his Department. Despite his multiple preoccupations, he devoted much of his time to supervise this project right to the end; I hope he finds this dissertation as a motive of satisfaction and encouragement in his career.

I acknowledge Prof. Dr. Wolfgang Engel for his continuous moral support which has been very helpful to carry this project to the end.

Members of Professor Pieler's group are thanked for sharing a very nice and productive time with me. I am particularly grateful to Marion Sölter (1) and Kristine Henningfeld (2) for their assistance in the previously quoted experiments and for the translation (1) and corrections (2) made to improve the quality of this dissertation. I address my acknowledgements to Yong Long Chen, and Thomas Hollemann for nicely introducing (1) me to the basic techniques in molecular embryology in *Xenopus*. The technical support of Tina Berneking and Ulrike Strube has been helpful.

I express my profound gratitude to Prof. Dr. François Xavier Etoa, Head of Department of Biochemistry, University of Yaoundé I for all the facilities provided, allowing my authorization to stay abroad during the accomplishment of this project. I am thankful to his staff and particularly to Drs. Jules Tchuinmogne Simo and John Payne Muluh for taking care of my teaching activities. The administration of the University of Yaoundé I and its hierarchy are thanked for releasing me for this period of studies.

Finally, I acknowledge my family, my family in law and my friends for their encouragement; I hope they find this thesis as a mean of happiness.

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Part of the results reported in this dissertation was/will be presented in the following meetings, conference, or congress:

1)- 9th Interntional Xenopus Conference, August 2002, Cambridge, UK.
2)- SFB meeting, April 2002, Rothenberg, Germany.
3)- 3rd German-Italian Xenopus Meeting, October 2001, Pontignano (Siena), Italy.
4)- 14th International Congress of developmental Biology, July 2001, Kyoto, Japan.

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