Target identification and molecular characterization of the RNA-binding protein XSeb4R in *Xenopus laevis*

Dissertation

zur Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultäten

der Georg-August-Universität zu Göttingen

vorgelegt von

Barbara Rust

aus Duderstadt, Deutschland

Göttingen 2008

D 7

Referent: Prof. Dr. Tomas Pieler

Korreferent: Prof. Dr. Ernst A. Wimmer

Tag der mündlichen Prüfung:

For my parents

Table of Contents

Acknowledgements

I would like to thank Prof. Pieler for giving me the opportunity to do my PhD thesis in his laboratory and to work on a very interesting project. Additionally I thank Prof. Wimmer, who kindly agreed to be second examiner of my thesis and Prof. Hardeland, Prof. Braus, Prof. Gatz and Prof. Ficner who agreed to be member of my board of examiners.

My special gratitude goes to Kris Henningfeld, who supported me a lot during my PhD thesis and invested a great deal of time to help with any kinds of problems. Thanks!

Further I want to thank the whole department for the great atmosphere and help with problems, especially the Neuro-Group (Kris, Katja, Frank and Marie). Special thanks go to Katharina Damianitsch, Katja Ditter and Juliane Melchert, who became next to colleagues also good friends.

I want to thank my friends, who where also there for me, when times were not very sunny, but and of cause also for all the good moments!

At last, I want to thank my family, especially my parents. I would not be where I am today, if they had not been so supportive over the years, in many ways.

List of Figures

List of Tables

Abbreviations

General abbreviations

aa	Amino Acid
BCIP	5-Bromo-4-chloro-3-Indolyl-Phosphate
BMB	Bohringer Mannheim Blocking Reagent
bp	base pairs
°C	Degree celcius
cap	7-methyl guanosine cap structure (m^7GpppG)
cap analog	guanosine(5')triphospho(5')adenosine (GpppA)
cDNA	Complementary DNA
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propansulphate
H_2O	water
DIG	Digoxigenin-11-2´-deoxyuridin-5´-triphosphate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleicacid
DNase	Deoxyribonuclease
dsDNA	double-stranded DNA
DTT	1,4-Dithiothreit
EDTA	Ethyle-di-amin-tetra-acetate
est	expressed sequence tag
h	hours
HEPES	4-(2-Hydroxyethyl)-1-piperazin
IRES	Internal ribosomal entry site
kb	Kilobase
l	Litre
LB	Luria-Bertani (medium)
M	Molar (mol/l)
MAB	Malic acid buffer
MEM	MOPS-EGTA-$MgSO_4$-Buffer

MEMFA	MOPS-EGTA-MgSO4-Formaldehyde-Buffer
min	minutes
MOPS	4-Morpholinpropanosulfonic acid
mRNA	messenger-RNA
NaAc	Sodium acetate
nt	Nucleotides
OD	Optical density
pCp	Cytidine 3',5'-Bis(Phosphate)
PBS	Phosphate buffed saline
PCR	Polymerase chain reaction
pH	Preponderance of hydrogen ions
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
RT-PCR	Reverse transcriptase –PCR
s	seconds
SSC	Standard Saline Citrate Buffer
ssDNA	single-stranded DNA
Taq	Thermus aquaticus
TBE	Tris-Borate-EDTA-Electrophoresis buffer
tRNA	transfer RNA
w/o	without
Xl	*Xenopus laevis*

Abbreviations of Deoxynucleic Acids

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

Abbreviations of Amino Acids

A	Alanin
C	Cystein
D	Aspartate
E	Glutamate
F	Phenylalanin
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophane
Y	Tyrosine

List of Publications

Jürgens K., Rust B., Pieler T., Henningfeld K.A. (2005) Isolation and comparative expression analysis of the Myc-regulatory proteins Mad1, Mad3, and Mnt during *Xenopus* development. Dev Dyn. 233 (4): 1554-9.

Klisch T.J., Souopgui J., Jürgens K., Rust B., Pieler T. and Henningfeld K.A. (2006) Mxi1 is essential for neurogenesis in *Xenopus* and acts by bridging the pan-neural and proneural genes. *Dev Biol* 292, 470-85.

Souopgui J., Rust B., Vanhomwegen J., Heasman J., Henningfeld K.A., Bellefroid E., Pieler T. (2008) The RNA-binding protein XSeb4R: a positive regulator of *VegT* mRNA stability and translation that is required for germ layer formation in *Xenopus*. Genes Dev. 1; 22 (17): 2347-52.

1. Introduction

1.1 Maternally localized RNAs involved in *Xenopus* germ layer formation

The polarization of the *Xenopus* oocyte is reflected by the subdivision into a darkly pigmented animal and lightly pigmented vegetal hemisphere. Fertilization of the egg within the animal hemisphere causes the cortex to loosen from the dense yolky core cytoplasm (Vogt *et al*., 1929; Smith *et al*., 1989; Weaver *et al*., 2004; Lane *et al*., 2006). Cortical rotation results in displacement of the vegetal cortex by 30° forming a structure referred to as the dorsal crescent or “graue Halbmond” (Spemann *et al*., 1924; Gerhart *et al*., 1989; Houliston *et al*., 1992; Rowning *et al*., 1997). This event coincides with translocation of maternal determinants located at the vegetal hemisphere (Fig.1A and B; Kikkawa *et al*., 1996; Sakai, 1996; Kageura, 1997). Both events depend on the assembly of parallel arrays, of microtubule bundles (Elinson *et al*., 1988; Houliston *et al*., 1992; Chang *et al*., 1999). The maternal vegetal determinants, as well as their transport to the future dorsal site by microtubules, have been shown to be essential for dorso-ventral axis formation (Scharf *et al*., 1983; Elinson *et al*., 1988; Holowacz *et al*., 1993; Kageura *et al*., 1997; Cha *et al*., 1999). Impairment of microtubule function by UV irradiation results in complete loss of dorsal structures. The observed phenotype is referred to as belly piece or “Bauchstück”, thus translocation of maternal determinants plays a crucial role in dorso-ventral axis formation (Harland *et al*., 1997; Heasman *et al*., 1997; Moon *et al*., 1998).

Several of the maternal, vegetally localized determents have been identified, among them *Wnt11* mRNA and Disheveled protein, both members of the Wnt signaling pathway (Fig. 1A and B; Sokol *et al*., 1995; Tao *et al*., 2005). Active Wnt signaling stabilizes cytoplasmic ß-catenin, which accumulates, translocates into the nucleus and regulates the transcription of target genes (Miller *et al*., 1999; Dominguez *et al*., 2001; Huelsken *et al*., 2002). *Vg1* mRNA, which encodes for a member of the TGF-ß ligand family, has also been found to localize to the vegetal cortex (Fig. 1A; Melton, 1987). However, its function in germ layer formation remains elusive. While Vg1 protein was found to be processed *in vitro*, the mature

form of Vg1 has never been detected during early cleavage stages *in vivo*, and wild type Vg1 does not induce mesoderm or endoderm when ectopically expressed in the prospective ectoderm (Dale *et al*., 1989; Tannahill *et al*., 1989; Joseph *et al*., 1998; Yasuo *et al*., 2001).

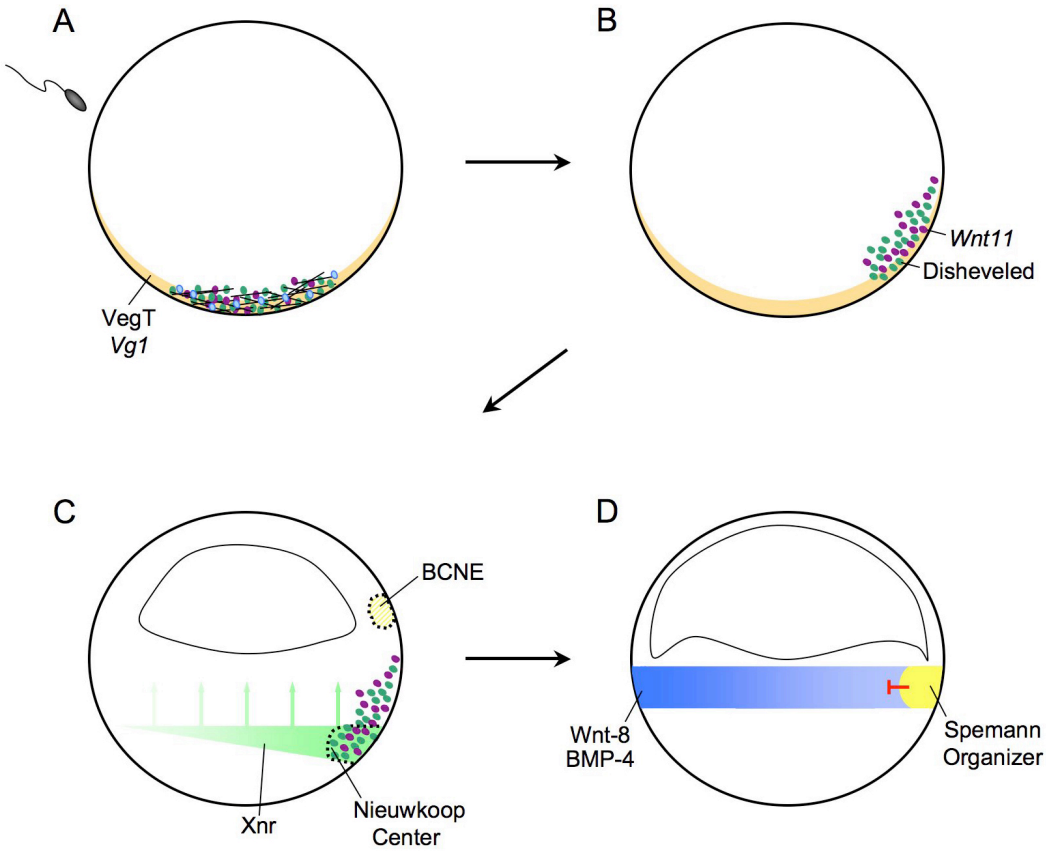


Fig. 1: Establishment of the dorso-ventral axis and mesoderm induction. **(A)** Maternal transcripts are anchored at the vegetal cortex of the egg (green, purple, blue). In addition, VegT protein and *Vg1* mRNA are localized to the vegetal hemisphere (orange). **(B)** During the first cell cycle, cortical rotation induces the shift of Disheveled protein and *Wnt11* transcripts (green, purple) to the future dorsal site of the embryo. **(C)** In the blastula stage, VegT induces Xnr-expression, which forms a dorso-ventral gradient within the future mesoderm, while Wnt11 and Disheveled (purple, green) activate Wnt signaling in the future dorsal site of the embryo. The region where both signals overlap forms the Nieuwkoop Center (black dotted line). Furthermore, a Chordin/Noggin expressing center is located in the dorsal animal hemisphere (BCNE, light yellow). **(D)** At the onset of gastrulation, the Spemann Organizer (yellow) is established within the dorsal mesoderm, which secretes antagonists of TGF-ß (BMP-4) and Wnt signaling (Wnt-8), forming a ventrodorsal TGF-ß/Wnt gradient (blue) (adapted from DeRobertis *et al*., 2000).

1.2 VegT is essential for germ layer formation

The T-box transcription factor VegT is an additional localized and essential for endoderm and mesoderm formation. *VegT* mRNA is localized to the vegetal cortex during oogenesis and becomes translated upon egg maturation (Fig. 1A and B; Lustig *et al*., 1996; Stennard *et al*., 1996; Zhang *et al*., 1996; Horb *et al*., 1997; Stannard *et al*., 1999). Maternal depletion of *VegT* results in loss of endoderm; the endodermal cells adopt a mesodermal cell fate while the ectoderm expands into the area that would develop into mesoderm (Fig. 2; Zhang *et al*., 1998; Heasman *et al*., 2001). In addition, the localization of other maternal determinants, such as *Vg1*, *Bicaudal-C* and *Wnt11* transcripts, is disrupted (Heasman *et al*., 2001).

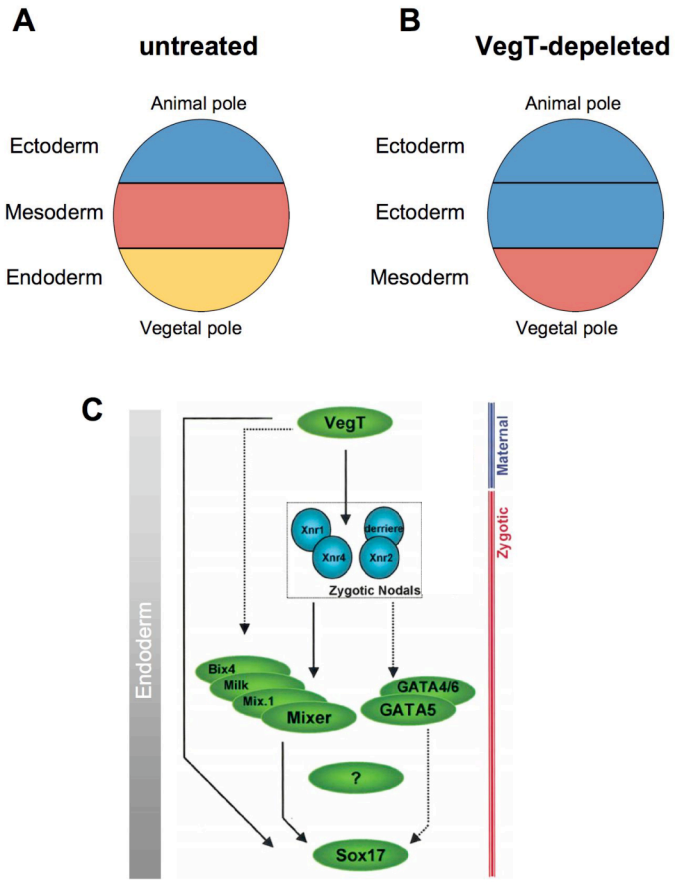


Fig. 2: Depletion of VegT alters germ layer identity. (A) A normal embryo will establish three germ layers. The animally located layer is the ectoderm, while the vegetal one will become endoderm, and the mesoderm is positioned in between. **(B)** VegT-depletion results in the change of germ layer identity; the mesoderm will adopt an ectodermal and the endoderm a mesodermal fate. **(C)** The maternally provided transcription factor VegT induces the expression of a cascade of transcription factors (green) and signaling molecules (blue) to induce endoderm establishment cell-autonomously (Zhang *et al*., 1998; Shivdasani, 2002; modified).

VegT induces an endodermal fate cell-autonomously through the activation of several transcription factors, such as Sox17, Xbra, Eomesodermin and GATA (Fig. 2 C; Ryan *et al*., 1996; Zhang *et al*., 1998; Clemens *et al*., 1999; Xanthos *et al*., 2001; Sinner *et al*., 2006). VegT results further in the induction of TGF-ß family ligands, like *derrière* and *Nodal-related* molecules, which mediate mesoderm induction (Zhang *et al*., 1998; Clemens *et al*., 1999; Xanthos *et al*., 2001; Sinner *et al*., 2006). Nodal-related, in combination with ß-catenin, promotes the formation of a signaling center within the dorsal endoderm, which is referred to as the Nieuwkoop center. This signaling center is a source of instructive signals generating an equatorial mesodermal compartment (Fig. 1 C; Nieuwkoop, 1969; Agius *et al*., 2000; Shivdasani, 2002; Kimelman, 2006). During gastrulation the Nieuwkoop center induces the establishment of the Spemann organizer in the overlaying dorsal mesoderm, which expresses numerous organizer-specific genes, notably secreted proteins that bind to growth factors in the extracellular space, mainly molecules of the TGF-ß and Wnt family (Fig. 1C, D; Smith *et al*., 1992; Sasai *et al*., 1994; Wylie *et al*., 1996; Heasman *et al*., 1997; DeRobertis *et al*., 2000). Degradation of maternal *VegT* transcripts at the onset of gastrulation is accompanied by the expression of a zygotic alternatively spliced version, which carries a different amino terminus and is referred to as Antipodean (Apod) but seems to exert identical activities in *Xenopus* gain of function assays (Stennard *et al*., 1996; Stennard *et al*., 1999). The function of VegT during gastrulation is much less understood, but has been suggested to activate the transcription of several inhibitors secreted by the Spemann organizer, such as Chordin, Noggin and Cerberus (Xanthos *et al*., 2002). Taken together, these findings identify VegT as a key-determinant in germ layer formation.

The three germ layers are finally established by cell movements during gastrulation, which starts with involution of the blastopore lip (Vogt *et al*., 1929; Spemann *et al*., 1938; Keller *et al*., 2003; Keller *et al*., 2008). Initially, bottle cells migrate into the blastocoel followed by the future mesoderm and finally the endoderm. While the outer cell layer, the ectoderm, gives rise to the epidermis and the nervous system, the endoderm will differentiate into the inner organs and the mesoderm into muscles, connective tissue, blood, heart, kidney and spleen.

1.3 Neural induction

The Spemann organizer secretes factors, such as Chordin, Follistatin, Cerberus and Noggin that act extracellulary to inhibit BMP signaling, which is crucial to induce a neural over an epidermal cell fate in the ectoderm (Fig. 1D and 3; Sasai *et al*., 1996; Zimmerman *et al*., 1996; DeRobertis *et al*., 2004). More recently it has been demonstrated that a blastula Chordin and Noggin expressing center (BCNE) is located in the dorsal animal cap of the embryo, which predisposes the prospective neuroectoderm to neural induction (Fig. 1C; DeRobertis *et al*., 2004; Kuroda *et al*., 2004). Furthermore, Fibroblast growth factor (FGF) and Insulin growth factor (IGF) signaling has been shown to inhibit BMP signaling by downregulating Smad1 activity, which is an intracellular transducer of BMP signaling (Fig. 3; Launay *et al*., 1996; Sasai *et al*., 1996; Richard-Parpaillon *et al*., 2002; Pera *et al*., 2003). BMP inhibition, however, appears not to be sufficient for establishment of the neural plate. Independent of inhibiting BMP signal transduction, low amounts of FGF are required for the formation of the neural plate (Baker *et al*., 1999; Delaune *et al*., 2005; Heeg-Truesdell *et al*., 2006). The events of neural induction induce the expression of transcription factors that initiate a neural cell fate, such as the Zic-related, Sox2, Sox3 and SoxD (Smith, 1989; Keller *et al*., 1992; Dale *et al*., 1999; Hardcastle *et al*., 2000; Kishi *et al*., 2000).

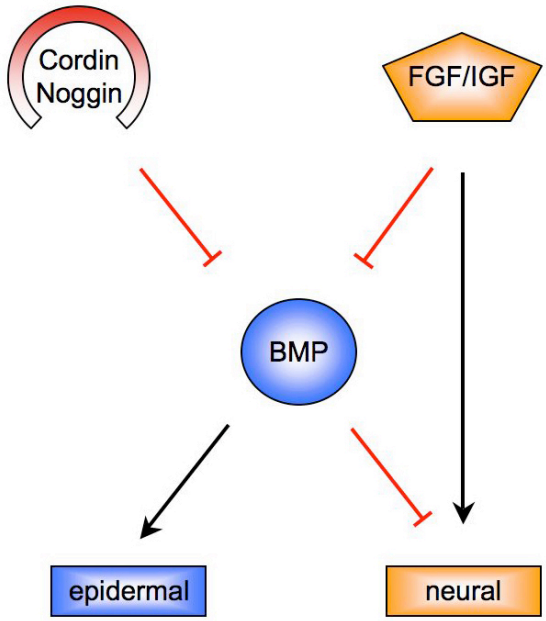


Fig. 3: Neural induction requires inhibition of BMP signaling. Ectodermal cells receiving BMP signaling will adopt an epidermal cell fate. Secreted molecules of the Spemann organizer, such as Chordin, Noggin, Follistatin or Cerberus, (red-white) prevent BMP signaling via sequestering the signal molecule extracellulary. FGF and IGF signaling additionally inhibit intracellular BMP signal transduction. Lack of BMP in combination with low amounts of FGF signaling induce a neural over an epidermal cell fate.

1.4 Neural determination and differentiation

Only a subset of cells within the induced neural ectoderm will undergo primary neurogenesis. These first neurons, termed primary neurons, are born bilateral in three longitudinal stripes along the dorsal midline and in the trigeminal placodes, which can be visualized by the expression of the neural-specific *type II ß-tubulin* (*N-tubulin*) (Fig. 4; Hartenstein, 1989; Oschwald, 1991; Moody *et al*., 1996). While the medial and intermediate stripe are located within the neural plate, the lateral stripe lies at the border, suggesting the requirement of an intermediate level of BMP activity for their formation (Hardcastle *et al*., 2000; Rossi *et al*., 2008). Maturation of the neuroectoderm activates expression of additional panneural factors, such as the nervous system specific RNP protein 1 (Xnrp1), the neural cell adhesion molecule (NCAM) and SoxD (Fig. 4; Kintner *et al*., 1987; Richter *et al*., 1990; Mizuseki *et al*., 1998b). In contrast to the HMG-box containing transcription factors Sox2 and Sox3, which enhance sensitivity of ectodermal cells to receive

extracellular neural-inductive factors, SoxD has been shown to be sufficient to induce neural determination (Mizuseki *et al.*, 1998a; Mizuseki *et al.*, 1998b Hardcstle *et al*., 2000).

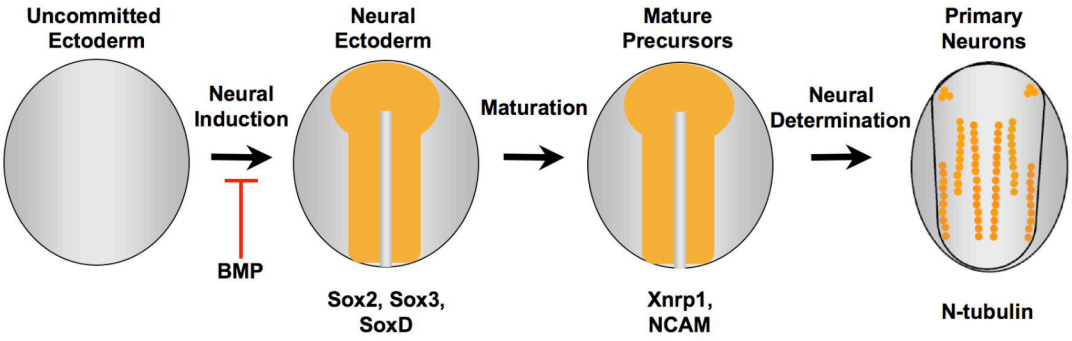


Fig. 4: Schematic cascade of the events leading to neuronal differentiation. At blastula stage, the animal half of the embryo consists of an uncommitted epidermal cell population. Upon BMP inhibition, the neural plate is induced (orange), demarcated by expression of panneural marker genes, such as Sox2, Sox3 and SoxD. Maturation of the neural plate induces additional panneurally expressed markers (Xnrp-1 and NCAM). Neural determination occurs in three longitudinal stripes (orange circles) to both sites of the midline and in addition in the trigeminal placodes (orange). The terminal differentiated neurons express N-tubulin.

The Zic-related family of zinc-finger transcription factors has been implicated to play an important role in restriction of the proneural domains within the neural plate. While Zic1 and Zic3 have been implicated to promote proneural properties, Zic2 has been reported to inhibit neuronal differentiation and to restrict the neuronal domains (Mizuseki *et al.*, 1998a; Nakata *et al.*, 1997; Brewster *et al*., 1998). In addition members of the homeobox containing family of Iroquois transcription factors, Iro1 and Iro2, are expressed at the borders of the restricted areas, induce proneural genes and thus further restrict the neural plate (Gomez-Skarmeta *et al.*, 1998). Iro3, on the other hand, promotes neurogenesis, but appears to inhibit neuronal differentiation, implicating a role similar to Zic2 in establishing a neuronal cell population and maintaining an undifferentiated state (Bellefroid *et al*., 1998).

It has been proposed that expression of the neural determination factor *Xngnr-1* is activated by Zic1 and Zic3, however the molecular mechanism remains to be characterized (Ma *et al*., 1996; Mizuseki *et al*., 1998a). Xngnr-1 belongs to the family of basic helix-loop-helix (bHLH) transcription factors, which are known to bind to E-box sequences on the DNA (Murre *et al*., 1989; Brennan *et al*., 1991; Lin

et al., 1991; Doyle *et al*., 1994; Powell *et al*., 2004). Xngnr-1 is expressed in all territories of primary neurogenesis where it activates transcription of other bHLH transcription factors like *NeuroD*, *Coe2*, *Ebf3* and *Mxi1* as well as the zinc-finger transcription factor *MyT1* (Lee *et al*., 1995; Bellefroid *et al*., 1996; Dubois *et al*., 1998; Pozzoli *et al*., 2001; Klisch *et al*., 2006). In addition, Xngnr-1 has been described to activate transcription of molecules involved in chromatin remodeling (*Brg1*) and RNA metabolism (*XSeb4R*) (Boy *et al*., 2004; Seo *et al*., 2005).

A further essential step for a neuronal precursor cell to undergo terminal differentiate is withdrawal from the cell cycle. Genes involved in cell-cycle arrest and subsequent neuronal differentiation during primary neurogenesis include the cyclin kinase inhibitor $p27^{Xic1}$, the p21 activated serine/threonine kinase *Pak3* and the growth arrest and DNA-damage induced gene *Gadd45*γ (Ohnuma *et al*., 1999; de la Calle-Mustienes *et al*., 2002; Souopgui *et al*., 2002; Vernon *et al*., 2003). All genes have been shown to be required for neuronal differentiation, but the exact epistatic relationships have not been determined.

1.5 Lateral inhibition

Cells that express Xngnr-1 not only express genes responsible for neural commitment, but at the same time also factors that inhibit the differentiation of the neighboring cell. Xngnr-1 induces expression of the membrane bound signal protein XDelta-1, which binds to the Notch-receptor of the adjacent cell (Fig. 5; Chitnis *et al*., 1995; Chitnis *et al*., 1996; Ma *et al*., 1996, Louvi *et al*., 2006). The binding activates a series of proteolytic cleavages in the signal-receiving cell, which allows the translocation of the Notch intracellular domain (NICD) into the nucleus where it further binds to the Suppressor of Hairless (Su(H)) (Fortini *et al*., 1994; Schroeter *et al*., 1998; Kadesch, 2004). Together, both factors activate the transcription of enhancer of split related (*ESR*) transcription factors that inhibit the expression and the function of Xngnr-1 (Chitnis *et al*., 1996; Ma *et al*., 1996). While the exact mechanism of how a cell escapes Notch signaling remains elusive, it has been shown that the zinc-finger transcription factor MyT1, an Xngnr-1 target gene, is required (Bellefroid *et al*., 1996; Chitnis *et al*., 1996). Results in the mammalian

system suggest that the signal-receiving cell adopts a neuroglial cell fate, whereas the signal-sending cell will become a neuron; if this is conserved in *Xenopus* remains to be determined (Wang *et al*., 2000a; Yoon *et al*., 2005; Louvi *et al*., 2006). Thus, the mechanism of lateral inhibition establishes a “salt and pepper” like pattern of neurons within the proneural domains (Fig. 4, N-tubulin expression; Sasai, 1998).

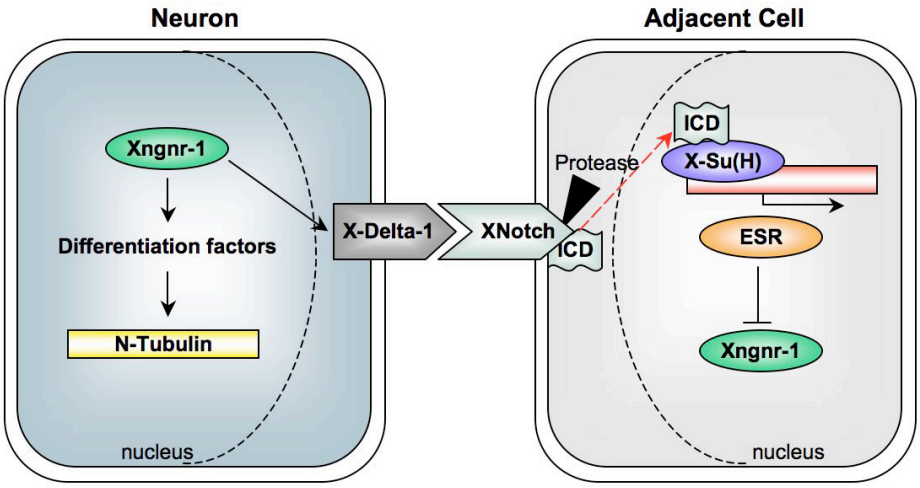


Fig. 5: Scheme of lateral inhibition. Xngnr-1 (green) induces X-Delta-1 (dark gray) and other proneural genes, such as *MyT1*, *Pak3*, *NeuroD* (differentiation factors), which in turn activate transcription of N-tubulin (yellow). The ligand Delta activates the Notch-receptor (light blue) of the neighboring cell, which results in the proteolytic cleavage of the Notch intracellular domain (ICD). That domain translocates into the nucleus and binds to X-Su(H) (purple), which induces the expression of *ESR*-genes (orange). ESR proteins inhibit Xngnr-1 transcription and function. The signal-receiving cell has been suggested to adopt a neuroglial cell fate, while the signal sending cell becomes a neuron.

1.6 Distinct protein-motifs mediate RNA-binding

Development of an embryo requires tight regulation of gene expression. While later developmental events, like cell specification, are widely controlled by complex cascades of transcription factors, early processes like axis determination and germ layer specification derive from a maternal pool of RNA. These RNAs are stored in the oocyte and are translationally silenced up to oocyte maturation and/or the first embryonic cleavages. In most cases, the 3’UTR of the RNA harbors motifs for interaction with RNA binding proteins (RBPs). Many eukaryotic proteins that have

been shown to bind RNA contain one or multiple copies of an RNA-binding region such as the arginine-rich motif (ARM), arginine-glycine-glycine box (RGG), double-stranded RNA-binding motif (DSRM), K homology (KH) or RNA recognition motifs (RRM or RNP) (Lutz-Freyermuth *et al*., 1990; Hall *et al*., 1992; Burd *et al*., 1994). The RRM is the most common and best-characterized domain, consists of 90 to 100 amino acids harboring an RNP1 and RNP2 consensus sequence and has a ß1-α1-ß2-ß3-α2-TI'-ß4 topology (Swanson *et al*., 1987; Dreyfuss *et al*., 1988; Bandziulis *et al*., 1989; Query *et al*., 1989; Kenan *et al*., 1991; Burd *et al*., 1994). Interaction with about four nucleotides of ssRNA through stacking, electrostatics and hydrogen bonding is mediated by the surface of an RRM ß-sheet (Stefl *et al*., 2005; Auweter *et al*., 2006; Lunde *et al*., 2007). In a few studies, the RRM has been shown to further communicate binding to single stranded DNA and protein-protein interaction (DeAngelo *et al*., 1995; Samuels *et al*., 1998; Ding *et al*., 1999; Johnston *et al*., 1999; Kielkopf *et al*., 2001; Selenko *et al*., 2003; Moran-Jones *et al*., 2005; ElAntak *et al*., 2007).

Furthermore, a small number of transcription factors has been reported to interact with RNA, for example the zinc-finger/knuckle transcription factor TFIIIA, which interacts with both, the 5S rRNA gene and 5S RNA (Picard *et al*., 1979; Guddat *et al*., 1990; Theunissen *et al*., 1992). The homeobox containing protein Bicoid harbors an ARM within helix III of its homeodomain that is needed for DNA as well as RNA recognition and has been shown to bind to the Bicoid-binding region (BBR) within the *caudal* 3'UTR, where it directly represses *caudal* translation (Dubnau *et al*., 1996; Rivera-Pomar *et al*., 1996; Niessing *et al*., 2002).

1.7 RNA binding proteins mediate translational activation

One of the best-characterized RRM containing proteins is the poly-adenylate binding protein (PABP) that interacts with the polyA-tail of RNAs and positively regulates translation (Standart *et al*., 1981; Grossi de Sa *et al*., 1988; Bernstein *et al*., 1989; Sachs *et al*., 1989; Sladic *et al*., 2004). PABP has also been shown to play an important role in RNA turnover and transport (Mangus *et al*., 2003). Structurally, PABP consists of four N-terminal RRM motifs and one PABP unique

motif in the C-terminus, which is important for protein-protein interactions (PAPC domain) (Bernstein *et al*., 1989; Siddiqui *et al*., 2007). Furthermore, PABP has been shown to bind to AU-rich elements (ARE) via RRM3 and RRM4, while RRM1 and RRM2 preferentially bind to stretches that contain only adenosines (Kühn *et al*., 1996; Voeltz *et al*., 2001; Bollig *et al*., 2003; Mullin *et al*., 2004; Sladic *et al*., 2004; Khanam *et al*., 2006). AREs can be bound by stabilizing and destabilizing factors that recruit components of the decay machinery such as exosomes and the polyA-specific ribonuclease (PARN) (Bevilacqua *et al*., 2003). Occupation of this motif by PABP is thought to prevent association of factors promoting degradation and so stabilizes the transcript (Chen *et al*., 1995; Chen *et al*., 2001; Lai *et al*., 2003; Gherzi *et al*., 2004).

The mechanism of PABP-function has been extensively studied in the context of translational initiation (Fig. 6). Multiple proteins bind to the polyA-tail of the transcript, the number of PABP proteins associating depends of the length of the homopolymeric polyadenylate tract (Mangus *et al*., 2003). At least 12 adenosines are required for PABP association (Baer *et al*., 1983; Sachs *et al*., 1986; Sachs *et al*., 1987). Responsible for binding to the polyA-tail are RRM1 and RRM2, which have also been shown to promote interaction with the scaffolding protein eukaryotic initiation factor 4G (eIF4G) that is part of the cap binding complex eIF4F (Kühn *et al*., 1996; Tarun *et al*., 1996; Tarun *et al*., 1997; Kessler *et al*., 1998; Imataka *et al*., 1998; Gingras *et al*., 1999; Otero *et al*., 1999; Prévôt *et al*., 2003; Brune *et al*., 2005; Dunn *et al*., 2005; Derry *et al*., 2006; Slepenkov *et al*., 2008). The core of eIF4F is further composed of the cap-binding protein (eIF4E) and the RNA helicase eIF4A. In addition, eIF4B or eIF4H stimulate the activity of eIF4A, functioning as processivity factors for the helicase (Rogers *et al*. 2002). However, the interaction between eIF4G and PABP provides a link between the 5' and 3' termini of the RNA and the "closed loop structure" is formed which enhances the assembly of other factors essential for translational initiation (Jacobson 1996; Tarun *et al*., 1996; Le *et al*., 1997; Amrani *et al*., 2008). The complex of cap-bound eIF4E together with eIF4G directs the 43S preinitiation complex to the 5'UTR, mediated by eIF4G interaction with eIF3, which in turn mediates binding of the 40S ribosomal subunit to Met-tRNA$_i^{Met}$ (Trachsel *et al*., 1977; Benne *et al*., 1978; Emanuilov *et al*., 1978; Gebauer *et al*., 2004). This complex then scans the mRNA until it reaches the start AUG, at which point the complex is revered to as the 48S

initiation complex (de Moor *et al.*, 2005; Piccioni *et al.*, 2005). The 60S ribosomal subunit joins to form the 80S ribosomal particle and translation begins.

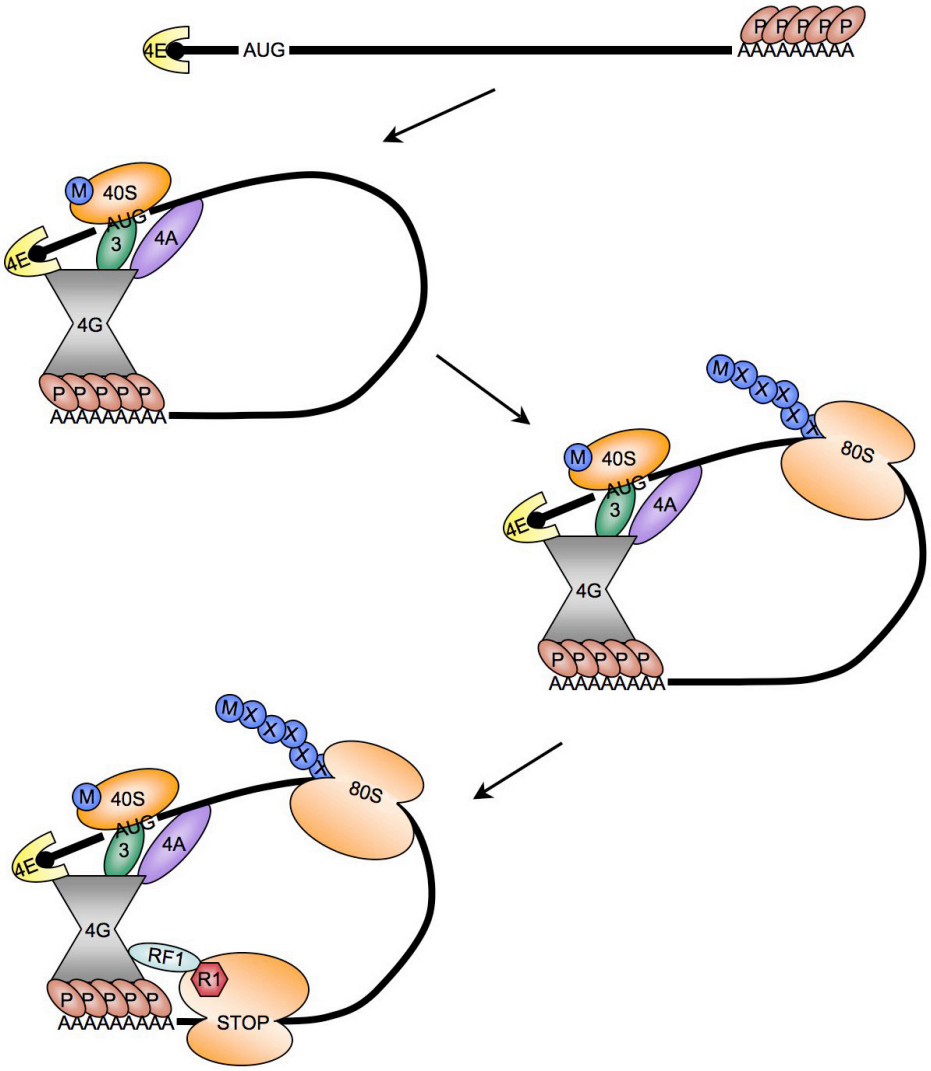


Fig. 6: Canonical translational activation. Eukaryotic initiation factor 4E (4E, yellow) binds to the m7GpppG cap (black circle) of the mRNA, while the polyA-binding protein (P, brown) interacts with the polyA tail. Both proteins are connected by eIF4G (4G, gray), closing the RNA loop, which further recruits eIF3 (green), promoting binding of the 43S subunit consisting of the 40S ribosomal subunit (40S, orange) and methionine (M, blue). Further, eIF4A is recruited (4A, purple). The 80S ribosomal particle is formed (80S, yellow) and translation starts (X indicating any amino acid, blue). Translation is terminated by eRF1 (RF1, light blue) and eRF1 (R1, red).

It has been shown that the PAPC domain can interact with eRF3 (eukaryotic polypeptide chain release factor 3), which is a GTPase that enhances eRF1 function to catalyze translational termination (Hoshino *et al.*, 1999; Welch *et al.*, 2000; Cosson *et al.*, 2002; Uchida *et al.*, 2002). This interaction between PABP

and eRF3 is thought to promote recycling of ribosomes from the 3' to the 5' end, thus facilitating multiple rounds of translation but also may link translation to transcript decay as eRF3 interferes with PABP to multimerize on the polyA-tail (Hoshino *et al*., 1999; Uchida *et al*., 2002). PABP multimerization and formation of the closed loop structure has been proposed to prevent access of deadenylases and can prevent decapping, thus providing enhanced transcript stability and prolonged half-life time (Bernsterin *et al*., 1989; Ross, 1995; Wormington *et al*., 1996; Ford *et al*., 1997; Körner *et al*., 1997; Körner *et al*., 1998; Tourrière *et al*., 2002; Amrani *et al*., 2008).

However, not all transcripts terminate in a polyA tail, like the replication-dependent *histone* mRNAs that end in a conserved stem-loop structure (DeJong *et al*., 2002; Zanier *et al*., 2002). These stem-loops are recognized by hairpin binding proteins (HBP) or stem-loop binding proteins (SLBP) (Marzluff *et al*., 2002). The binding of SLBP to the stem-loop is mediated by a centrally located RRM motif and activates cap-dependent target translation (Wang *et al*., 1996; Wang *et al*., 1999; Ling *et al*., 2002). The mechanism of translational activation by SLBP is not entirely understood, but is suggested to be a functional mimic of PABP (Ling 2002; Sànchez *et al*., 2002; Sànchez *et al*., 2004; Gorgoni *et al*., 2005).

1.8 RNA binding proteins fulfill essential functions during development

RNA binding proteins are involved in virtually all steps of RNA metabolism and play essential roles during embryogenesis. In the oocyte, complexes of several RBPs are required for nuclear export, localization and translational repression of the maternal transcripts, mediating polarity early in development (Guddat *et al*., 1990; Schnapp *et al*., 1997; Oleynikov *et al*., 1998; Reverte *et al*., 2001; Kwon *et al*., 2002; King *et al*., 2005; Kloc *et al*., 2005; Czaplinski *et al*., 2006). Factors such as Vg1RBP/Vera facilitate transport of the target transcripts, while CPEB inhibits translation of the mRNA. RNA binding proteins are also required for later functions in development. The stem-loop binding protein (SLBP) for example has been shown to recognize specific secondary structures (stem-loops) of the *Histone* RNA

and activate its translation during mitosis (Sànchez *et al*., 2002; Zheng *et al*., 2003; Sànchez *et al*., 2004; Gorgoni *et al*., 2005). XDazl encodes an RBP that has been shown to be essential for migration and differentiation, while Dead end is required for migration and survival of primordial germ cells (Houston *et al*., 2000; Weidinger *et al*., 2003; Horvay *et al*., 2006). The zipcode RNA binding proteins mediate transport of transcripts in several tissues including neurons, where they facilitate axonal transport of actin mRNA (Oleynikov *et al*., 1998; Twiss *et al*., 2006; Lin *et al*., 2007). The function of an RNA binding protein in a specific aspect of RNA metabolism appears to be independent of their RNA binding motif. Both, Bicaudal-C in *Drosophila* and Hermes in *Xenopus* oocytes inhibit translation of their target transcripts, while the first harbors a KH domain and the latter an RRM motif (Mahone *et al*., 1995; Saffman *et al*., 1998; Song *et al*., 2007).

Furthermore, RNP function appears to be uncorrelated to the number of RRM motifs within the protein sequence. Several small nuclear ribonucleoprotein particles (snRNPs), which are components of spliceosomes, contain between one to three RRM motifs (Query *et al*., 1989; Lutz-Freyermuth *et al*., 1989; Scherly *et al*., 1990; Keane 1991). The splicing factor sex lethal (sxl, two RRM motifs) binds specifically to the transformer protein 2 (tra-2, one RRM motif), which also encodes a splice factor (Amrein *et al*., 1988; Bell *et al*., 1988; Baker *et al*., 1989; Goralski *et al*., 1989; Inoue *et al*., 1990). Both proteins are required for proper sex determination during *Drosophila* development.

RRM-containing proteins are not only involved in general RNA processing but also display target specificity. Musashi, which harbors two RRM motifs, bindings to uridine-rich elements and has been reported not only to repress but also activate translation of its targets (Imai *et al*., 2001; Sakakibara *et al*., 2001; Okano *et al*., 2002; Sakakibara *et al*., 2002; Okano *et al*., 2005; Charlesworth *et al*., 2006). Another example of such factors are the Elav/HuR proteins, harboring three RRM motifs and recognizing AU-rich elements within the 3'UTR (Peng *et al*., 1998). They have been shown to function as translational repressors, as well as influencing alternative splicing, polyadenylation, export, transcript stability, and localization in several organisms (Abdelmohsen *et al*., 2007; Arthur *et al*., submitted; Hinman *et al*., 2008).

1.9 Messenger RNA stability and decay

While mRNAs are generally relatively stabile, transcripts harboring A/U-rich elements (AREs) in the 3'UTR are more rapidly degraded (Greenberg, 1972; Caput *et al*., 1986; Shaw *et al*., 1986; Chen *et al*., 1995; Brewer, 2002; Bolognani *et al*., 2008). The ARE motif represents the most common and best characterized family of instability sequences and generally promotes deadenylation-dependent decay (Fig. 7). Here, deadenylases, such as PARN, remove the polyA-tract from the 3'UTR to generate a deadenylated transcript, which allows 5' to 3' and/or 3' to 5' degradation by exoribonucleases (Körner *et al*., 1998; Brewer, 2002; Meyer *et al*., 2004; Jacobson, 2004). Removal of the cap structure by decapping enzymes, such as Dcp1, further exposes the RNA to 5' to 3' decay (Jacobson, 2004; Liu *et al*., 2006; Simon *et al*., 2006).

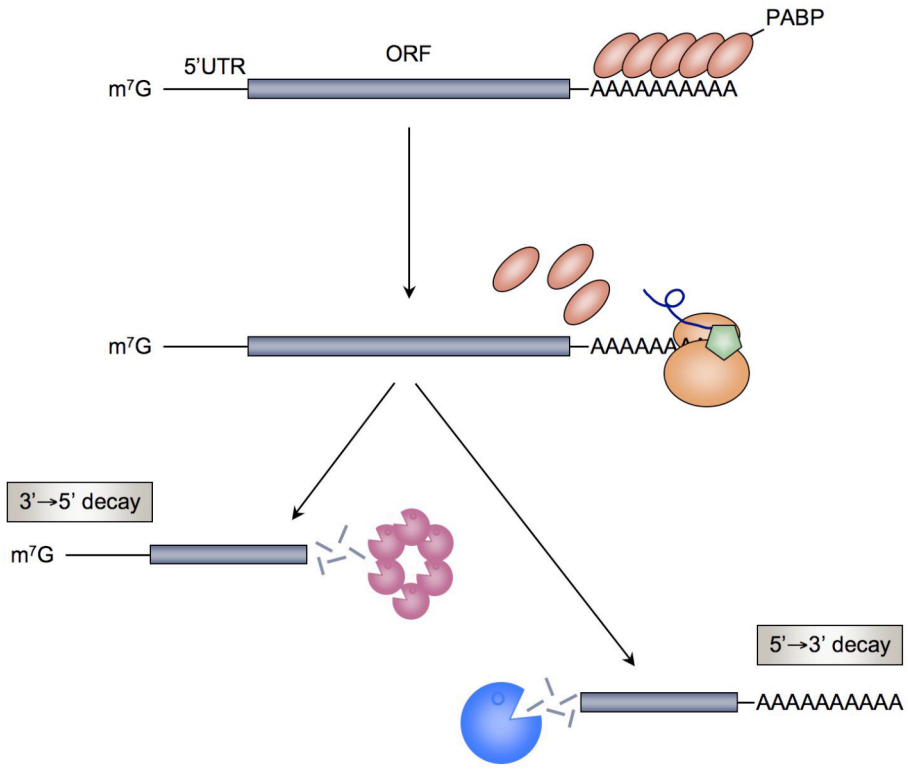


Fig. 7: Deadenylation-dependent RNA degradation. A transcript harboring a cap structure and a polyA-tract are stabile and protected against degradation. Binding of PABP stabilizes the polyA-tail. Relive of PABP renders the A-tail to deadenylating enzymes (pink), which promotes 3' to 5' degradation. Removal of the cap structure by decapping enzymes (blue) further activates 5' to 3' decay (Garneau *et al*., 2007, modified).

Several classes of ARE binding proteins have been identified, among them AUF1/hnRNP, TTP and Elav/HuR, while the latter represent one of the best-characterized class of ARE binding RNPs (Brewer, 1991; Ma *et al*., 1997; Lai *et al*., 1999; Wang *et al*., 2000b; Chen *et al*., 2001; Wilson *et al*., 2001; Brewer, 2002; Gorospe, 2002; Hinman *et al*., 2008). While AUF1 and TTP are involved in transcript destabilization, Elav/HuR proteins facilitate mRNA stability by competition with the destabilizing factors (Hinman *et al*., 2008). Furthermore, stabilization of transcripts by Elav/HuR have been shown to play important roles during developmental events such as muscle cell differentiation and development of the nervous system (Bolognani *et al*., 2007; van der Giessen *et al*., 2007; Hinman *et al*., 2008).

1.10 RNA-binding proteins in neural development

The transcriptional network of neural induction and neuronal differentiation has been extensively studied. Modifications at the mRNA level however, are much less understood. One of the best described families of RNA binding proteins involved in neurogenesis are lethal abnormal vision (Elav/HuR) proteins. In vertebrates, four members have been identified (HuR/HuA, HuB/Hel-N1, HuC/PLE21 and HuD), but only HuB, HuC and HuD are expressed in neuronal tissues (Dalmau *et al*., 1992; Hinman *et al*., 2008). Hu proteins are found to be involved in several developmental processes, like neurite outgrowth, neural plasticity and memory (Kasashima *et al*., 1999; Anderson *et al*., 2000; Mobarak *et al*., 2000; Anderson *et al*., 2001; Quattrone *et al*., 2001; Pascale *et al*., 2004; Akamatsu *et al*., 2005; Bolognani *et al*., 2007). Several neural target mRNAs have been described, like *Gap43*, *Tau*, *neurofilament M*, *GLUT1* and *MYCN* transcripts (Chung *et al*., 1997; Jain *et al*., 1997; Antic *et al*., 1999; Manohar *et al*., 2002; Atlas *et al*., 2004). An additional family of RNA binding proteins involved in neural development is Musashi (Msi). Mammalian Musashi1 and 2 are coexpressed predominantly in proliferating neural precursors and are found to be developmentally regulated (Sakakibara *et al*., 1996; Sakakibara *et al*., 1997; Keyoung *et al*., 2001; Sakakibara *et al*., 2001; Okano *et al*., 2002). While the exact mechanism of Musashi function

remains obscure, it has been implicated in stem-cell maintenance, differentiation and tumorgenesis by repressing translation of specific target transcripts, such as *mNumb* through interaction with its 3'UTR (Wilson *et al*., 1999; Imai *et al*., 2001; Sakakibara *et al*., 2002). The *Xenopus* homologue Xnrp1 has been suggested to play a role in the nucleo-cytoplasmic transport of transcripts (Good *et al*., 1993).

1.11 The Seb-family and XSeb4R

Recently, a novel family of RNA binding proteins has been identified; the Seb-proteins (Anyanful *et al*., 2004). XSeb4R belongs to the Seb-family of RNA binding proteins and harbors one RNA recognition motive in its N-terminus (see also below) (Boy *et al*., 2004). The other described member of this family, XSeb4, reveals high sequence homology within the RRM domain (92%), whereas the C-terminus is less conserved (56% homology) (Fig. 8A; Fetka *et al*., 2000; Boy *et al*., 2004). However, the expression of both proteins is quite divergent (Fedka *et al*., 2000; Boy *et al*., 2004). While XSeb4 expression is located to the mesoderm and later exclusively detected in muscular tissue, XSeb4R displays a more dynamic expression pattern (Fig. 8B). At gastrulation, XSeb4R is expressed in a ring around the blastoporus in the mesoderm, while transcripts at neurula stages are detected in the mesoderm as well as in the territories of primary neurogenesis. Neural expression is maintained until tadpole stages, as during tailbud stages additional stainings are detected in the liver and the tail tip. Overexpression of XSeb4R during primary neurogenesis promotes neuronal differentiation and targeted expression to retinal progenitors induces premature differentiation of ganglion cells (Boy *et al*., 2004).

The XSeb4R homolog in *Caenorhabditis elegans*, Sup-12, has been implicated in alternative splicing of the *unc-60* and *egl-15* mRNA, while the human homologue, RNPC1, appears to be involved in stabilization of the p21 transcript (Anyanful *et al*., 2004; Shu *et al*., 2006; Kuroyanagi *et al*., 2007). The function of XSeb4R in RNA metabolism, however, is unknown.

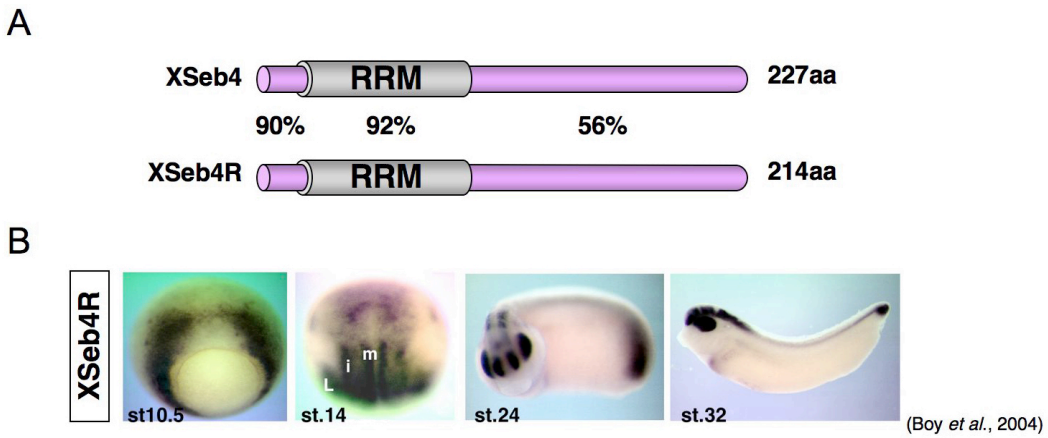


Fig. 8: XSeb4 and XSeb4R in *Xenopus*. (A) Protein sequence comparison of XSeb4 and XSeb4R. Identical amino acids of the different domains are indicated (N-terminus 90%, RRM motif 91.9%, C-terminus 55.7%), as well as protein length. **(B)** Spatial expression of XSeb4R determined by whole mount *in situ* hybridization. m: medial stripe, i: intermediate stripe, L: lateral stripe.

1.12 Aims

RNA binding proteins are involved in all aspects of RNA metabolism and have been extensively studied in the context of general processing and turnover of transcripts. However, very few examples of target specific RNA binding proteins involved in embryogenesis have been characterized so far. XSeb4R is expressed in the territories of primary neurogenesis and the previous functional studies revealed proneural activities. Expression of *XSeb4R* in the mesoderm suggests an additional function in embryogenesis besides a role in neural development. Therefore, a molecular characterization of XSeb4R function in RNA metabolism was performed and target RNAs identified.

2. Material and Methods

2.1 Organisms

2.1.1 *Xenopus laevis*

The African clawed frog *Xenopus laevis* was used as experimental organism. Albino and pigmented frogs were purchased from NASCO (Ft. Atkinson, USA). Staging of the embryos was according to Nieuwkoop and Faber (1967).

2.1.2 *Escherichia coli*

XL1-Blue recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac[F' proAB, laclqZDM15, Tn10(Tetr)]c (Bullock *et al*., 1987)

BL21 Star (DE3)pLysS: F- *ompT hsdS*B (rB-mB-) *gal dcm rne131* (DE3) pLysS (Cam^R) (Invitrogen)

BL21 Star (DE3): F- *ompT hsdS*B (rB-mB-) *gal dcm rne131* (DE3) (Invitrogen)

2.2 Kits

All kits were used according to the manufacturer's protocol.

RNeasy Kit (Qiagen); Miniprep Kit (Qiagen); Midiprep Kit (Qiagen); Nucleotide Removal Kit (Qiagen); Oligotex™ mRNA Spin-Column (Qiagen); mMessagemMachine Sp6/T3/T7 Kit (Ambion); NucAway Spin Columns (Ambion); RNAqueous®-Micro Kit (Ambion); TnT® Coupled Reticulocyte Lysate System (Promega); Dual-Luciferase Reporter Assay System™ (Promega)

2.3 Oligonucleotides

2.3.1 RT-PCR oligonucleotides

Table 1: RT-PCR oligonucleotides

primer	orientation	sequence	T_A	cycles
Firefly Luc	for	5´-GCA ACTGCATAAGGCTATG-3´	56°C	26
Firefly Luc	rev	5´-CGTGTACATCGACTGAAATC-3´	56°C	26
GATA 4	for	5´-GTGCCACCTATGCAAGCCC-3´	56°C	30
GATA4	rev	5´-TAGACCCACCCGGCGAGAC-3´	56°C	30
GATA 6	for	5´-CAGTCTATGTGCCCACTAGC-3´	60°C	35
GATA 6	rev	5´-GACGGCTGTGAAGCATAGTG-3´	60°C	35
G14	for	5´-GTTGTACTTTCACCTTGCCG-3´	60°C	38
G14	rev	5´-CTTCGGGTGAAAGCAGC-3´	60°C	38
H4	for	5´-CGGGAT AACATTCAGGGTATCACT-3´	56°C	25
H4	rev	5´-ATCCATGGCGGTAACTGTCTTCCT-3´	56°C	25
N-tubulin	for	5´-ACACGGCATTGATCCTACAG-3´	56°C	32
N-tubulin	rev	5´-AGCTCCTTCGGTGTAATGAC-3´	56°C	32
ODC	for	5´-GCCATTGTGAAGACTCTCTCCATTC-3´	56°C	25
ODC	rev	5´-TTCGGGTGATTCCTTGCCAC-3´	56°C	25
Renilla Luc	for	5´-GGCCAGATGTAAACAAATG-3´	56°C	33
Renilla Luc	rev	5´-CATCCCATGAATCAATCAC-3´	56°C	33
Sox3	for	5´-GCGCACATGAACGGCTGGACTA-3´	56°C	25
Sox3	rev	5´-GTGTGGGAGGTGATGGCTGGAG-3´	56°C	25
Sox17ß	for	5´-TATCAGTCCCAGAAGACGGTC-3´	56°C	32
Sox17ß	rev	5´-CATGTCACATCCACAAGAGTG-3´	56°C	32
VegT RT	for	5´-CAAGTAAATGTGAGAAACCG-3´	56°C	29
VegT RT	rev	5´-CAAATACACACACATTTCCC-3´	56°C	29
Wnt8	for	5´-TGTGGCCGGGTCTGAACTTATTTT-3´	56°C	32
Wnt8	rev	5´-GTCATCTCCGGTGGCCTCTGTTCT-3´	56°C	32
Xbra	for	5´-GGATCGTTATCACCTCTG-3´	56°C	26
Xbra	rev	5´-GTGTAGTCTGTAGCAGCA-3´	56°C	26
X-ngnr-1	for	5´-CAAGAGCGGAGAAACTGTGT-3´	56°C	35
X-ngnr-1	rev	5´-GAAGGAGCAACAAGAGGAAG-3´	56°C	35

2.3.2 General oligonucleotides

Table 2: General oligonucleotides

primer	sequence	T_A
GR9	5´-ACCTCCAACAGTGACACCAGG-3´	56°C
RR67	5´-GGAGAGCTTGGGCGACCTCACC-3´	56°C
SeqCMVLuc	5´-CTTACCGGAAAACTCGAC-3´	56°C
SeqMS2	5´-GGCCATCGCAGCAAAC-3´	56°C
SP6	5´-TATTTAGGTGACACTATAG-3´	56°C
T3	5´-AATTAACCCTCACTAAAGGG-3´	56°C
T7	5´-TAATACGACTCACTATAGGGCGA-3´	56°C
T7 (pCS2+)	5´-TCTACGTAATACGACTCACTATAG-3´	56°C

2.4 Constructs

2.4.1 Overexpression constructs

Flag-XSeb4RpCS2+ (Boy *et al*., 2004); **NLS LacZ** (Chitnis *et al*., 1995); **MT-GFP** (Klymkowsky, 1999); **pLucMS2** (Collier *et al*., 2005); **XPABPpETMS2** (Gray *et al*., 2000); **PV-IRES-Luc-MS2** (Gorgoni *et al*., 2005); **CrPv-IRES-Luc-MS2** (Gorgoni *et al*., 2005); **CSFV-IRES-Luc-MS2** (Gorgoni *et al*., 2005); **His-XSeb4R** (Suoupgui *et al*., 2008); **GST-XSeb4R** (Suoupgui *et al*., 2008)

Flag-XSeb4RΔRRMpCS2+ harbors the truncated coding sequence of *XSeb4R* (accession-number AY289193) lacking 57 aa of the N-terminus containing the RNA recognition motif (RRM). The fragment was generated by PCR amplification using XSeb4RpCS2+ (Boy *et al*., 2004) as template, 5'EcoRI Seb4R ΔRRM CC GAA TTC CAT GTC AGA CGA GCT and 3'XhoI Seb4R CCC CTC GAG TTA CTG CAT CCG GTC AGG CTG as primers and inserted into the EcoRI/XhoI sites of Flag-pCS2+ (D. Turner and R. Rupp, http://sitemaker.umich.edu/dlturner.vectors). For sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

MS2-XSeb4RpET harbors the full coding sequence of *XSeb4R* (accession-number AY289193). The fragment was generated by PCR amplification using XSeb4RpCS2+ as template, Seb4R BamHI F new CC GGA TCC C ATG CAC ACC and ΔRRM R BamHI CG GGA TCC TTA CTG CAT CCG GTC as primers and inserted into the BamHI site of pETMS2 (Coller *et al*., 1998) For sense RNA, the construct was linearized with HindIII and RNA transcribed with T7 RNA polymerase.

MS2-XSeb4RΔRRMpET harbors the truncated coding sequence of *XSeb4R* (accession-number AY289193) lacking 57 aa of the N-terminus containing the (RRM). The fragment was generated by PCR amplification using XSeb4RpCS2+ as template, ΔRRM F BamHI new CC GGA TCC G CTT GCG TAC TTA GGA G and ΔRRM R BamHI CG GGA TCC TTA CTG CAT CCG GTC as primers and inserted into the BamHI site of pETMS2 (Coller *et al*., 1998) For sense RNA, the construct was linearized with HindIII and RNA transcribed with T7 RNA polymerase.

MS2-XSeb4RRRMpET harbors RRM of *XSeb4R* (accession-number AY289193) lacking the C-terminal coding sequence. The fragment was generated by PCR amplification using XSeb4RpCS2+ as template, Seb4R F BamHI new CC GGA TCC G CTT GCG TAC TTA GGA G and XSeb4R RRM R BamHI CG GGA TCC GTT GAC GTT AGC TTT G as primers and inserted into the BamHI site of pETMS2 (Coller *et al*., 1998) For sense RNA, the construct was linearized with HindIII and RNA transcribed with T7 RNA polymerase.

VegT 3'UTRpBKCMVLuc harbors the complete 3'UTR of *VegT* (accession-number AAB93301). The fragment was generated by PCR amplification using VegTpSPORT1 as template, VegT 3' UTR 5' BamHI CC GGA TCC TCC TAA ATG GGT TAA GGG and VegT 3' UTR 3' NotI CC GCG GCC GC TTT GAA ATA AGG AAA AC as primers and inserted into the BamHI/NotI sites of pBKCMVLuc. For sense RNA, the construct was linearized with NotI and RNA transcribed with T3 RNA polymerase.

VegT 3'UTRpBKCMV harbors the complete 3'UTR of *VegT* (accession-number AAB93301) and was generated by restriction of VegT 3'UTRpBKCMVLuc with SpeI and SacI that excises the firefly luciferase coding sequence. Subsequently, the construct was treated with a KLENOW fragment and religated.

VegT 3'UTR F1 pBKCMVLuc harbors the partial 3'UTR of *VegT* (accession-number AAB93301). The fragment was generated by PCR amplification using VegT3'UTRLuc as template, VegT 3'UTR III BamHI FWD CCC GGA TCC ATG GGT TAA GGG AAA TGT G and VegT 3'UTR III NotI RWD CC GCG GCC GC AGG GGC AAC CTC TTT G as primers and inserted into the BamHI/NotI sites of pBKCMVLuc. For sense RNA, the construct was linearized with NotI and RNA transcribed with T3 RNA polymerase.

VegT 3'UTR F2 pBKCMVLuc harbors the partial 3'UTR of *VegT* (accession-number AAB93301). The fragment was generated by PCR amplification using VegT3'UTRLuc as template, VegT 3'UTR II BamHI FWD CCC GGA TCC GTG CTT GTG ATC AGG and VegT 3'UTR II NotI RWD CC GCG GCC GC GAA CAC CAA ATT TTG C as primers and inserted into the BamHI/NotI sites of pBKCMVLuc. For sense RNA, the construct was linearized with NotI and RNA transcribed with T3 RNA polymerase.

VegT 3'UTR F3 pBKCMVLuc harbors the partial 3'UTR of *VegT* (accession-number AAB93301). The fragment was generated by PCR amplification using VegT3'UTRLuc as template, VegT 3'UTR I BamHI FWD CCC GGA TCC CAT CTA AAG CAA AGC and VegT 3'UTR I NotI RWD CC GCG GCC GC TTT GAA ATA AGG AAA AC as primers and inserted into the BamHI/NotI sites of pBKCMVLuc. For sense RNA, the construct was linearized with NotI and RNA transcribed with T3 RNA polymerase.

VegT 3'UTR F3 multipGEMTeasy harbors the partial 3'UTR of *VegT* (accession-number AAB93301). The fragment was generated by PCR amplification using VegT3'UTRILuc as template, VegT 3' UTR I BglII FW: CC AGA TCT CAT CTA AAG CAA AGC and VegT 3' UTR I BamHI NotI: CA GCG GCC GC GGA TCC TTT GAA ATA AGG A as primers and inserted into pGEMTeasy.

VegT 3'UTR F3 multipBKCMVLuc harbors three repeats of *VegT* 3'UTR F3 (accession-number AAB93301). The insert is obtain by BglII/NotI restriction of VegT3'UTRImultipGEMTeasy and ligation into the BamHI/NotI sites of pBKCMVLuc. The next insert is cloned into the BamHI/NotI sites introduced by the previous insert. For sense RNA, the construct was linearized with NotI and RNA transcribed with T3 RNA polymerase.

Xngnr-1 3'UTRpBKCMVLuc harbors the complete 3'UTR of *Xngnr-1* (accession-number U67778). The fragment was generated by PCR amplification using pCL2EGFP-ngn3' 1.5 (KH38) as template, Ngn3'UTR FW BamHI CAC GGA TCC ACT CCT GTT GGA CTA TG and Ngn3'UTR RW NotI CC GCG GCC GC TCG ACT CGA TCA CC as primers and inserted into the BamHI/NotI sites of pBKCMVLuc. For sense RNA, the construct was linearized with NotI and RNA transcribed with T3 RNA polymerase.

Xngnr-1A 3'UTRpBKCMVLuc harbors the complete 3'UTR of *Xngnr-1* (accession-number U67778). The fragment was generated by PCR amplification using Xngnr-1 3'UTRpBKCMVLuc as template, Ngn3'UTR FW BamHI CAC GGA TCC ACT CCT GTT GGA CTA TG and Ngn3'UTR A NotI RW CT GCG GCC GC GAA GTT TTG GTT TGA C as primers and inserted into the BamHI/NotI sites of pBKCMVLuc. For sense RNA, the construct was linearized with NotI and RNA transcribed with T3 RNA polymerase.

Xngnr-1B 3'UTRpBKCMVLuc harbors the complete 3'UTR of *Xngnr-1* (accession-number U67778). The fragment was generated by PCR amplification using Xngnr-1 3'UTRpBKCMVLuc as template, Ngn3'UTR FW B BamHI CAC GGA TCC CTC TCT GAT GTG CAC and Ngn3'UTR RW NotI CC GCG GCC GC TCG ACT CGA TCA CC as primers and inserted into the BamHI/NotI sites of pBKCMVLuc. For sense RNA, the construct was linearized with NotI and RNA transcribed with T3 RNA polymerase.

Xngnr-1C 3'UTRpBKCMVLuc harbors the complete 3'UTR of *Xngnr-1* (accession-number U67778). The fragment was generated by PCR amplification using Xngnr-1 3'UTRpBKCMVLuc as template Ngn3'UTR C BamHI FW CAC GGA TCC GGT AAT GTC ATT TGA and Ngn3'UTR C NotI RW CT GCG GCC GC CAA CAT GTC TTG CTG as primers and inserted into the BamHI/NotI sites of pBKCMVLuc. For sense RNA, the construct was linearized with NotI and RNA transcribed with T3 RNA polymerase.

Xl G14pCS2+ harbors the full coding sequence of *Xl G14* (accession-number BC086468; obtained from the ImaGENES, IRBHp990B0572D in pCMV-SPORT6). The fragment was generated by PCR amplification using Xl G14pBSK as template, Xl G14EcoRI+1 FWD CA GAA TTC A ATG AGC GGC TCT AC and Xl G14XbaI RW CC TCT AGA TTT CTT GTT GAA GGC as primers and inserted into the EcoRI/XbaI sites of pCS2+. For sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

Xl G14 3'UTRpBKCMVLuc harbors the complete 3'UTR of *XL G14* (accession-number BC086468). The fragment was generated by PCR amplification using IRBHp990B0572D in pCMV-SPORT6 (ImaGENES) as template G14 3'UTR BamHI FW CCC GGA TCC GTT GTA CTT TCA CC and G14 3'UTR NotI RW CA GCG GCC GC TTT AAG AAA AAT GG as primers and inserted into the BamHI/NotI sites of pBKCMVLuc. For sense RNA, the construct was linearized with NotI and RNA transcribed with T3 RNA polymerase.

2.4.2 Constructs for whole mount *in situ* hybridization

N-tubulin (Chitnis *et al*., 1995); **X-ngnr-1** (Ma *et al*., 1996)

Sox3 pBSK harbors the full length ORF of *Xenopus Sox3* (accession-number Y07542) and was obtained in a cDNA library screen for *XSox1* (accession-number NM_001095674)(unpublished).

Xl G14pBSK harbors the full open reading frame of *Xl G14* (accession-number BC086468). Xl G14 was obtained by restricting IRBHp990B0572D (in pCMV-SPORT6; ImaGENES) with SalI and NotI. The insert was ligated into the SalI and NotI sites of pBSK.

2.5 Sequence analysis

MayMolly Tera, Version 3.10 (Analyze and Align) (Soft gene GmbH, Berlin) and EnzymeX Version 3 (Mekentosj.com, Amsterdam) were utilized for the analysis of DNA- and protein sequences. For gene- and protein annotation the following BLAST programs were used:
GenBank-Sequenzen (http://www.ncbi.nlm.nih.gov/BLAST/), *Xenopus laevis* EST-Projekt (http://*Xenopus*.nibb.ac.jp/), TIGR *Xenopus laevis* Gene Index (XGI) (http://www.tigr.org/tdb/tgi/xgi/index.html), *Xenopus tropicalis* Genomprojekt (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html) and Ensembl *Xenopus tropicalis* (http://www.ensembl.org/*Xenopus*_tropicalis/index.html).

2.6 Embryo culture and microinjections

Agar dishes: 60 mm Petri dishes, coated with 0.7% agar made with 0.8X MBS AC
Dejelly solution: 2% (w/v) L-cysteine hydrochloride in 0.1X MBS, pH 8.0
Ficoll: 10% (w/v) Ficoll
HCG: 2000 U/mL human chrorionic gonadotropin (HCG) (Sigma)
Injection buffer: 1% (w/v) FICOLL in 1X MBS
10X MBSH Salts: 880 mM NaCl, 10 mM KCl, 10 mM $MgSO_4$, 25 mM $NaHCO_3$, pH 7.8
5X MBSH AC: 880 mM NaCl, 10 mM KCl, 10 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.05 mM $CaCl_2$, 1.65 mM $Ca(NO_3)_2$, pH 7.8
1X MBSH: 1X MBS Salts, 0.7 mM $CaCl_2$
Nile blue staining: 0.01% (w/v) Nile Blue chloride, 89.6 mM Na_2HPO_4, 10.4 mM NaH_2PO_4, pH~7.8

Embryos were obtained from female, adult *Xenopus laevis* by HCG induced egg-laying using 800 U HCG. Spawn was *in vitro* fertilized and embryos staged according to Nieuwkoop and Faber, 1956. Embryos were dejellied and injected in one blastomere of the two-cell stage or one dorsal of the four-cell stage as described. As lineage tracer, 75 pg nuclear *lacZ* mRNA was coinjected. For ectodermal explants and western blotting experiments, both blastomeres were injected omitting nuclear *lacZ* mRNA.
Animal caps were dissected from stage 8.5-9 embryos in agar dishes in 0.8X MBSH AC. Animal caps were cultured in 0.8X MBSH AC in agar dishes until sibling controls reached the desired stage, then shock frozen and stored at -80°C.

2.7 Oocyte preparation

Collagenase-buffer: 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES pH 7.5
10X MBSH Salts: 880 mM NaCl, 10 mM KCl, 10 mM $MgSO_4$, 25 mM $NaHCO_3$, pH 7.8
1X MBSH: 1X MBSH Salts, 0.7 mM $CaCl_2$

To isolate the oocytes, adult, female *Xenopus laevis* were operated on under anesthesia with 0.25% 3-aminobenzoic acid ethyl ester solution (20 min anesthesia). Oocytes were separated by treatment with 1 mg/ml liberase blendzyme (Roche) in collagenase-buffer and agitation up to 120 min. Oocytes were extensively washed using 1x MBSH, incubated at 18°C and staged according to Dumont, 1972.

2.8 MS2 tethering assay

2.8.1 Oocytes

Stage V-VI oocytes were injected with 50 nl (500 ng/µl) of *MS2-XSeb4R* mRNA and incubated for 5 to 6 hours at 18°C. Accordingly, 10 nl (10 ng/µl) *Luc-MS2* and 10 nl (0.35 ng/µl) *Renilla luciferase* mRNA were injected and incubated over night at 18°C. Two batches of 15 oocytes were collected, frozen in liquid nitrogen and assayed for luciferase activity with the Dual-Luciferase Reporter Assay System™ (Promega) following the manufacturer's protocol.

2.8.2 Embryos

Stage 2 embryos were injected with 100 pg/blastomere of *MS2-XSeb4R* mRNA together with 100 pg/blastomere *Luc-MS2* and 25 pg/blastomere *Renilla* l*uciferase* mRNA. Embryos were cultured over night at 18°C. Two batches of 15 embryos were collected and analyzed as described above.

2.9 RNA Methods

2.9.1 RNA extraction and cDNA synthesis

2.9.1.1 Total RNA extraction of animal cap explants

Total RNA was isolated with the RNAqueous®-Micro Kit (Ambion). To lyse the cells, 20 to 50 animal caps or three whole embryos were macerated with a 29-Gauge syringe in 100 µl lysis buffer and centrifuged at maximum speed for 2 minutes. The lysis buffer containing the total RNA was removed without debris. Further purification was performed following the manufacturer's protocol. The RNA was eluted in 30 µl, 75°C pre-heated elution buffer.

2.9.1.2 Total RNA extraction of embryos

Total RNA was extracted using the TRIZOL reagent (Invitrogen). To lyse the cells, 10 to 13 whole embryos were macerated with a 24- followed by a 29-Gauge syringe in 400 µl TRIZOL reagent and centrifuged at maximum speed for 2 minutes. The reagent containing the total RNA was removed without any debris. Further purification steps were performed following the manufacturer's protocol. The RNA was re-suspended in 50 µl RNase-free H_2O and subject to DNAseI

treatment for one hour at 37°C to digest genomic DNA. DNAseI was inactivated by heating the sample to 70°C for 10 minutes followed by treatment with 1/10 vol. DNAse inactivation solution (Ambion).

2.9.1.3 Total RNA extraction of oocytes

5x RNA extraction buffer: 250 mM Tris-HCl (pH 7.5), 25 mM EDTA (pH 8.0), 400 mM NaCl, 2.5% SDS

Xenopus total RNA of collagenated stage V to VI oocytes was extracted by macerating 10 oocytes with a 24- and 29-Gauge syringe in 1 ml of 1x extraction buffer containing 1.25 µl Proteinase K. Following 45 minutes incubation at 37°C, the sample RNA was further extracted with the TRIZOL reagent (as described above). The pellet was resuspended in 30 µl RNase-free H_2O.

2.9.1.4 Enrichment of polyA+ RNA from total RNA

PolyA+ RNA was isolated from total RNA using the Oligotex™ mRNA Spin-Column (Qiagen) following the manufacturer's protocol. 0.75 mg total RNA was added to 45 µl of the Oligotex suspension and eluted two times with 40 µl elution OEB (Qiagen).

2.9.2 *In vitro* synthesis of RNA

2.9.2.1 Capped sense RNA

For synthesis of capped mRNA used for microinjection, the mMessage-mMachine™ Kit (Ambion) was used according to the manufacturer's protocol. In a 20 µl reaction, 1 µg linearized plasmid was used. Transcription was carried out at 37°C for 3 hours. The DNA template was removed by addition of 5 U DNaseI to the reaction mix and incubation at 37°C for 30 min. The reaction was purified with the RNeasy™ Mini Kit (Qiagen), eluted in 30 µl of RNase-free H_2O and 2 µl aliquots stored at -80°C.

2.9.2.2 Antisense RNA

Preparation of antisense RNA for probes in whole mount *in situ* hybridization, 1 µg linearized template was used in a total reaction volume of 25 µl containing 1 mM ATP, 1 mM GTP, 1 mM CTP, 0.64 mM UTP, 0.36 mM digoxigenin-UTP, 0.03 µM DTT, 1.6 U RNase OUT (Invitrogen), 0.05 U Pyruphosphatase, 0.8 U RNA polymerase in 1X transcription buffer. After 3 hours at 37°C, the DNA template was digested by addition of 5 U DNAseI. The mix was incubated at 37°C for 30 min and purified using the RNeasy Mini Kit (Qiagen). The RNA was eluted twice with 50 µl RNase-free H_2O and stored in Hybridization Mix (see whole mount *in situ* hybridization) at -20°C.

2.9.3 XSeb4R target RNA identification - cloning of small, low abundant RNA-fragments

2.9.3.1 RNA Immuno-Precipitation (RIP)

DEPC-H_2O: 1 ml DEPC per 1 L H_2O, dissolve with mixing, autoclave
RNA-IP buffer: 0.1% Nonidet-P40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM NaF, 1 mM DTT, 10 mM EDTA, 40 U/100 µl buffer RNase Out (Invitrogen)

Around 10.000 *Xenopus* embryos were injected with 100 pg FLAG-XSeb4R mRNA and allowed to develop until open neural plate stage (stage 13 to 15). Embryos were cross-linked in 0.1% PBS-FA for 10 minutes at room temperature followed by two times washing in 1x PBS. Aliquots of 100 embryos were frozen in liquid nitrogen and stored at -80°C. RNA-protein-complexes were extracted by maceration using a 24- and 29-Gauge syringe of the embryos in 1 ml of RIP-lysis buffer. Samples were centrifuged for 5 minutes at maximum speed at 4°C. The supernatant was transferred into a new reaction tube and pre-incubated with 10 µl RNase-free γ-Sepharose (Amersham) for 15 minutes at 4°C on a rotating disc to avoid precipitation of proteins binding unspecifically to the Sepharose beads. Subsequently, the samples were centrifuged for 5 minutes at 3,000 rpm at 4°C, the supernatant transferred into a new reaction tube and incubated with 2 µg anti-FLAG antibody (0.8 µg/µl, Sigma) for 2 hours at 4°C on a rotating disc. 15 µl of γ-Sepharose was added and further incubated at 4°C on the rotating disc for another hour. Beads were centrifuged at 3.000 rpm for 5 minutes at 4°C, the supernatant removed without disturbing the pellet and beads washed three times with 100 µl lysis buffer. The crosslink of RNA to protein was reversed by heating the sample to 70°C for 45 minutes in 25 µl RNase-free 50 mM Tris-HCl (pH 7.5). RNA was extracted using the TRIZOL reagent (see above) and a DNaseI digestion was performed. Quality was determined by a pCp 3' labeling of the RNA. Therefore, 20 µl RNA was labeled with 0.1 µl $^{32}\gamma$P Cytidine-3'-,5'-Bisphosphate (pCp, Amersham, 10 µCi/µl) using T4-RNA-Ligase in 1x T4-RNA-Ligase buffer (Fermentas), 1 mM ATP and 12% DMSO overnight at 4°C. Unincorporated pCp

was removed by purification over an RNeasy column (Qiagen) following the manufacturer's protocol and eluted with 30 µl RNase free H_2O.

2.9.3.2 Adaptor labeling

5' Adaptor: 5'-ACGGAATTCCTCACTaaa-3' (Prologo)
3' Adaptor: 5'-uuuAACCGCGAATTCCAg (Amin)-3' (Proligo)

Both Adaptors consist of 15 desoxyribonucleotides (capital letters) and three ribonucleotides (small letters, underlined). The 3' Adaptor harbors in addition a 3' amin block (Amin). Two µl of 3' and 5' Adaptor (10 µM) were 5' phosphorylated using 20 U T4-Polynucleotide Kinase (T4 PNK, 10 U/µl, Fermentas), 2 µl 10x PNK buffer A (Fermentas), 1 µl $^{32}\gamma$ATP (10µCi/µl, Amersham) and 1 µl RNase Out (Invitrogen). The reaction was incubated for 1 hour at 37°C. Unincorporated ATP was removed by purification utilizing NucAway spin columns (Ambion) following manufacturer's protocol. Labeled adaptor was purified on a 10% denaturing polyacrylamide gel electrophoresis (PAGE), the adaptor excised with a scalpel and incubated shaking in 3 volumes of 0.3 M NaCl for 1 hour at 50°C followed by centrifugation at maximum speed for 1 minute. Hereafter, the Supernatant was transferred into a 2 ml reaction tube and precipitated with 1 ml isopropanol for I hour at -80°C. The elution step was repeated and precipitated over night at -20°C. Pellets were washed twice with 500 µl 70% ethanol, both fractions were pooled during the second washing step. The dried pellet was resuspended in 20 to 30 µl RNase free H_2O.

2.9.3.3 3' Adaptor and 5' Adaptor ligation

Prior to ligation the RNA was treated with alkaline phosphatase (CIAP, Fermentas) to avoid circulation of the RNA as product of the ligation reaction. 25 µl RNA was incubated with 1 µl CIAP (10 U/µl, Fermentas), 3 µl 10x CIAP buffer (Fermentas) and 1 µl RNase Out (40 U/µl, Invitrogen) for 1 hour at 37°C. RNA was purified on a

10% denaturing PAGE and 18 fractions excised from the gel. Further purification was carried out as described above. The pellet was resuspended in 25 µl RNase free H_2O. Ligation was performed in a total volume of 40 µl, containing 23.2 µl RNA, 2 µl of the recovered, labeled 3' Adaptor, 1x T4-RNA-Ligase buffer (Fermentas), 1 mM ATP, 12% DMSO, 1 µl RNase Out and 2 µl T4-RNA-Ligase (20 U/µl, Fermentas) overnight at 4°C. Removing unligated Adaptor, RNA was purified on a 10% denaturing PAGE as described above and the pellet resuspended in 25 µl RNase free H_2O. To enable 5' Adapter ligation it was essential to transfer a phosphate back to the 5' end of the RNA, which was accomplished by T4-PNK treatment as described above except the use of 3 µl ATP (1 mM). The reaction was purified with NucAway spin columns (Ambion) following the manufacturer's protocol. 5' Adaptor was ligated as described above and purified using NucAway spin columns.

2.9.3.4 Amplification and sub-cloning of RNA-fragments

RNA was translated into cDNA by Reverse Transcription (RT). Five µl of the primed RNA was incubated with 1 µl of 100 µM RNase free 3' Adaptor primer and 8 µl of RNase free H_2O for 2 minutes at 80°C followed by short cooling on ice. 3 µl 10x PCR-buffer II (Qbiogene), 7 µl dNTPs (2 mM each base) and 6 µl $MgCl_2$ (25 mM) were added and heated to 48°C for 2 minutes. As negative control 3 µl of the reaction were saved and 3 µl MuLV Reverse Transcriptase (50 U/µl, Roche) added to the remaining reaction that was carried out as described above. Subsequently 10 µl cDNA were taken for amplifying in a polymerase chain reaction (PCR) with 4 µl 10x PCR-buffer II, 1.25 µl $MgCl_2$ (25 mM), 0.5 µl 5' Adaptor primer (100 µM), 0.5 µl 3' Adaptor primer (100 µM), 32.75 µl H_2O and 1 µl Taq (Qbiogene). Initial denaturation was performed for 60 s at 94°C, followed by 50 cycles of denaturation (45 s at 94°C), annealing (45 s at 50°C) and elongation (2 min at 72°C). Obtained fragments were purified on a 3% agarose gel. Each fraction was subcloned into the pGEMTeasy vector (Promega) using 0.5 µl vector, 2.5 µl 2x Ligation buffer (Promega), 0.5 µl T4-DNA-Ligase (3 U/µl, Promega) and 1.5 µl cDNA fragments. The reaction was incubated for at least 2 hours at room temperature followed by transformation into chemical competent bacteria as described above. Positive

clones were identified by blue-white screen of the obtained colonies with a subsequent test digest using the vector internal EcoRI sides. XSeb4R targets were annotated by sequencing, performed as described above.

2.9.3.5 XSeb4R RNA Immuno-precipitation (RIP) with subsequent RIP-RT-PCR

GST-XSeb4R and GST-XSeb4RΔRRM were prepared as described below. The total lysate was incubated for 1 hour at 4°C on a rotating disc with 400 µl Glutathione agarose (Sigma) that was treated according to the manufacturer's protocol. Beads were centrifuged 5 minutes at 3,000 rpm at 4°C and washed 3 times with 1 ml 1x RIP binding buffer. To avoid unspecific binding of RNA to agarose and protein, the beads were pre-incubated with 40 µg tRNA (5 mg/ml) for 30 minutes at 4°C on a rotating disc followed by 3 washing steps with 1 ml 1x RIP binding buffer. Likewise, total and polyA+ RNA, purified as described above, was pre-cleared with 20 µl Glutathione agarose at 4°C for 30 minutes on a rotating disc. 50 µl of GST-fusion proteins attached to the Glutathione agarose was incubated with either 10 µg of polyA+ RNA or 40 µg total RNA for 2 hours at 4°C on a rotating disc followed 3 times washing with 500 µl 1x RIP binding buffer. Bound RNA was extracted using TRIZOL reagent (Invitrogen) as described above. For cDNA synthesis 4 µl RNA was incubated together with 2 µl $MgCl_2$ (25 mM), 1.0 µl 10x PCR buffer II (Qbiogene), 0.4 µl dNTPs (25 mM), 0.5 µl Random Hexamer (50 µM), 0.5 µl Oligo dT Primer (50 µM), 0.2 µl RNase Out (40 U/µl), 0.4 µl MuLV (50 U/µl, Roche) and 1.5 µl RNase free H_2O. The RT reaction and the subsequent PCR were carried out as described above.

2.9.4 Nucleic acid binding assay

Na_2HPO_4: 1 M Na_2HPO_4*2 H_2O

NaH_2PO_4: 1 M NaH_2PO_4*1 H_2O

IP-buffer: 10 mM Tris-HCl pH 7.5, 2.5 mM $MgCl_2$, 0.5% Triton X-100, 0.2 M NaCl

Phosphate buffer: Na_2HPO_4 and NaH_2PO_4 were mixed to pH 7.3

Tris-EDTA (TE): 1 M Tris-HCl pH 7.5, 0.5 M EDTA

Poly (A) and Poly (U) Sepharose (Pharmacia): Sepharose was swelled in 0.01 M phosphate buffer and 0.5 M NaCl, followed by three washing steps with 0.1% Triton X-100 for 1 minute at 37°C. Beads were re-equilibrated in IP-buffer.

Poly-Cytidylic acid agarose and Poly-Guanylic acid agarose (Sigma): Agarose was swelled in IP-buffer.

Deoxyribonucleic acid cellulose single stranded and Deoxyribonucleic acid cellulose double stranded (Sigma): The experiment was carried out under RNase-free conditions. Cellulose was swelled in TE-buffer and re-equilibrated in IP-buffer.

XSeb4R and XSeb4RΔRRM proteins were *in vitro* synthesized employing the TnT-Kit (2.11.2). 1 µl of the TnT-reaction was mixed with 1 ml IP-buffer and ribonucleic acids of 1.5 mg/ml ssDNA cellulose, 2.0 mg/ml dsDNA cellulose, 0.22 mg/ml poly (U) Sepharose, 0.8 mg/ml poly (A) Sepharose, 0.24 mg/ml poly (C) agarose or 0.21 mg/ml poly (G) agarose. The reaction was further incubated for 2 hours at 4°C. Samples were centrifuged for 10 minutes at 3,000 rpm at 4°C and wash twice with IP-buffer containing 2 mg/ml heparin, followed by two washings with IP-buffer without heparin. Samples were separated by SDS-PAGE and binding determined by phosphoimager analysis.

2.9.5 UV-crosslink assay

5x crosslink buffer: 1% glycerol, 50 mM KCl, 10 mM DTT, 5.2 mM HEPES (pH 7.9), 1 mM MgCl2, 0.1 mM EDTA, 5 mg/ml Heparin, 40 µg/ml carrier RNA (yeast tRNA)
2x protein loading buffer: 100 mM Tris pH 6.8, 200 mM DDT, 4% SDS, 0.2% bromophenol blue, 20% glycerol

0.5 µg His-XSeb4R was combined with 2 µl 5x crosslink buffer, competitor mRNA and/or H_2O to a volume of 9 µl. The mix was incubated for 10 minutes at room temperature. Thereafter, 1 µl of ^{32}P-αUTP (Perkin Elmer) labeled RNA was added and further incubated for 10 minutes at room temperature followed by a 10 minute UV-crosslink (4 cm from a 254 nm light source, Stratagene). Unbound RNA was removed by a 20 minutes RNase A (6 mg/ml) digest at 37°C. The reaction was stopped by adding 10 µl 2x protein loading buffer and denatured for 3 minutes at 100°C. Samples were separated by 12% SDS-PAGE and binding was analyzed by phosphoimager analysis.

2.10 DNA Methods

2.10.1 cDNA synthesis

For cDNA synthesis, 75 ng to 100 ng per 10 µl reaction mix of total RNA was used, the mix containing 5 mM $MgCl_2$, 2.5 ng random hexamer, 5 mM dNTP mix, 0.8 U RNase OUT (Invitrogen), 20 U MuLV reverse transcriptase (Roche) in 1X Taq incubation buffer without $MgCl_2$ buffer (Qbiogene). After an initial incubation at 20°C for 20 min to anneal the random hexamer primers, cDNA synthesis was carried out for 1 hour min at 42°C and terminated by heating to 95°C for 5 min.

2.10.2 RT-PCR analysis

For semi-quantative RT-PCR, 5 µl cDNA was used in a total volume of 25 µl containing 0.2 mM RT primers each, 1.5 mM $MgCl_2$, 0.5 U Taq polymerase in 1X Taq incubation buffer without $MgCl_2$ buffer (Qbiogene). A Histone H4 RT-PCR was carried out to control equal cDNA concentrations and test for DNA contamination using cDNA and total RNA, respectively.

2.11 Protein methods

2.11.1 Protein isolation

Protein homogenization buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 15% Glycerol. Before use, 1 tablet complete protease inhibitors (Roche) was added per 50 ml Homogenization buffer.
SDS: 10% SDS

15 to 20 embryos were used for stabilization experiments. For each embryo 10 µl homogenizing buffer was added and homogenized. After a 20 min centrifugation at maximum speed at 4°C, the supernatant was collected and centrifuged for 10 min at 10,000 rpm at 4°C. The supernatant was removed and protein concentration was measured using the Bradford method (Bradford, 1976). Equal amounts of total protein were separated by denaturing SDS-polyacrylamide gel electrophoresis according to Sambrook and Russell, 2001 and subject to Western Blot analysis.

2.11.2 TnT (*in vitro* transcription and translation)

In vitro translation was performed in a 12.5 µl reaction with the TnT® Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's user manual. Proteins were separated by denaturing SDS-polyacrylamide gel electrophoresis according to Sambrook and Russell, 2001. The dried gel was exposed to an autoradiography sensitive phosphoimager screen and scanned the following day (Amersham).

2.11.3 Western blotting

Antibody solution 1 (Western Blot): 5% (w/v) milk powder, 1:2,000 to 1:10,000 dilution of primary antibody in PTw
Antibody solution 2 (Western Blot): 5% (w/v) milk powder, 1:10,000 to 1:20,000 dilution of secondary antibody coupled to horseradish peroxidase (HRP) in PTw
Blocking buffer: 5% (w/v) milk powder in PTw
Transfer Buffer: 39 mM glycine, 48 mM Tris-HCl, pH 7.5, 0.04% SDS, 20% Methanol
PBS: 8% NaCl, 2% KCl, 65 mM Na_2HPO_4, 18 mM KH_2PO_4; adjust pH 7.2
PTw: 0.1% Tween-20 in 1X PBS

Proteins were separated by denaturing SDS-polyacrylamide gel electrophoresis according to Sambrook and Russell, 2001 and transferred for 1 hour to a nitrocellulose membrane (0.45 µm, Schleicher & Schuell) for 1 hour using the semi-dry blotting method (Sambrook and Russell, 2001). The membrane was blocked overnight in blocking buffer. After two brief washes in PTw, the membrane was incubated with 1° AB solution for 2 hours. After two brief washes in PTw, one 20 min in PTw and two brief washes in PTw, the membrane was incubated in 2° AB solution for 1 hour. After two brief washes and one for 20 min in PTw, the ECL DirectTM nucleic acid labeling and detection system was used to visualize the proteins (Amersham).

2.11.4 Expression of recombinant protein

Coomassie-staining solution: 0.05% (w/v) Coomassie Brilliant Blue R 250; 40% (v/v) ethanol; 10% (v/v) acetic acid; 50% H_2O
Distaining solution: 40% (v/v) methanol; 10% (v/v) acidic acid; 50% H_2O
Bradford-stock-reagent: 5× concentrated Coomassie Brilliant Blue R250 solution: 0.5 mg/ml Coomassie Brilliant Blue R250 in 42.5% phosphoric acid and 25% methanol. The solution was stored in a dark bottle at 4°C.
Bradford-assay-reagent: Assay reagent is prepared by diluting 1 volume of the Bradford-stock-reagent with 4 volumes of distilled H_2O. The solution should appear brown, and have a pH of 1.1.

GST- and His-fusion proteins were synthesized following the manufacturer's protocol. Purification of GST-fusion proteins was performed utilizing G-Sepharose (Amersham) and His-fusion proteins using Ni-NTA Agarose (Qiagen) following the manufacturer's protocol. Protein-quality was tested with a Coomassie stained SDS-PAGE and protein-concentrations were determined employing the Bradford method (Bradord, 1976).

2.12 Chemical treatments

2.12.1 Dexamethasone treatment

500x Dexamethasone (Dex): 20 mM dexamethasone in ethanol, stored in the dark, stable for up to 3 month

Embryos were injected with inducible mRNA constructs. Animal caps and embryos were treated with fresh 1X DEX at various stages and continuously kept in solution until fixation.

2.12.2 X-gal staining

Dent's solution: 20% (v/v) DMSO in methanol

$K_3Fe(CN)_6$: 0.5 M in H_2O, stored in the dark.

$K_4Fe(CN)_6$: 0.5 M in H_2O, stored in the dark.

10X MEM: 1 M Mops, 20 mM EGTA, 10 mM $MgSO_4$, pH 7.4, sterile filtered and stored in the dark

MEMFA: 4% (v/v) formaldehyde in 1X MEM

10X PBS: 1.75 M NaCl, 1 M KCl, 65 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4

X-gal: 40 mg/mL 5-Bromo-4-chloro-3-indolyl-b-D-galactosidase (X-gal) in formamide and stored in the dark at -20°C.

X-gal staining solution: 1 mg/ml X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in 1X PBS

Embryos were grown to the desired stage and fixed for 20 min in MEMFA. After washing three times for 10 min in 1x PBS, embryos were transferred to X-gal staining solution until staining was sufficient, typically 10 to 20 min. The reaction was stopped by washing the embryos three times in 1x PBS and afterwards fixed in MEMFA for 25 min. For whole mount *in situ* embryos were dehydrated with absolute ethanol and stored at -20°C. For pH3 staining, embryos were dehydrated with methanol, transferred to Dent's solution and stored at least 24 hours at -20°C.

2.13 Whole mount *in situ* hybridization

Antibody solution: 2% BMB, 20% heat inactivated horse serum, 1:2000 dilution of anti-digoxigenin antibody coupled to alkaline phosphatase (Roche) in 1X MAB

APB: 100 mM Tris-HCl, pH 9.0, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% TWEEN-20

BCIP: 50 mg/mL in 100% Dimethylformamide; stored at -20°C

Color reaction solution: 80 µg/ml NBT, 175 µg/ml BCIP in APB

EtOH series: 100%, 75%, 50% ethanol in H_2O, respectively, 25% ethanol in PTw

Hybridization Mix (Hyb Mix): 50% Formamide (deionized), 1 mg/ml Torula-RNA, 10 µg/ml Heparin, 1X Denhardt's, 0.1% Tween-20, 0.1% CHAPS, 10 mM EDTA in 5X SSC

5X MAB: 500 mM maleic acid, 750 mM NaCl, pH 7.5

MAB/BMB: 2% BMB in 1X MAB

MAB/BMB/HS: 2% BMB, 20% heat inactivated horse serum in 1X MAB

MeOH series: 100%, 75%, 50% methanol in H_2O, respectively and 25% methanol in 1X PBS

NBT: 100 mg/mL in 70% Dimethylformamide; stored at -20°C

PBS: 8% NaCl, 2% KCl, 65 mM Na_2HPO_4, 18 mM KH_2PO_4; adjust pH 7.2

Proteinase K: 5 µg/ml Proteinase K in 0.1X PBS

PTw: 0.1% Tween-20 in 1X PBS

PTw/MEMFA: 4% (v/v) formaldehyde in PTw.

RNase Solution: 10 µg/ml RNase A, 0.01 U/ml RNase T1 in 2X SSC

20X SSC: 3 M NaCl, 0.3 M Na-citrate, pH 7.2

Tween-20: 25% (w/v) Tween-20 (not autoclaved)

Whole mount *in situ* hybridization (WMISH) was performed essentially as described (Harland, 1991; Hollemann *et al*., 1999) using antisense RNA labeled with digoxigenin-11-UTP. Double *in situ* hybridization was performed according to Knecht *et al*. (1995). All steps were performed at ambient temperature with mild shaking. Embryos were rehydrated with the EtOH series to PTw, washed three times in PTw for 10 min and subjected to Proteinase K treatment to allow better penetration of the RNA probe. Stage 15 embryos were incubated for 6 min, later stage embryos were incubated for no longer than 15 min in Proteinase K. Embryos were washed twice in 0.1M triethanolamine, pH 7.5, to stop Proteinase K digestion and acetylated by adding 25 µl acetic anhydrite to fresh triethanolamine. After 5 min, another 25 µl acetic anhydrite was added. Then embryos were fixed in PTw/MEMFA for 25 min, washed five times in PTw, transferred to Hyb Mix and incubated for 5 hr at 65°C in a water bath. Hyb Mix was exchanged for the antisense RNA probe and incubated overnight at 65°C in a water bath. The next day, the RNA probe was collected and stored -20°C for reuse. After washes in Hyb Mix for 10 min at 65°C, three times in 2X SSC for 15 min at 65°C, non-hybridized RNA probe was removed by RNase digestion for 1 hour at 37°C in RNase solution.

The digested probe removed by washing once in 2X SSC at 37°C and twice 0.2X SSC at 65°C. After exchanging the buffer to MAB, embryos were blocked in MAB/BMB for 20 min and MAB/BMB/HS for 40 min to minimize unspecific binding of the antibody. The solution was replaced with antibody solution and incubated for 5 hours. The embryos were washed three times for 10 min with MAB and then overnight in MAB. After three rinses with MAB for 5 min, the caps were exchanged and the embryos transferred to APB. After three washes in APB for 5 min each, alkaline phosphatase was detected in color reaction solution. Embryos were kept at 4°C in the dark until staining was sufficient. The embryos were transferred to 100% Methanol to stop the reaction and to minimize background staining. Then embryos were rehydrated with the MeOH series to MEMFA and fixed overnight in MEMFA.

2.13.1 Sections

Gelatin/albumin: 4.88 mg/ml gelatin, 0.3 g/ml bovine serum albumin, 0.2 mg/ml sucrose in PBS. The gelatin was dissolved by heating the solution to 60°C. Albumin and sucrose was added, filtered with a 0.45 µm filter (Sartorius) and stored at -20°C.

Mowiol: 5 g Mowiol was stirred overnight in 20 ml PBS. After addition of 10 ml glycerol, the solution was stirred again overnight. Not dissolved Mowiol was collected by centrifugation for 30 min at 20,000 g. The supernatant was pH adjusted to pH~7.0 (using pH strips) and stored at -20°C.

Specimens were transferred to PBS and after equilibration in gelatin/albumin for 20 min, mounted by addition of glutardialdehyde. Sections (30 µm) were cut on a Leica VT1000M vibratome and mounted in Mowiol (Hollemann *et al*., 1999).

3. Results

3.1 XSeb4R is an RNA binding protein

The RNA-binding protein XSeb4R harbors a single RNA recognition motif (RRM), containing two ribonucleoprotein motifs (RNPs) (Boy *et al*., 2004; Fig. 5A). While best characterized as an RNA binding motif, the RRM has also been shown to bind single- and double-stranded DNA, as well as mediate protein-protein interactions (Query *et al*., 1989; Lutz-Freyermuth *et al*., 1990; Hall *et al*., 1992; DeAngelo *et al*., 1995; Samuels *et al*., 1998; Kielkopf *et al*., 2001; Selenko *et al*., 2003; ElAntak *et al*., 2007).

An *in vitro* binding assay was performed to determine XSeb4R nucleic acid specificity (Fig. 9). *In vitro* synthesized ^{35}S-labeled XSeb4R and an XSeb4R mutant lacking the N-terminal RRM motif (XSeb4RΔRRM) were incubated with immobilized nucleic acid polymers. Pulled-down proteins were separated by SDS-PAGE and visualized by phosphoimager analysis. XSeb4R bound exclusively to polyG-Sepharose, demonstrating substrate specificity. This binding was dependent on the RRM motif of XSeb4R, as XSeb4RΔRRM failed to interact with any nucleic acid. Importantly, XSeb4R did not bind to single- or double-stranded DNA suggesting its RRM domain exclusively interacts with RNA.

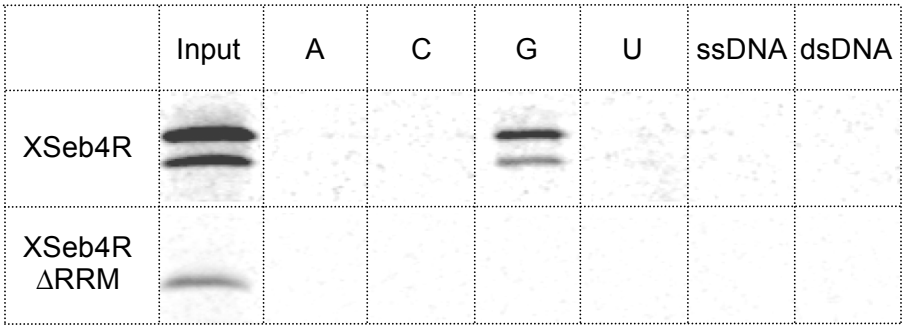


Fig. 9: XSeb4R specifically bound polyG-Sepharose *in vitro*. *In vitro* synthesized, ^{35}S-labeled XSeb4R and XSeb4RΔRRM were evaluated for binding to immobilized ribonucleic acids. XSeb4R was detected in double-bands; while the upper bands represented the predicted size of the full-length protein, the band below reflected most likely a degraded fragment. Pulled-down samples were separated by 12% SDS-PAGE, followed by phosphoimager analysis. A: polyA-Sepharose; C: polyC-Sepharose; G: polyG-Sepharose; U: polyU-Sepharose; ssDNA: single-stranded DNA-cellulose; dsDNA: double-stranded DNA-cellulose.

3.2 XSeb4R activates translation in *Xenopus* oocytes

RNA binding proteins are involved in virtually all steps of RNA metabolism, including splicing, modification, export, localization, stabilization, translation and degradation of transcripts (Fedoroff, 2002; Colegrove-Otero *et al*., 2005; Minakhina *et al*., 2005; Sanchez-Diaz *et al*., 2006; Lewis *et al*., 2007; Lin *et al*., 2007). As a first approach, a possible function for XSeb4R as a translational regulator was tested employing the MS2-tethering assay (Coller *et al*., 1998; Gray *et al*., 2000; Gorgoni *et al*., 2004; Collier *et al*., 2005; Gorgoni *et al*., 2005). In this assay, the firefly *luciferase* gene harboring MS2 binding sites in its 3'UTR is used as a reporter and the protein of interest is targeted to the reporter RNA by fusion to the MS2 coliphage coat protein. Thus, this assay allows testing for translational regulation by XSeb4R without prior knowledge of a specific target RNA.

XSeb4R was fused to the MS2 RNA binding protein and the corresponding mRNA coinjected with a firefly *luciferase* reporter mRNA harboring three MS2 binding sites (Coller *et al*., 1998; Collier *et al*., 2005). In addition, *Renilla luciferase* transcripts lacking the MS2-binding sites were coinjected to monitor the applied mRNA amounts and facilitating normalization of the firefly luciferase activities. First, the effector mRNA was injected and incubated for 5 to 6 hours allowing translation of the MS2-fusion proteins. Then the reporter mRNA was injected and after 15 hours, luciferase activity measured from cell lysates.

Overexpression of MS2-XSeb4R in oocytes resulted in a 4.5-fold activation of the luciferase reporter containing MS2 binding sites (Luc-MS2) relative to the reporter alone (Fig. 10). Remarkably, the observed effect by MS2-XSeb4R was stronger than the activation due to the well-characterized activator of translation, the PolyA-binding protein (XPAB1P) that induced a 2.5-fold activation of the reporter in this assay (Gray *et al*., 2000; Collier *et al*., 2005). MS2-XSeb4RΔRRM also showed enhanced luciferase activity (3.2-fold). In this experiment, XSeb4R is recruited to the target RNA via the MS2-domain, making the XSeb4R RRM motif dispensable. The C-terminal end of XSeb4R is essential for the activation, as a deletion construct (MS2-XSeb4RΔC) failed to activate the reporter, localizing the domain responsible of mediating the activation to the C-terminus of the protein. The activation was specific, as XSeb4R without the MS2-fusion only moderately enhanced Luc-MS2 activity. Additionally, MS2-XSeb4R failed to increase

luciferase activity of a luciferase reporter lacking the MS2-binding sites (Luc-ΔMS2). Both, the failure of untethered-XSeb4R to activate Luc-MS2 and MS2-XSeb4R to activate a reporter lacking the MS2-binding sites demonstrated a *cis*-binding requirement for XSeb4R translational activation. Interestingly, the MS2-domain itself seemed to inhibit translation. This may be due to binding of the MS2-protein to the MS2 binding sites of the reporter and blocking the attachment of other endogenous, unspecific stem-loop binding activators, thereby resulting in a reduced basal activity.

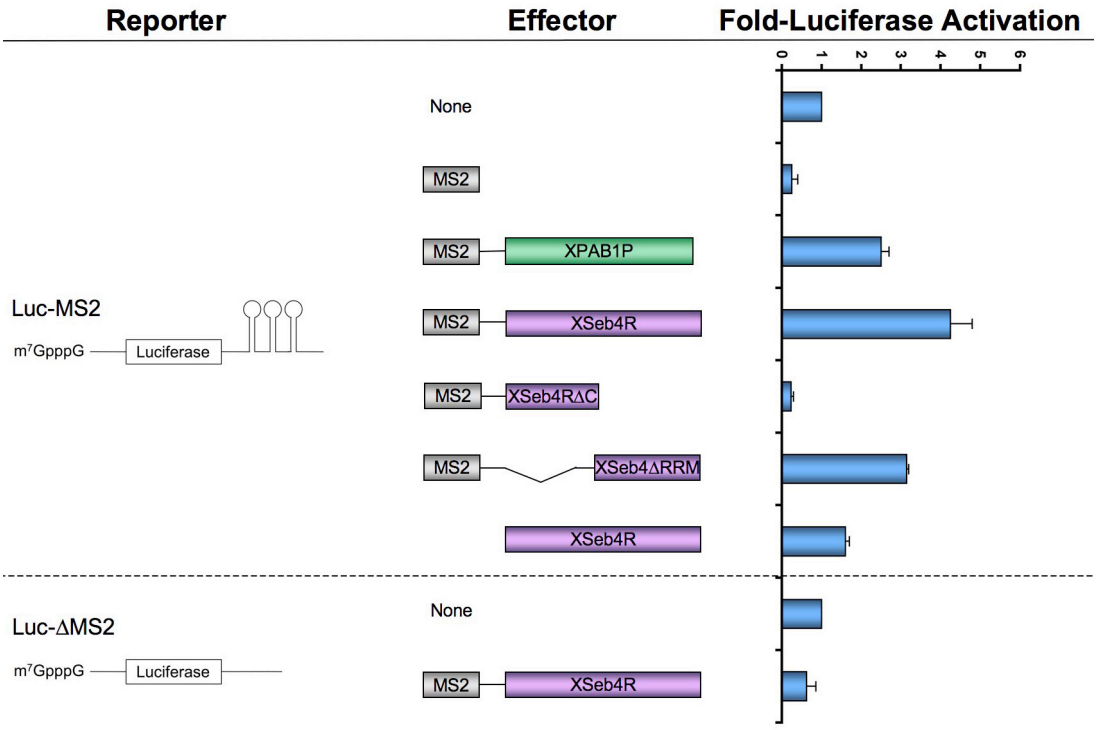


Fig. 10: MS2-XSeb4R activated translation in *Xenopus* oocytes. Stage V-VI oocytes were injected with 25 ng effector mRNAs and incubated for 5 to 6 hours. Thereafter, 100 pg of the *firefly Luc-MS2* reporter mRNA were injected together with 3.5 pg *Renilla luciferase* mRNA. Oocytes were further incubated overnight, harvested in two pools with 15 oocytes each and evaluated for luciferase activity. The firefly luciferase values were normalized to *Renilla* luciferase activity; shown is the fold-activation relative to firefly Luc-MS2 reporter activity alone.

To determine, whether the enhanced luciferase activity observed in the MS2-tethering assay by XSeb4R was the result of translational enhancement of the reporter or due to alterations of mRNA stability, reporter transcript amounts were evaluated by semiquantitative RT-PCR. Total RNA of oocytes coinjected with *MS2-XSeb4R* and the *Luc-MS2* reporter mRNA was isolated and cDNA synthesized (Fig. 11 B). Several cDNA-dilutions were evaluated to ensure the chosen PCR conditions were within the linear amplification range. Importantly, none of the tethered proteins, including MS2-XSeb4R, did not have a stabilizing effect on reporter transcripts. Firefly *Luc-MS2* and *Renilla luciferase* reporter RNA concentrations varied between different injections; this was most likely attributed to injection artifacts (Fig, 11 B, compare band intensities of *Luc-MS2* in respect to *Renilla* for each injection). This conclusion was supported through quantitation of the band intensity and normalization of the Luc-MS2 relative to that of *Renilla* luciferase values (Fig. 11 B). The moderate increases in reporter transcript levels observed were unlikely to cause the increased luciferase activity, as MS2-XSeb4RΔC and untethered XSeb4R failed to enhance the activation in spite of the slightly increased amount of Luc-MS2 reporter. Taken together, the enhanced firefly luciferase activity observed by XSeb4R in the MS2-tethering assay is indeed attributed to an increase in translational activation of the reporter and not due to influencing reporter RNA stability.

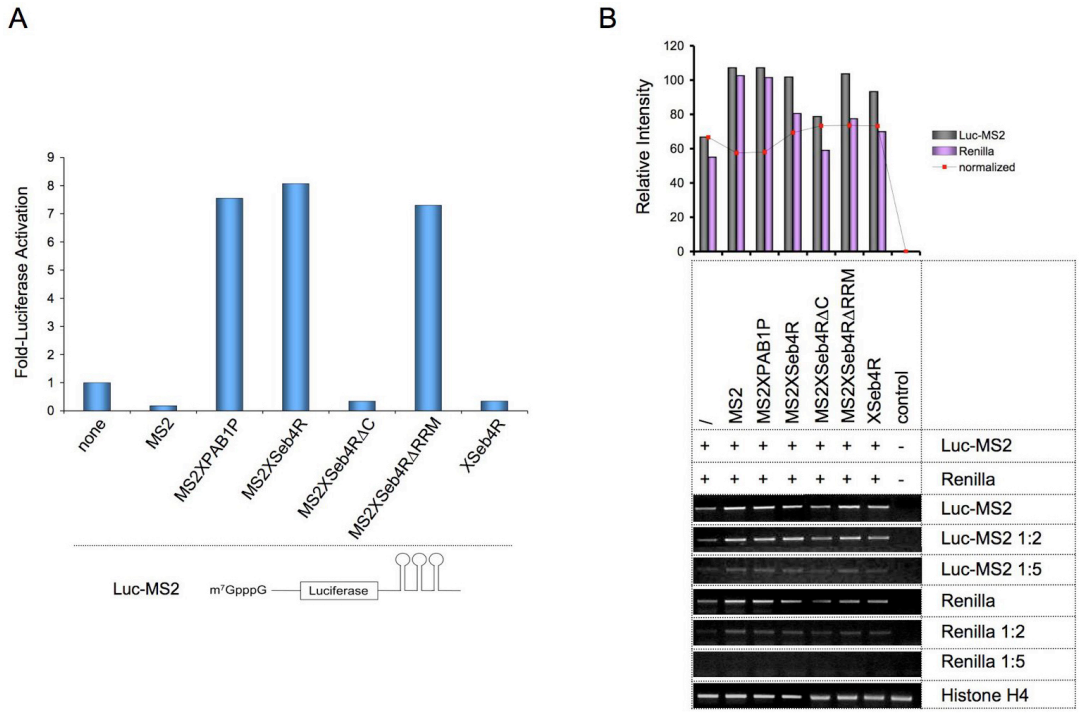


Fig. 11: MS2XSeb4R did not influence *Luc-MS2* reporter RNA stability. (A) Stage V-VI oocytes were injected with 25 ng MS2-fusion effector mRNAs and incubated for 5 to 6 hours. Thereafter, 100 pg of the *firefly Luc-MS2* reporter mRNA together with 3.5 pg *Renilla luciferase* mRNA was injected. Oocytes were further incubated overnight, harvested in two pools with 15 oocytes each and evaluated for luciferase activity. The firefly luciferase values were normalized to *Renilla* luciferase activity; shown is the fold-activation relative to the Luc-MS2 reporter activity alone. **(B)** Five oocytes of the experiment described in (A) were harvested the following day (16h incubation). All samples were subjected to total RNA extraction, followed by cDNA synthesis and RT-PCR analysis. cDNA dilutions of 1:2 and 1:5 for both reporters were performed, ensuring the PCR cycle number being in the linear range. *Renilla luciferase* serves as injection control and *Histone H4* as loading control. Five oocytes were harvested directly after reporter mRNA injection. Band intensity was quantified utilizing the ImageQuant software (Amersham). Displayed are intensities of firefly Luc-MS2 (gray bars), *Renilla* luciferase (violet bars) relative to the background and firefly Luc-MS2 values normalized to *Renilla* luciferase (red squares).

To demonstrate, that semiquantitative RT-PCR is sufficient to detect differences in mRNA levels of the magnitude represented by the difference in luciferase activity, firefly *luciferase-MS2* mRNA was titrated in the range of 10 to 1000 ng per oocyte, and incubated overnight. Oocyte lysates were tested for luciferase activation and in parallel, total RNA was extracted and reporter mRNA levels were detected by RT-PCR.

As shown in Fig. 12, the luciferase activity increased in correlation with the amount of reporter mRNA injected. Importantly, reporter RNA levels that afforded 3- to 5-fold differences in luciferase activity were distinguished by RT-PCR (Fig. 12 B).

Similar differences in luciferase activity were detected in the previous tethering assay by MS2-XSeb4R, demonstrating that semiquantitative RT-PCR assays can detect differences in mRNA levels of the magnitude represented by the differences in luciferase activity.

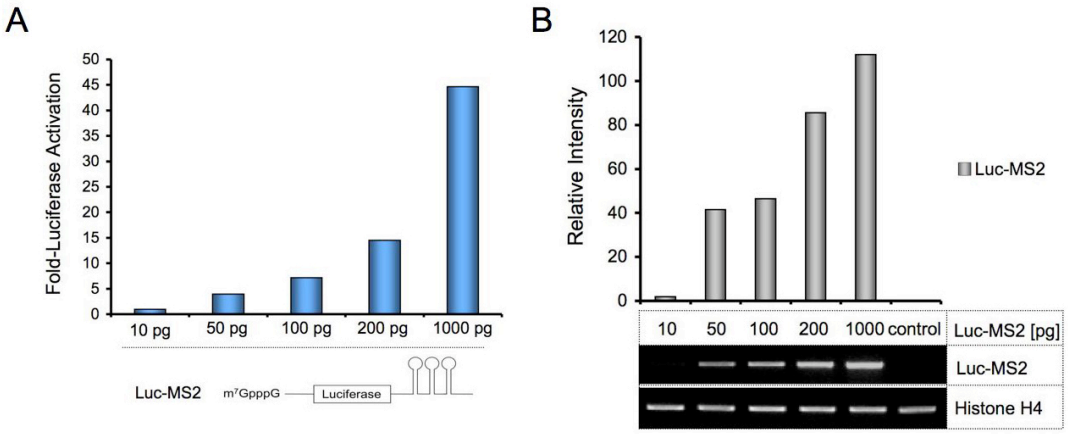


Fig. 12: Differences in reporter construct concentrations and their effects on luciferase activity. (A), (B) Stage V-VI oocytes were injected with 10, 50, 100, 200 or 1000 pg *Luc-MS2* mRNA in combination with 3.5 pg *Renilla luciferase* mRNA and incubated overnight. **(A)** Two pools with 15 oocytes were harvested and cell lysates evaluated for luciferase activity. The firefly luciferase values were normalized to *Renilla* luciferase activity; shown is the fold-activation relative to the 10 pg Luc-MS2 reporter mRNA. **(B)** Five oocytes of the experiment described in (A) were harvested and subjected to total RNA extraction, followed by cDNA synthesis and RT-PCR analysis. *Histone H4* served as loading control. Additionally, band intensity was quantified utilizing the ImageQuant software (Amersham). Displayed are intensities of firefly Luc-MS2 (gray bars) relative to the background.

3.3 MS2-XSeb4R activates translation in *Xenopus* embryos

To determine whether XSeb4R activates translation during *Xenopus* embryogenesis, the MS2-tethering assay was also performed in embryos. Several adjustments of the experimental procedure were necessary. First, the MS2-fusion constructs could not be injected in advance allowing the effector-proteins to be synthesized as embryos undergo mitosis. Furthermore, the dosage of the effector had to be significantly reduced, because concentrations used in oocytes would be lethal to the developing embryo. Due to observed higher basal activity, the dosage of the reporters also had to be decreased.

As shown in Fig. 13, MS2-XSeb4R and MS2-XSeb4RΔRRM activated translation of the *Luc-MS2* reporter in embryos, albeit weaker than observed in assays performed in oocytes (Fig. 10). Similar to the experiments in oocytes, MS2-XSeb4RΔC or untethered XSeb4R failed to enhance luciferase activity, indicating identical activities of XSeb4R in both, oocytes and developing embryos.

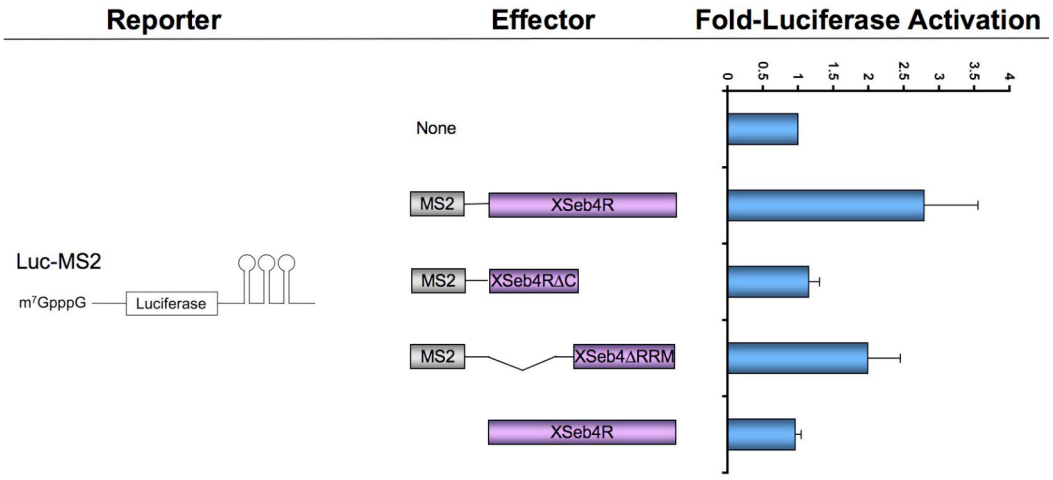


Fig. 13: MS2-XSeb4R activated the *Luc-MS2* reporter in *Xenopus* embryos. Embryos were injected with 100 pg effector mRNA animally into both blastomeres at the 2-cell stage, in combination with 100 pg firefly *Luc-MS2* and 25 pg *Renilla luciferase*. Embryos were cultured overnight (15 hours) at 18°C. Two pools of 15 embryos were harvested and subjected to luciferase activity measurement. The firefly luciferase values were normalized to *Renilla* luciferase activity; shown is the fold-activation relative to the Luc-MS2 reporter activity alone.

3.4 Functional mechanism of XSeb4R-mediated translational activation

Previous results indicated XSeb4R functions as a translational activator, but the underlying mechanism remained elusive. During canonical translational activation, the cap-binding protein eIF4E binds to the 5' cap structure of the mRNA and PABP to the polyA-tail (Fig. 14; Tarun *et al*., 1997; Kessler *et al*., 1998; Imataka *et al*., 1998; Gingras *et al*., 1999; Otero *et al*., 1999; Mangus *et al*., 2003; Prévôt *et al*., 2003; Brune *et al*., 2005; Dunn *et al*., 2005; Slepenkov *et al*., 2008). The scaffolding protein eIF4G interacts with both proteins, promotes the formation of the closed-loop structure and recruits additional initiation factors (eIF4E, eIF4A,

eIF4B and eIF4H). The requirement of cap associated proteins for translational enhancement can be determined by employing different virally derived internal ribosomal entry sites (IRES), which are able to recruit host factors involved in different steps of the canonical translational initiation (Pestova *et al*., 1998; Belsham *et al*., 2000; Roberts *et al*., 2000). The poliovirus *IRES* (*PV-IRES*) initiates translation by recruiting the complete cap-binding complex, with exception of the cap-binding protein eIF4E (Belsham *et al*., 2000; Roberts *et al*., 2000). The classical swine fever virus *IRES* (*CSFV-IRES*), however, activates translation by direct recruitment of the 43S initiation complex (Pestova *et al*., 1998).

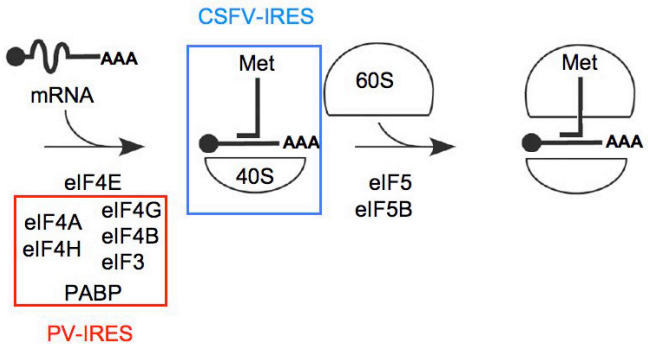


Fig. 14: Scheme of translational initiation by the canonical pathway, PV-IRES and CSFV-IRES. The canonical translational initiation is shown in black, while factors recruited for translational activation by the poliovirus *IRES* is indicated by a red box and the factors bound by the classical swine fever virus *IRES* in blue. eIF: eukaryotic initiation factor, 40S: 40S ribosomal subunit, 60S : 60S ribosomal subunit, PABP: polyA binding protein, AAA: polyA tail, Met: methionine, PV: polio virus, CSFV: classical swine fever virus, IRES: internal ribosomal entry site (adapted from the website http://www.wormbook.org/chapters/www_mechregultranslation/mechregultranslation.html).

To determine whether MS2-XSeb4R mediates translational activation in a cap-dependent manner and at which step of translational initiation XSeb4R is acting, constructs harboring either the *PV-IRES* or the *CSFV-IRES* and a cap analog at the 5' region of *Luc-MS2* were evaluated for translational activation in *Xenopus* oocytes. Furthermore, *Luc-MS2*, containing a canonical cap-structure or a cap analog was also tested. Absolute luciferase values were significantly reduced for the cap analog Luc-MS2 reporter construct compared to the same reporter containing the canonical cap-structure (Fig. 15). However, the fold-luciferase activation induced by MS2-PAB1P and MS2-XSeb4R was similar with both reporter constructs (Fig. 16).

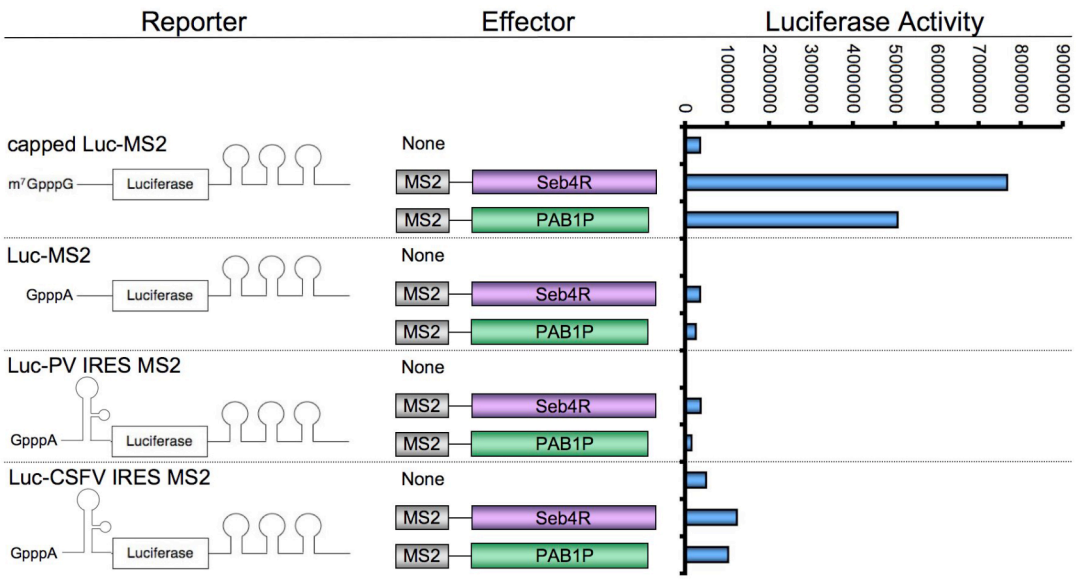


Fig. 15: MS2-XSeb4R activated the *PV-IRES Luc-MS2* luciferase reporter in *Xenopus* oocytes a. Stage V-VI oocytes were injected with 25 ng MS2-fusion mRNAs and incubated for 5 to 6 hours. Thereafter, 100 pg of the *firefly Luc-MS2* reporter mRNA were injected together with 3.5 pg *Renilla luciferase* mRNA. Oocytes were further incubated overnight, harvested in two pools with 15 oocytes and cell lysates evaluated for luciferase activity. The firefly luciferase values were normalized to *Renilla* luciferase activity; shown is the luciferase-activation relative to the Luc-MS2 or the IRES-Luc-MS2 reporter activity alone. PV: poliovirus, CSFV: classical swine fever virus, IRES: internal ribosomal entry site, m^7GpppG: 7-methyl guanosine cap structure, GpppA: cap structure analog.

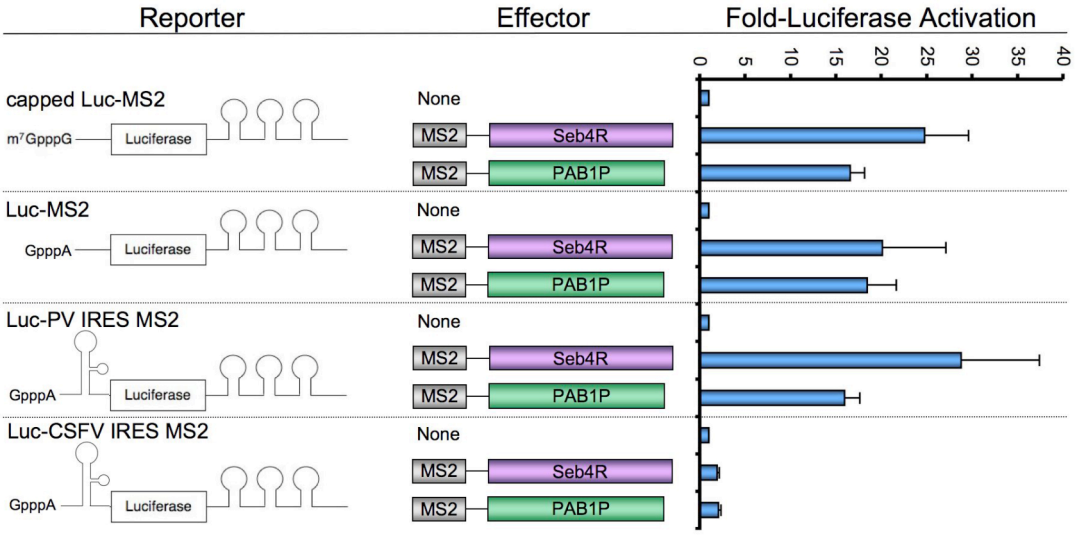


Fig. 16: MS2-XSeb4R activated the *PV-IRES Luc-MS2* luciferase reporter in *Xenopus* oocytes b. Stage V-VI oocytes were injected with 25 ng MS2-fusion effector mRNAs and incubated for 5 to 6 hours. Thereafter, 100 pg of the firefly *Luc-MS2* reporter mRNA were injected together with 3.5 pg *Renilla luciferase* mRNA. Oocytes were further incubated overnight, harvested in two pools with 15 oocytes and cell lysates evaluated for luciferase activity. The firefly luciferase values were normalized to *Renilla* luciferase activity; shown is the fold-activation relative to the Luc-MS2 reporter activity alone. PV: poliovirus, CSFV: classical swine fever virus, IRES: internal ribosomal entry site, m^7GpppG: 7-methyl guanosine cap structure, GpppA: cap structure analog.

Compared to the capped *MS2-Luc* construct, absolute luciferase activities of the IRES-Luc-MS2 reporters, harboring a cap analog, were also significantly decreased (Fig. 15). While MS2-XSeb4R and MS2-PAB1P failed to enhance luciferase activation of the *CSFV-IRES*, both MS2-fusion proteins induced a fold-induction of the *PV-IRES-Luc-MS2* reporter comparable to the MS2-Luc reporters (Fig. 16).

MS2-PAB1P, which is a known interaction partner of eIF4G, has been shown to stimulate translation through the *PV-IRES* (Gorgoni *et al*., 2005), implicating a similar functional mechanism for XSeb4R. Taken together, these results suggest that XSeb4R interacts with cap-associated proteins but if XSeb4R binds directly to these factors or through cofactors remains to be determined.

3.5 Identification of XSeb4R-targets through a candidate gene approach

3.5.1 XSeb4R activates expression of genes from all three germ layers

XSeb4R has previously been shown to function as a positive regulator of *Xenopus* neurogenesis (Boy *et al.,* 2004). To determine whether XSeb4R is sufficient to drive neuronal differentiation in non-neural ectoderm, an animal cap assay was performed. Animal cap explants, cut at blastula stage, represent a mass of pluripotent precursor cells that will differentiate into atypical epidermis. Cell fate decision of this cell population can be altered by the injection of mRNA, DNA, proteins or incubation in proteins or chemicals to drive differentiation into derivates of all three germ layers (Fig. 17 A). To control the timing of XSeb4R activity, a hormone inducible construct, XSeb4R-GR, was used in the animal cap assay. Here, the glucocorticoid receptor ligand-binding domain (GR domain) is fused to the C-terminus of a protein. After translation, the GR-fusion protein is sequestered in the cytoplasm by the heatshock protein Hsp90 (Jakob *et al*., 1995; Scheibel *et al*., 1997; Fig, 17 B). Upon addition of dexamethasone (Dex), a conformational

change of the fusion protein takes place and binding by Hsp90 is relieved (Pratt *et al.*, 1997).

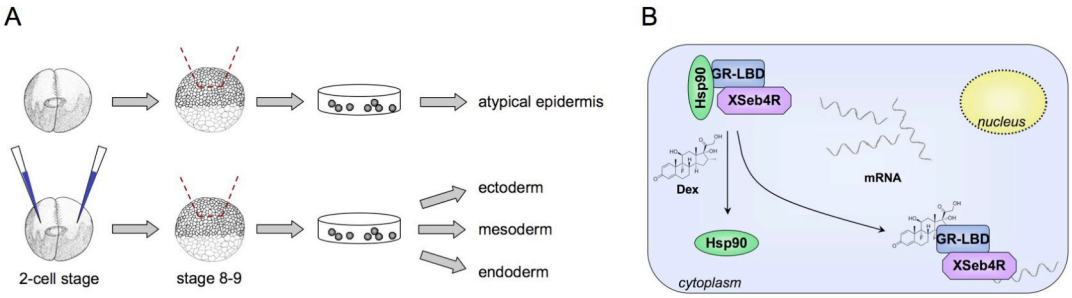


Fig. 17: Scheme of the animal cap assay and function of the XSeb4R-GR fusion construct. (A) Animal caps dissected at blastula stage (stage 8 to 9) develop into atypical epidermis, while manipulated animal caps have the potential to develop into derivates of all three germ layers**. (B)** XSeb4R fused to the glucocorticoid receptor ligand binding domain (XSeb4R-GR) is bound to the heatshock protein 90 (Hsp90) in the cytoplasm. Addition of dexamethasone (Dex) relieves the binding and allows XSeb4R function.

XSeb4R-GR was injected animally into both blastomeres of 2-cell stage embryos and animal caps were dissected at blastula stage (Fig. 18 A). As control embryos reached stage 9, a pool of caps was harvested prior to XSeb4R-GR induction (time 0h). The remaining caps were split into two batches and one half was induced with Dex, while the other half was kept untreated and served as a control. Pools of induced and uninduced caps were harvested after 13 and 25 hours of Dex treatment. Total RNA from both samples was isolated and analyzed by RT-PCR.

As shown in Fig. 18 B, XSeb4R-GR was indeed found to activate genes characteristic for neuronal differentiation, such as the neural determination factor *Xngnr-1* and the marker for terminal differentiated neurons, *N-tubulin*. While *Xngnr-1* transcripts were present in the uninjected animal cap at stage 9, transcript levels decreased over time and were no longer detected after 25 hours of culture. However, in XSeb4R-GR injected caps, the *Xngnr-1* signal persisted and increased over time (Fig. 18 B, compare 13h, 25h XSeb4R-GR injected induced and 13h, 25h of control caps induced). Transcripts of *N-tubulin* were only detected after 13 hours and 25 hours after XSeb4R-GR induction. Surprisingly, an induction of molecular markers for the endodermal and mesodermal germ layers, such as *VegT*, *Wnt8* and *Sox17ß*, was also observed. These same findings were also observed by J. Souopgui (Souopgui *et al.*, 2008). As previously described, *VegT*

was present in uninjected animal caps at stage 9 (Clements *et al*., 1999; Cao *et al*., 2007; Fig. 18 B, 0h XSeb4R-GR and control caps). Similar to the *Xngnr-1*, transcript levels of *VegT* appeared not only to be stabilized, but also increased over time. The mesodermal marker *Wnt8* and the endodermal marker *Sox17ß*, downstream targets of VegT, were not present in the uninduced animal cap at stage 9 but could be detected at 13 hours and 25 hours after induction.

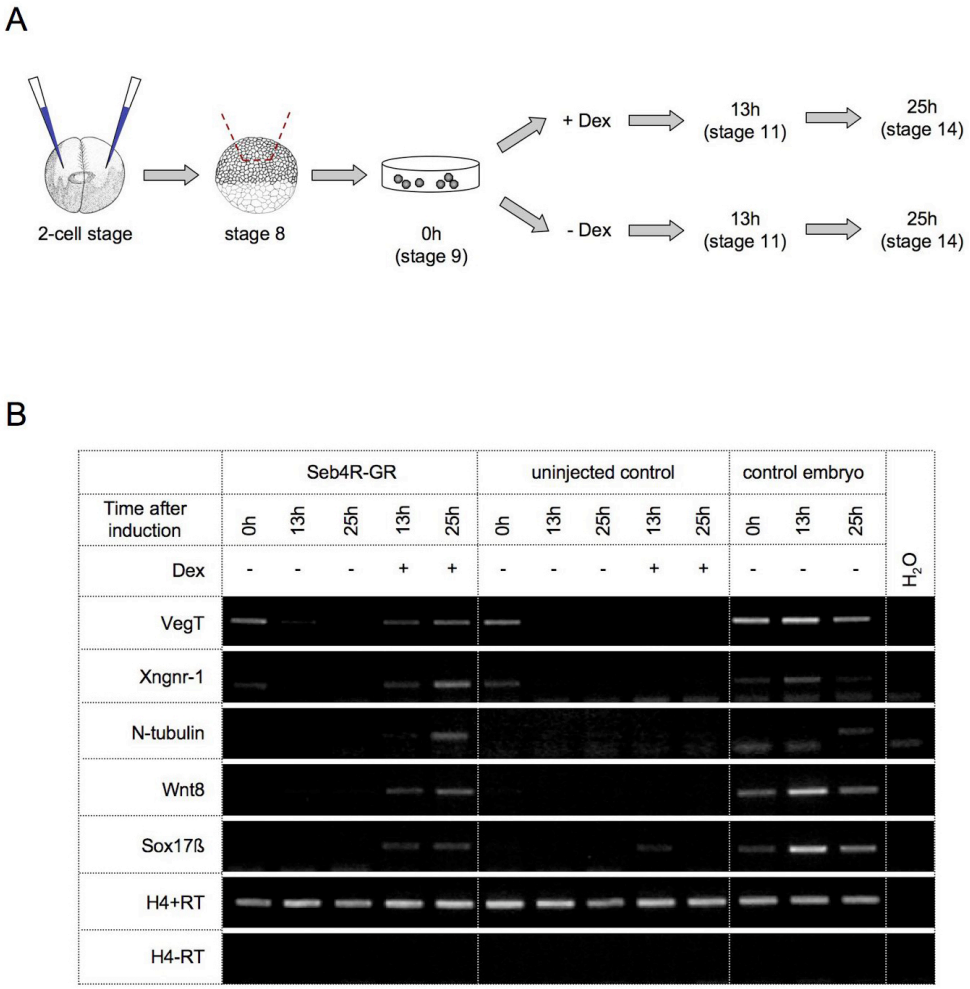


Fig. 18: XSeb4R-GR activated transcription of *Xngnr-1*, *VegT* and their downstream targets between 0 and 13 hours after induction. (A) Scheme of XSeb4R-GR time-course. XSeb4R-GR (50 pg) was injected animally into both blastomeres of the 2-cell stage. Caps were cut at stage 8 and one half treated with dexamethasone at stage 9. Explants were cultured until control embryos reached the indicated stages and subjected to RT-PCR analysis. **(B)** RT-PCR analysis of XSeb4R-GR injected animal caps. Molecular markers for the three germ layers were evaluated: mesoderm (*Wnt8*), endoderm (*VegT* and *Sox17ß*) and neural ectoderm (*Xngnr-1* and *N-tubulin*). *Histone H4* served as loading control.

The remarkably broad pattern of endo- and mesodermal gene induction as observed in XSeb4R-GR injected animal caps is similar to that described to occur

upon ectopic expression of VegT in the same explant system (Clements *et al*., 1999). These results suggested that XSeb4R might be operating via VegT in the context of meso- and endoderm induction. Similarly, the activation the neuronal markers could occur by induction of the VegT-induced mesoderm.

3.5.2 Verification of putative XSeb4R-targets by RNA-IP

The RT-PCR analysis suggested *VegT* and *Xngnr-1* may be targets of XSeb4R. Therefore, an *in vitro* binding assay with immobilized, recombinant XSeb4R and purified RNA was performed to verify XSeb4R binding. Total and polyA+ RNA were separately tested for binding to GST-XSeb4R and GST, the latter serving as negative control to monitor unspecific RNA interactions. The precipitated RNA was analyzed by RT-PCR (Fig. 19 A), the employed proteins were recovered after the RNA-IP, separated by SDS-PAGE and concentrations were monitored by Western Blot analysis (Fig. 19 B).

VegT and *Xngnr*-1 mRNAs were both specifically precipitated by GST-XSeb4R, as they were not bound by GST alone. In addition, Sox3 transcripts were detected in the GST-XSeb4R RNA immunoprecipitation but were also present in weaker amounts in the negative control, indicating unspecific interaction. *Histone H4*, and *ornithine carboxylase* (*ODC*), serving as negative controls, showed signals in both, the GST-XSeb4R and GST RNA-IP, while the signal for ODC was weaker in the GST pull-down, further suggesting unspecific interaction with the GST-tag. *N-tubulin*, an indirect Xngnr-1 target, was not precipitated in the experiment, similar to *Kinesin 4B*, serving as an additional negative control. The VegT target, *Xbra*, was weakly precipitated by GST-XSeb4R.

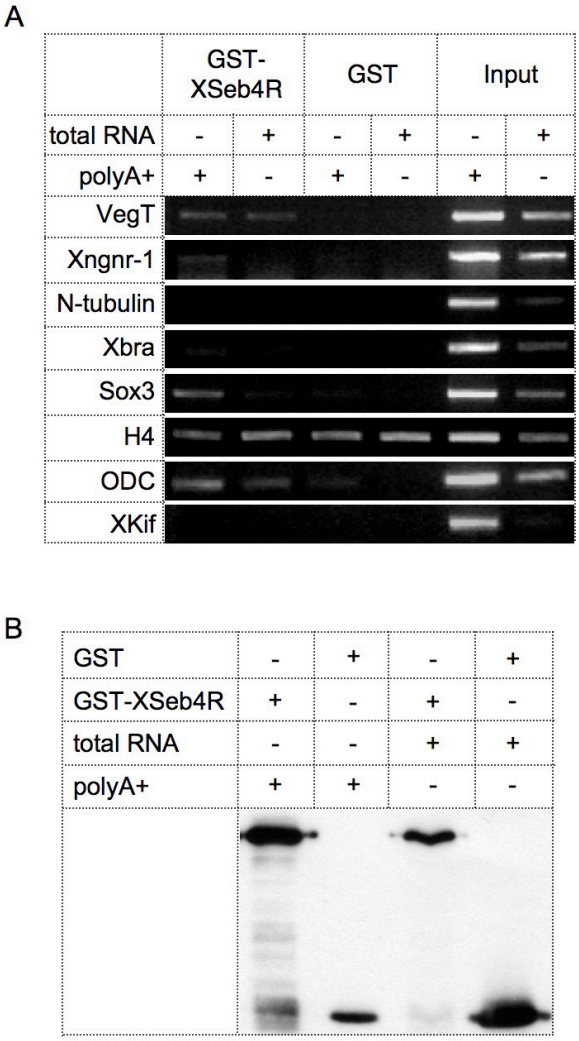


Fig. 19: RNA immunoprecipitation confirmed *VegT*, *Xngnr-1* and *Xl G14* as XSeb4R targets. (A) RT-PCR analysis of GST-XSeb4R and GST-tag precipitated RNAs. *Histone H4*, *ODC* and *Kinesin4B* served as negative controls. 1% of the RNA-IP-input was used as positive control. **(B)** Western Blot analysis of GST-tagged proteins recovered after the RNA-IP, detected by using the anti-GST antibody. XKif: *Xenopus* Kinesin 4B.

3.6 XSeb4R binds to the *VegT* 3'UTR and activates VegT translation

The observations of both, the animal cap induction assay and the XSeb4R RNA-IP, are compatible with the idea that the ability of XSeb4R to induce endoderm- and mesoderm-specific gene expression occurs via and/or in cooperation with VegT. Since *VegT* mRNA can be detected in all three germ layers of blastula stage

embryos, these effects could be either due to XSeb4R stabilizing the maternal *VegT* mRNA, the result of XSeb4R-mediated translational activation or a combination of both (Clements *et al*., 1999; Cao *et al*., 2007; Souopgui *et al*., 2008).

In order to further unravel the mechanistic correlation of XSeb4R and VegT activities, the ability of XSeb4R to bind to the *VegT* 3'UTR was evaluated using an UV-crosslinking assay. Recombinant His-XSeb4R was incubated *in vitro* with ^{32}P-labeled *VegT* 3'UTR and RNA-protein interactions stabilized by UV-crosslinking. Unprotected RNA was removed by RNaseA digestion, RNPs separated by SDS-PAGE and binding determined by phosphoimager analysis. As shown in Fig. 20 B, His-XSeb4R bound directly to the *VegT* 3'UTR. To identify the region within the *VegT* responsible for binding to XSeb4R, the 3'UTR was delineated into three fragments (F1-F3; Fig. 20 A) and assayed for their ability to compete with the full-length *VegT* 3'UTR for binding by XSeb4R. While F1 only weakly competed with the full-length *VegT* 3'UTR for XSeb4R-binding, strong competition was observed with F2 and F3 (Fig. 20 B). These data indicate XSeb4R interacts with the *VegT* 3'UTR through multiple independent binding sites. This was further supported by the results of J. Souopgui, who observed strong binding of XSeb4R to F2, F3 and very weak interaction with F1 (Souopgui *et al*., 2008). XSeb4R-VegT interaction was specific, as ^{32}P-labeled LacZ mRNA failed to compete for binding (Souopgui *et al*., 2008).

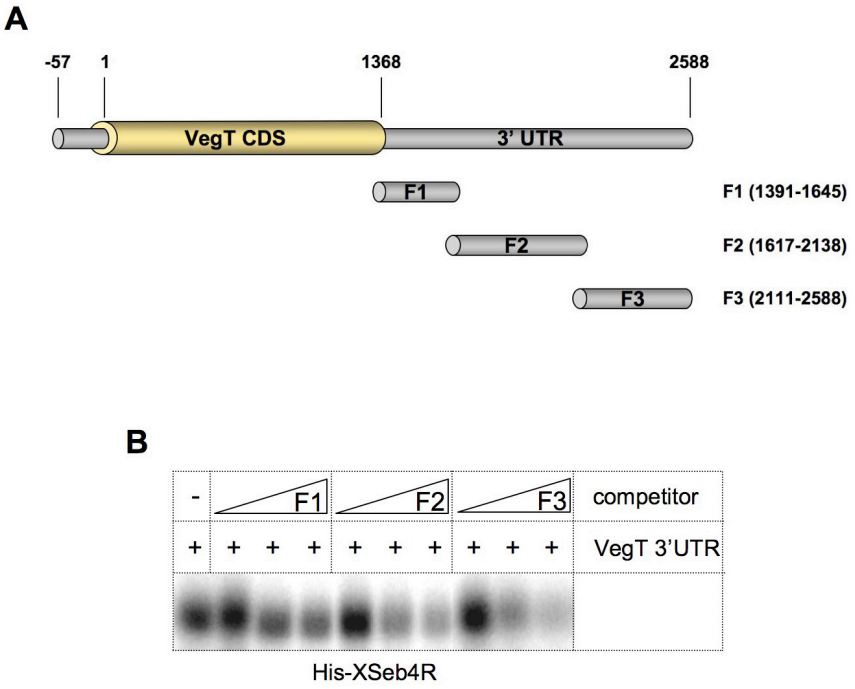


Fig. 20: XSeb4R binding to the full-length *VegT* 3'UTR could be effectively competed with *VegT* 3'UTR fragments F2 and F3 but not F1. A) Scheme of *VegT* mRNA structure. Nucleotides are indicated in respect to the ATG. Fragment F1 consists of 254 nucleotides of the *VegT* 3'UTR 5'-terminus. F2 is 521 nucleotides long and covers the middle part of the 3'UTR. The 477 nucleotide long fragment F3 harbors the 3' terminus of the *VegT* 3'UTR. **B)** Bacterially purified His-XSeb4R protein was tested for binding in an *in vitro* UV-crosslinking assay to ^{32}P-labeled full-length *VegT* 3'UTR mRNA. Competition was performed with equimolar, increasing amounts of unlabeled *VegT* 3'UTR F1, F2 and F3 RNA. Binding was determined by phosphoimager analysis.

The crosslinking experiments described above provide strong evidence for a direct interaction of the *VegT* 3'UTR and the XSeb4R protein. Moreover, through the MS2-tethering assay, it was demonstrated that XSeb4R could increase translation when recruited to the 3'UTR of a reporter mRNA. Taken together, these results would predict that fusion of the *VegT* 3'UTR to the luciferase ORF should result in a reporter construct that is translationally activated by the XSeb4R wildtype protein.

Thus, the ability of XSeb4R to activate translation of a *firefly luciferase* reporter mRNA harboring the *VegT* 3'UTR was investigated in *Xenopus* oocytes. As shown in Fig. 21, XSeb4R, but not XSeb4RΔRRM, activated translation of the *Luc-VegT* 3'UTR reporter, demonstrating *cis*-binding for the observed activity. While XSeb4R stimulated translation via F2 and F3, it failed to enhance luciferase activity via F1. This activity correlated well with their ability to bind with XSeb4R (Fig. 20 B and Fig. 21). XDead-end, an RNA binding protein also harboring a single RRM motif,

failed to induce activation of the *Luc-VegT* 3'UTR reporter, indicating the observed translational activation by XSeb4R was specific (Fig. 21).

The Luc-MS2 reporter harbors three repeats of the MS2-binding sites, allowing multiple binding of the MS2-effector proteins and thus, potentially amplifying translational activation. Therefore, a reporter construct of three copies *VegT* 3'UTR F3 downstream of the firefly *luciferase* coding sequence was evaluated (Fig. 21, Luc-VegT 3'UTR F3 3X). Injection of the multimerized construct lead to a stronger translational activation compared to the reporter harboring a single copy of F3. The obtained 6-fold activation was even stronger than that observed by XSeb4R in the MS2-tethering assay (4.5-fold).

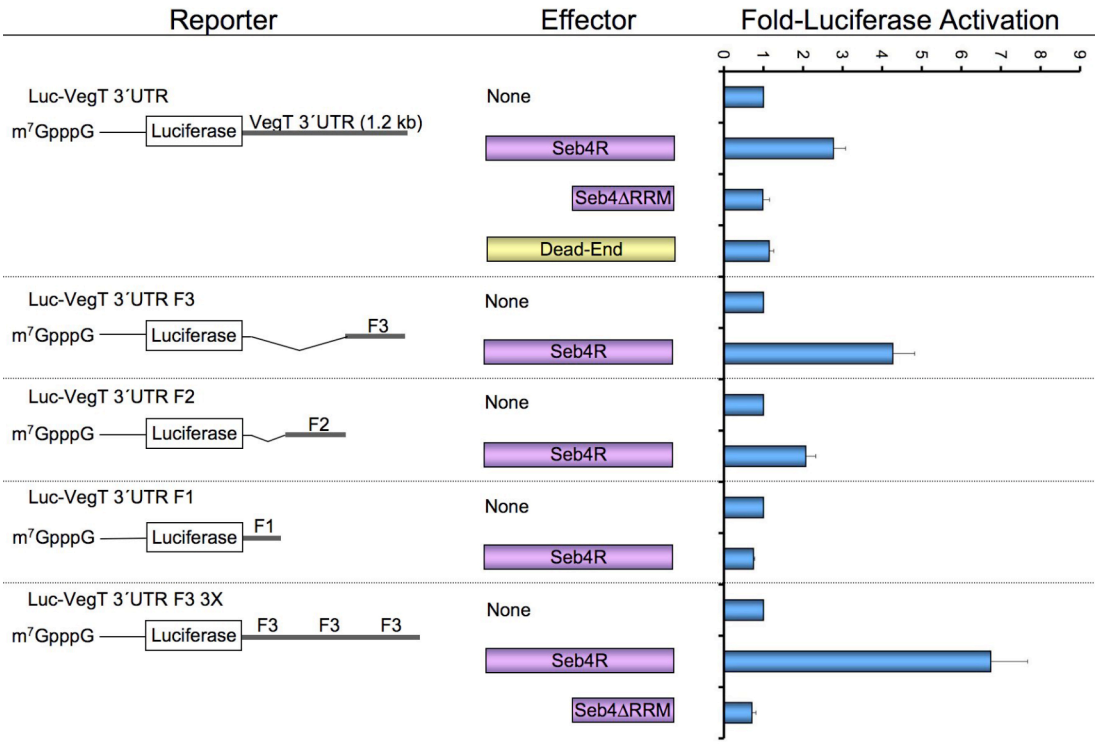


Fig. 21: XSeb4R facilitated translation activation through the *VegT* 3'UTR. Stage V-VI oocytes were injected with 25 ng effector mRNAs and incubated for 5 to 6 hours at 18°C. Thereafter, 100 pg of the *VegT* 3'UTR-Luc reporter mRNA was injected together with 3.5 pg *Renilla luciferase* mRNA. Oocytes were further incubated overnight at 18°C, harvested in two pools with 15 oocytes each and evaluated for luciferase activity. The firefly luciferase values were normalized to *Renilla* luciferase activity and shown is the fold-activation relative to the VegT 3'UTR-Luc reporter activity alone.

To demonstrate that the enhanced luciferase expression observed in the oocyte translation assay was indeed a result of translational activation and not due to influencing reporter RNA stability, luciferase activity was determined and reporter concentrations monitored by semiquantitative RT-PCR analysis.

As shown in Fig. 22 A-D, oocytes were injected as described above and analyzed for luciferase activation. In parallel, total RNA was extracted, evaluated by RT-PCR and the PCR products quantified (Fig. 22 E-H). While XSeb4R enhanced the luciferase activity via the full-length *VegT* 3'UTR (Fig. 22 A), no significant alterations in reporter mRNA concentrations could be detected (Fig. 22 E). Similar results were obtained by F3 (Fig. 22 D and H), indicating that the enhanced luciferase activation was due to translational activation rather than prolonged reporter RNA stability. The *luciferase* reporter harboring F1, which failed to undergo XSeb4R-induced translational activation (Fig. 22 B and F), were slightly destabilized by XSeb4R (Fig. 22 C and G). However, these mild effects on reporter transcript stability cannot account for the observed translational activation, which has already been demonstrated for the MS2-fusion proteins in Fig. 11 and Fig. 12.

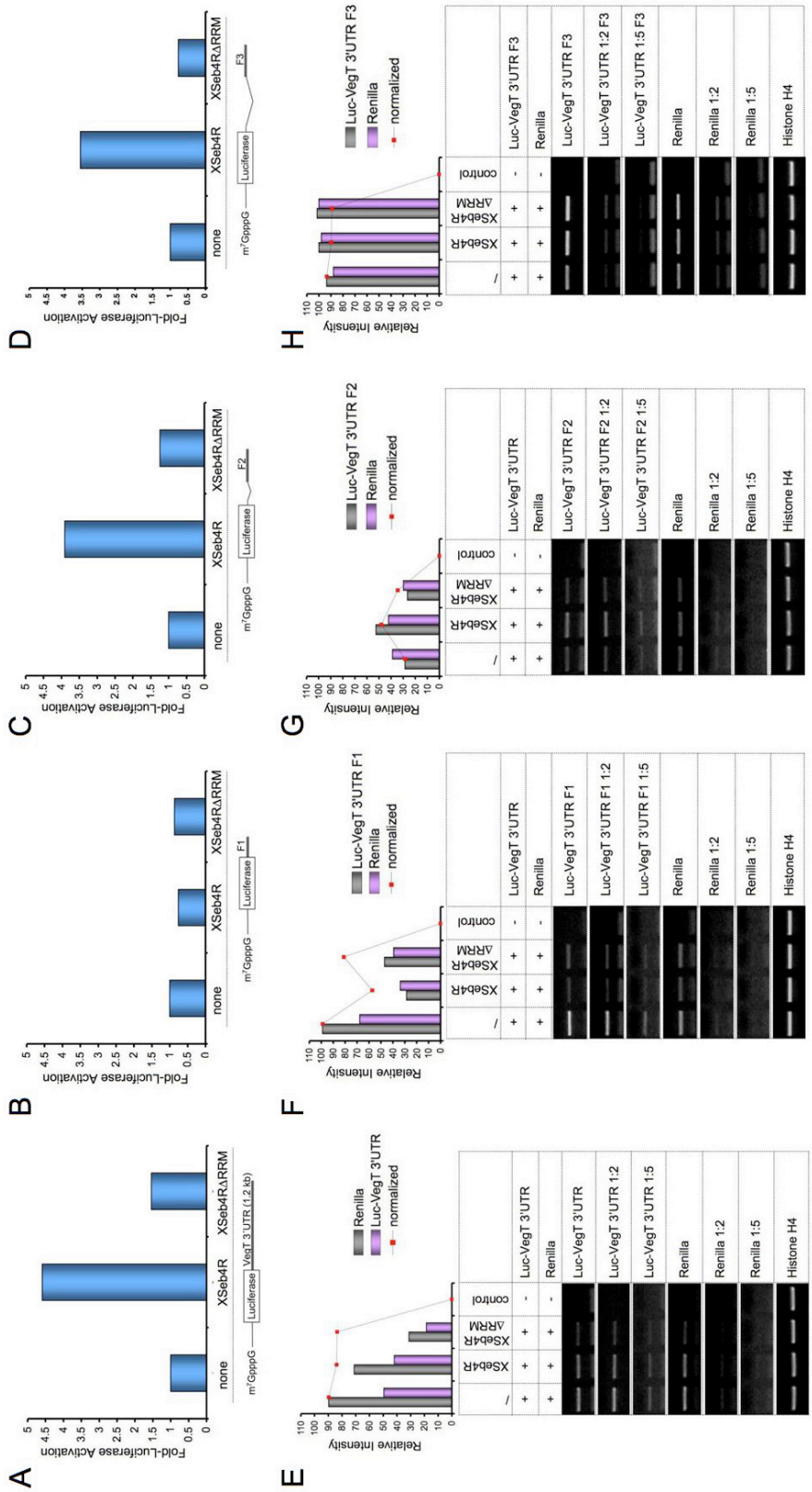


Fig. 22: XSeb4R effect on translation was not based upon reporter RNA stability. (A-D) Stage V-VI oocytes were injected with 25 ng effector mRNAs and incubated for 5 to 6 hours. Thereafter, 100 pg of the firefly *Luc-VegT* reporter mRNA **(A)** full-length *VegT* 3'UTR, **(B)** F1, **(C)** F2 and **(D)** F3 were injected together with 3.5 pg *Renilla luciferase* mRNA. Oocytes were further incubated overnight, harvested in two pools with 15 oocytes each and evaluated for luciferase activity. All Luc-VegT values were normalized to *Renilla* luciferase activity; shown is the fold-activation relative to the Luc-VegT reporter activities alone. **(E-H)** Five oocytes of the experiment corresponding to (A-D) were harvested the following day (16h incubation). All samples were subjected to total RNA extraction, followed by cDNA synthesis and RT-PCR analysis **(E)** full-length *VegT* 3'UTR, **(F)** F1, **(G)** F2 and **(H)** F3. cDNA dilutions of 1:2 and 1:5 for both reporters were performed, insuring the PCR cycle number being in the linear range. *Renilla luciferase* serves as injection control and *Histone H4* as loading control. Five oocytes were harvested directly after reporter mRNA injection. Band intensity was quantified utilizing the ImageQuant software (Amersham). Displayed are the measured intensities of VegT 3'UTR-Luc (gray bars), *Renilla* luciferase (violet bars) relative to the background and Luc-VegT values normalized to that of *Renilla* luciferase (yellow triangles).

To test whether XSeb4R also facilitates translational activation of *VegT* during embryogenesis, XSeb4R was coinjected with *VegT* reporter mRNA into 2-cell stage embryos, incubated for 15 hours and luciferase activity of the cell lysates measured (Fig. 23).

XSeb4R activated translation through the *VegT* 3'UTR in embryos only moderately (1.5-fold), whereas XSeb4RΔRRM did not influence luciferase activity. Multimerization of *VegT* 3'UTR F3 (*Luc-VegT* 3'UTR F3 3x) increased the translational activation (2-fold). Injection of XSeb4RΔRRM had no effect on translation of the multimerized *VegT* 3'UTR F3 reporter. These findings indicate a similar mechanism of XSeb4R-mediated translational regulation of the *VegT* 3'UTR, as observed in oocytes, also during *Xenopus* embryogenesis.

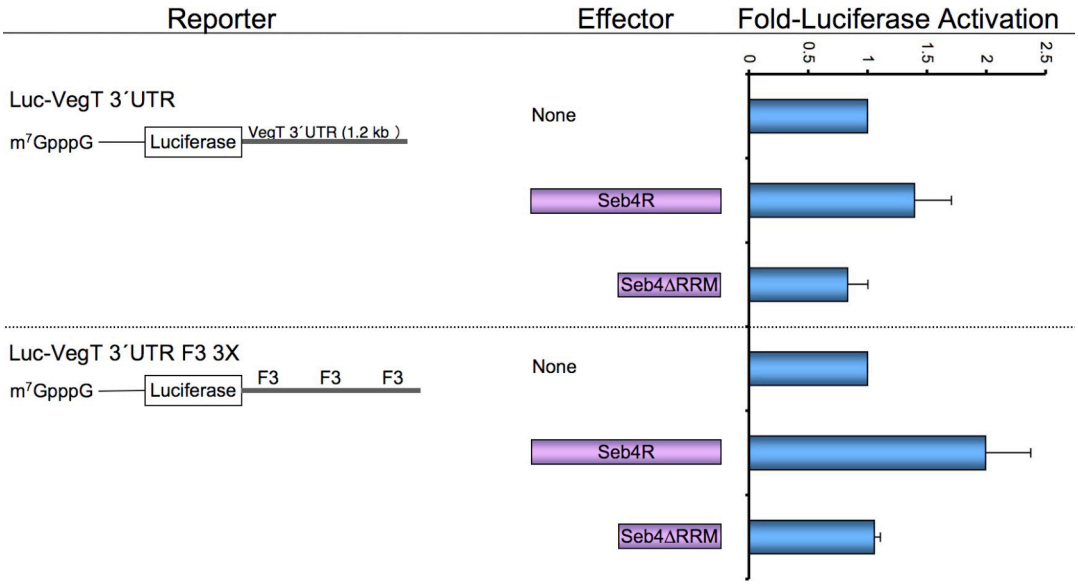


Fig. 23: XSeb4R moderately activated translation through the *VegT* 3'UTR reporter in *Xenopus* embryos. XSeb4R or XSeb4RΔRRM mRNA (100 pg each) were injected into the animal hemisphere of both blastomeres of 2-cell stage embryos in combination with 100 pg firefly *VegT* 3'UTR-Luc and 25 pg *Renilla luciferase*. Embryos were cultured overnight. Two pools of 15 embryos were harvested and subjected to luciferase activity measurement. All firefly luciferase values were normalized to *Renilla* luciferase and shown is the fold-activation relative to the VegT 3'UTR-Luc reporter activity alone.

3.7 XSeb4R binds to the *Xngnr-1* 3'UTR and activates Xngnr-1 translation

XSeb4R displays a highly dynamic expression pattern that is only partially overlapping with *VegT*, suggesting additional XSeb4R-target mRNAs (Lustig *et al*., 1996; Stannard *et al*., 1996; Zhang *et al*., 1996; Horb *et al*., 1997; Boy *et al*., 2004). The neural determination factor *Xngnr-1* shows overlapping expression with *XSeb4R* in the territories of primary neurogenesis. XSeb4R has previously been shown to activate *Xngnr-1* expression in *Xenopus* embryos as well as in the animal cap assay, similar to *VegT* (Fig. 18; Ma *et al*., 1996; Boy *et al.,* 2004). Moreover, XSeb4R weakly pulled-down *Xngnr-1* transcripts in the RNA-IP experiment (Fig. 19). These results provide evidence that *Xngnr-1* mRNA may also be an XSeb4R target.

To determine if XSeb4R could indeed interact specifically with the *Xngnr-1* mRNA, an *in vitro* UV-crosslinking experiment was performed (Fig. 24 A and B). As shown in Fig. 24 B, full-length *Xngnr-1* 3'UTR bound to XSeb4R. The interaction was specific, as binding could be effectively competed by cold *Xngnr-1* 3'UTR but not with *LacZ* RNA. To determine the region responsible for XSeb4R interaction, the *Xngnr-1* 3'UTR was delineated into two fragments (F1 and F2) as well as a third fragment (F3), partially overlapping with F1 and F2 (Fig. 24 A). These fragments were then tested for binding in the UV-crosslinking assay. XSeb4R bound to all fragments tested, indicating multiple XSeb4R-binding sites harbored within the *Xngnr-1* 3'UTR (Fig. 24 C-E). The interactions were specific, as binding could be effectively competed by cold RNA of the corresponding *Xngnr-1* 3'UTR fragments but not with *LacZ* RNA.

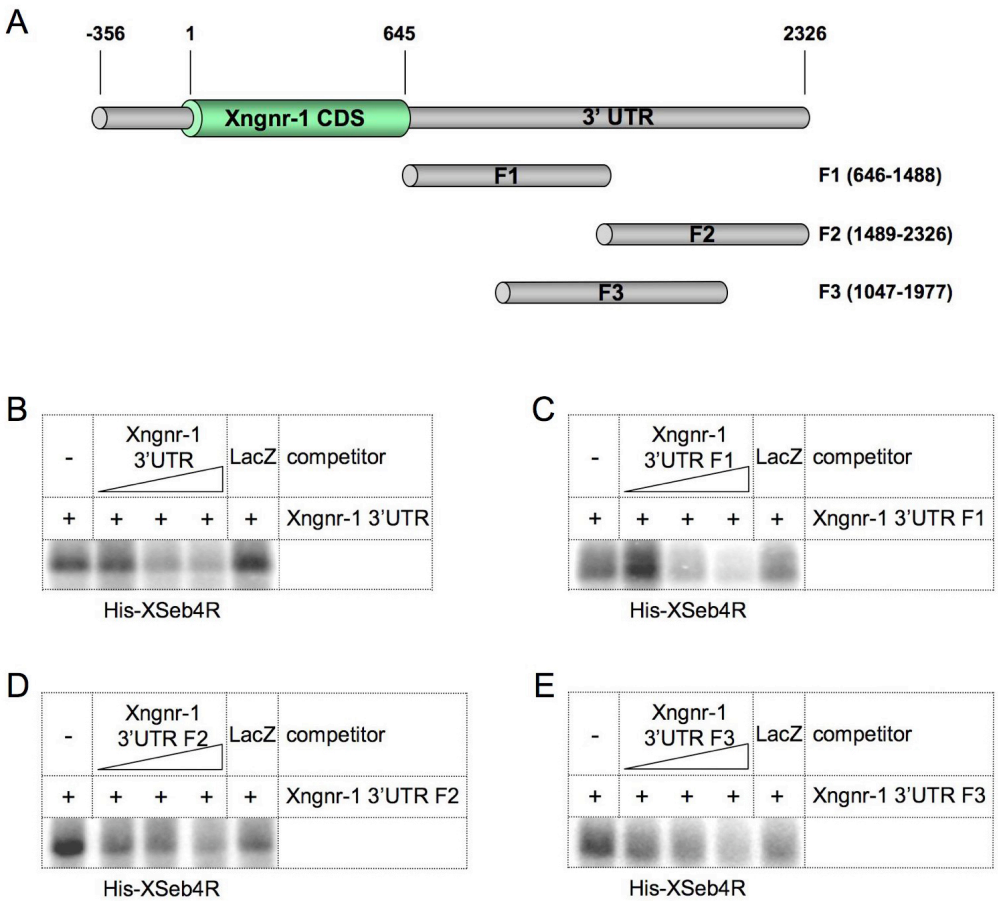


Fig. 24: XSeb4R interacted with the *Xngnr-1* 3'UTR *in vitro*. (A) Scheme of *Xngnr-1* mRNA structure. Nucleotides are indicated in respect to the start ATG. F1 consists of 842 nucleotides of the *Xngnr-1* 3'UTR. F2 is 837 nucleotides long and the 930 nucleotide long F3 consists partially of sequences harbored in F1 and F2. **(B)-(E)** Bacterially purified His-XSeb4R protein was tested for binding in an *in vitro* UV-crosslinking assay to ^{32}P-labeled full-length *Xngnr-1* 3'UTR **(B)**, F1 **(C)**, F2 **(D)** and F3 **(E)** RNA. Competition was performed with increasing amounts (100, 500 and 1000 pg) of unlabeled *Xngnr-1* RNA or the fragments respectively. *LacZ* RNA (1000 pg) served as negative control. Binding was determined by phosphoimager analysis.

To determine whether XSeb4R can also activate translational of *Xngnr-1*, the full-length 3'UTR, as well as the three fragments, were fused behind the firefly *luciferase* coding sequence and injected into *Xenopus* oocytes. Coinjection of *XSeb4R* together with *Xngnr-1* 3'UTR reporter RNA resulted in moderate translational activation (1.3-fold) (Fig. 25), while XSeb4RΔRRM failed to activate translation. Interestingly, luciferase activity was not enhanced when F1 was evaluated; despite the ability of XSeb4R to interact *in vitro* with this fragment, indicating XSeb4R binding to a target transcript is not sufficient to activate translation. XSeb4R increased luciferase activity of reporter mRNAs containing F2

(2.5-fold) and F3 (2-fold), stronger than the full-length 3'UTR. Taken together, the previous *in vitro* binding assay and the *in vivo* luciferase assay suggest *Xngnr-1* is also a target of XSeb4R.

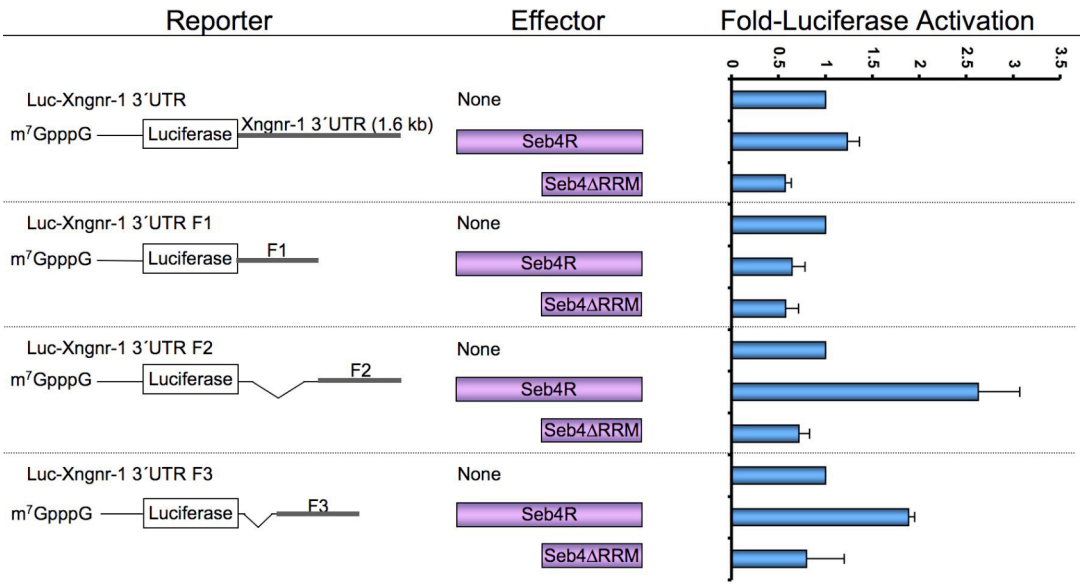


Fig. 25: XSeb4R activated translation through the *Xngnr-1* 3'UTR. Stage V-VI oocytes were injected with 25 ng effector mRNAs and incubated for 5 to 6 hours. Thereafter, 100 pg of the firefly *Xngnr-1* 3'UTR-Luc reporter mRNA was injected together with 3.5 pg *Renilla luciferase* mRNA. Oocytes were further incubated overnight, harvested in two pools with 15 oocytes and cell lysates were evaluated for luciferase activity. All firefly Xngnr-1-Luc values were normalized to *Renilla* luciferase and shown is the fold-activation relative to the Xngnr-1 3'UTR reporter activity alone.

3.8 Identification of additional XSeb4R target mRNAs

3.8.1 Cloning of low abundant, small RNA-fragments by XSeb4R RNP-immunoprecipitation

So far, only a few target-specific RNA binding proteins have been characterized and even less have been identified as positive translational regulators (Gorgoni *et al.*, 2005; Charlesworth *et al.*, 2006; Vasudevan *et al.*, 2007). XSeb4R activates translation of VegT and Xngnr-1 through binding sites harbored in their 3'UTR. However, the XSeb4R expression pattern implicates additional unknown target

mRNAs. A method for target identification of RNA binding proteins is RNA immunoprecipitation (RNA-IP or RIP), where endogenous RNPs are isolated (Niranjanakumari *et al*., 2002; Gilbert *et al*., 2004; Peritz *et al*., 2006; Townley-Tilson *et al*., 2006). As no suitable XSeb4R antibody was available, an overexpression strategy employing an epitope-tagged XSeb4R-construct was chosen (Fig. 26). *Flag-XSeb4R* mRNA was injected into both blastomeres of 2-cell stage embryos in a concentration that was previously shown to promote neurogenesis (Boy *et al*., 2004). A high number of embryos (3000) were injected to compensate for a predicted low amount of XSeb4R-RNA complexes. In addition to *Flag-XSeb4R*, pools of embryos were also injected with mRNA encoding *Flag-XSeb4RΔRRM* or *Flag-GFP* to identify possible unspecific interacting RNAs. At the open neural plate stage crosslinking by formaldehyde was performed to stabilize the protein-RNA complexes. Embryonic extracts were prepared and cell lysates precleared with Sepharose to remove transcripts that unspecifically bind to the beads. Flag-XSeb4R-RNA complexes were immunoprecipitated with a Flag antibody, the crosslink was reversed and the protein associated RNA extracted.

To evaluate the complexity of the RNA targets, the recovered RNA was 3' terminally labeled with ^{32}pCp, separated by denaturing PAGE and visualized by phosphoimager analysis. Flag-XSeb4R pulled down a wide range of RNAs of different length as shown in Fig. 27. The significantly weaker pattern of RNA obtained by Flag-XSeb4RΔRRM, Flag-GFP and the uninjected control IP partially resembled that obtained by Flag-XSeb4R, suggesting unspecific binding to RNAs. However, the RNA IP of Flag-XSeb4R contained multiple RNAs that were unique (Fig. 27, Flag-XSeb4R, red asterisks), indicating putative specific XSeb4R interaction.

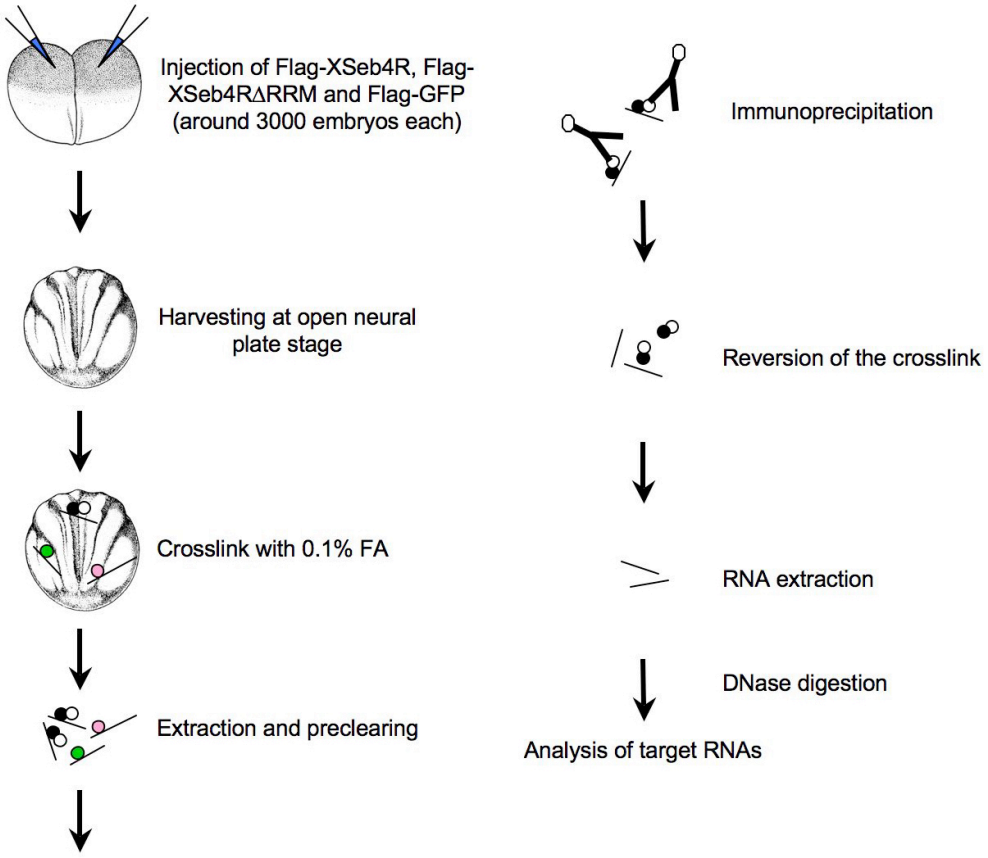


Fig. 26: Scheme of XSeb4R target RNA immunoprecipitation. Embryos were injected animally with 100 pg *Flag-XSeb4R*, *Flag-XSeb4RΔRRM* or *Flag-GFP* into both blastomeres at the 2-cell stage and grown until open neural plate stage (stage 13 to 14). Protein-RNA complex were stabilized by a crosslink using 0.1% FA. Embryonic lysate was subjected to immunoprecipitation against the FLAG-tag. The crosslink was reversed by heating the extracts to 70°C for 45 minutes. The bound RNA was recovered by TRIZOL extraction, with subsequent DNaseI digestion.

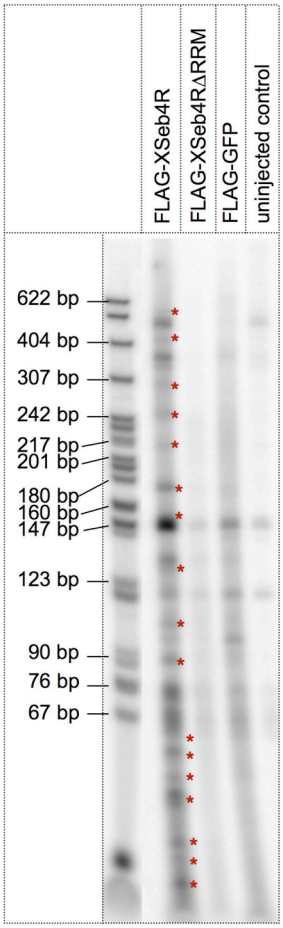


Fig. 27: Visualization of FLAG-XSeb4R, Flag-XSeb4RΔRRM, Flag-GFP and uninjected control bound RNAs. Obtained RNA of the Flag-XSeb4R, Flag-XSeb4RΔRRM, Flag-GFP and uninjected control RNA IP was 3' terminal labeled utilizing ^{32}pCp. Fragments were separated by an 8% denaturing PAGE, followed by phosphoimager analysis. RNA obtained by FLAG-XSeb4R precipitation displayed several unique bands that are labeled with a red asterisk.

Identification of XSeb4R target mRNAs by sequence analysis requires the transcription into DNA (cDNA). Due to the extraction procedure, the target RNAs might be fragmented or partially degraded, thus lacking a polyA-tail. Therefore, not all RNA fragments would be efficiently transcribed into cDNA by conventional reverse transcription employing oligo dT primers and/or random hexamers, as oligo dT primers would lack their docking site and random hexamers would shorten the transcripts sequence due to internal binding.

Thus, an approach employing adaptor ligation was performed (Fig. 28; Pfeffer *et al*., 2003; Chen *et al*., 2005). The adaptors are RNA-DNA hybrids, consisting of 15 desoxyribonucleotides and three terminal ribonucleotides to increase ligation

efficiency. The 3' adaptor carries an amine-block at the 3' terminus to prevent multimerization of the adaptor during the ligation processes. In addition to the precipitated RNA, *VegT* mRNA, as well as a control without RNA, were cloned in parallel, serving as positive and negative control during the cloning procedure.

First, the precipitated RNA was treated with alkaline phosphatase to remove the 5' phosphate group of the RNA and thus, preventing recirculation during the adaptor ligation. The 3'adaptor was then 5'-P^{32} labeled and subsequently ligated to the recovered RNA. The resulting RNA-fragments were further rephosphorylated and ligated to the 5'-P^{32} labeled 5'adaptor. The isolated RNA containing 5' and 3' adaptors was separated by denaturing PAGE and subdivided into 16 fractions followed by RT-PCR, using adaptor specific primers. The PCR-products from the 16 fractions were separated by agarose gel electrophoresis; 26 individual bands were isolated and cloned.

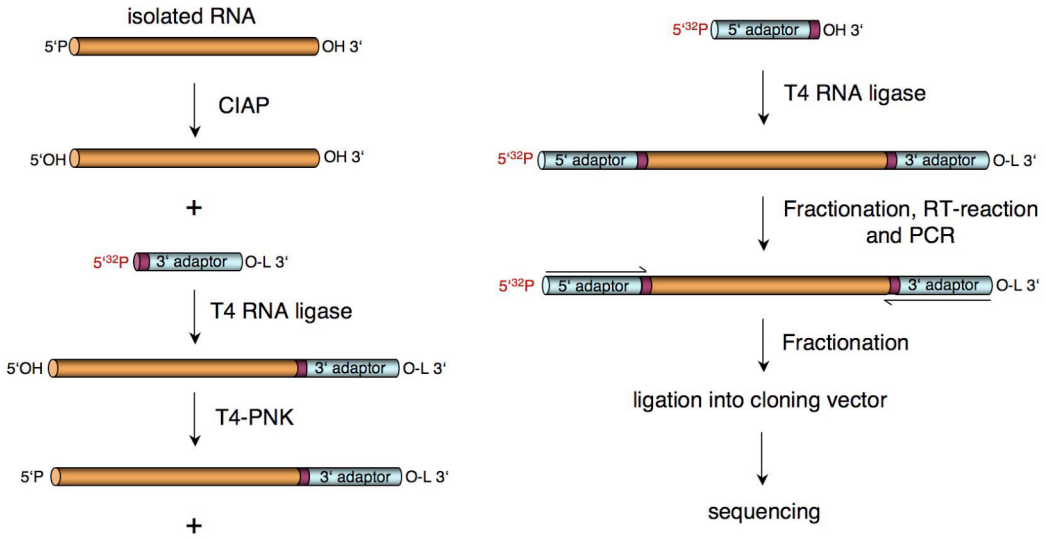


Fig. 28: Scheme of XSeb4R target RNA cloning and identification. O-L: 3'amine-block

The potential XSeb4R targets were identified by sequence analysis. In total 53 clones were sequenced, representing at least two individual clones of each fraction, if possible. Several fractions resulted in no only one clone after ligation into the cloning vector. The sequence of the cloned *VegT* mRNA, which served as positive control during the screen, was determined and reflected the expected length. Ten clones could not be analyzed as the sequences revealed multiple, overlapping signals and five clones represented the empty vector. Several clones

contained multiple inserts, which is indicated in Table 3. Insert consisting of the adaptors without a fragment are indicated with an insert length of 0. Five clones were identified as ribosomal RNA (Table 3, BR 01, BR 02, BR 04, BR 05 and BR 08). As ribosomal RNA is the most abundant RNA, this most likely was an artifact and a possible interaction of XSeb4R and the ribosomal subunits was not further investigated. The vast majority of clones (74.3%) consisted of fragments shorter than 20 nucleotides, which made annotation impossible. However, one clone was annotated to an EST-sequence (Table 3, accession number BC086468, highlighted in blue) and is further referred to as Xl G14 (a complete list with clones, the corresponding fractions and sequences is shown in the appendix).

Table 3: Sequenced clones of the Flag-XSeb4R RNA IP

clone	annotation	multiple inserts	insert length w/o adaptors [nt]
BR 01	28S rRNA	no	159
BR 02	28S rRNA	no	85
BR 03	G14 (BC086468)	no	85
BR 04	18S rRNA	no	46
BR 05	28S rRNA	no	43
BR 06	unknown	yes (2)	43, 0
BR 07	unknown	yes (2)	35, 20
BR 08	28S rRNA	no	31
BR 09	unknown	yes (2)	18, 0
BR 10	unknown	no	17
BR 11	unknown	yes (2)	16,15
BR 12	unknown	yes (2)	17,7
BR 13	unknown	yes (2)	16, 0
BR 14	unknown	no	14
BR 15	unknown	yes (2)	14, 0
BR 17	unknown	no	13
BR 18	unknown	yes (2)	9, 2
BR 19	unknown	yes (4)	8, 5, 2, 0
BR 20	unknown	yes (2)	5, 5
BR 21	unknown	yes (3)	5, 0, 0
BR 22	unknown	yes (2)	4, 3
BR 23	unknown	yes (2)	4, 2
BR 24	unknown	yes (2)	2, 2
BR 25	unknown	yes (2)	2, 2
BR 26	unknown	yes (2)	2, 1
BR 27	unknown	yes (2)	2, 0
BR 28	unknown	yes (2)	2, 0
BR 29	unknown	yes (2)	2, 0
BR 30	unknown	yes (2)	2, 0
BR 31	unknown	yes (2)	2, 0
BR 32	unknown	yes (2)	2, 0
BR 33	unknown	yes (2)	2, 0
BR 34	unknown	no	2
BR 35	unknown	yes (2)	0, 0
negative control	H_2O	-	6
positive control	VegT	-	~ 500

3.8.2 Modification of the XSeb4R target RNP-IP

The first RNA-IP resulted in clones consisting of a relative short sequence, indicating the necessity to optimize the experimental setup. Therefore, 10,000 embryos were injected with *Flag-XSeb4R* to increase the amount of RNPs. To avoid competition between the different sized RNA-fragments in the ligation reaction, the recovered RNA was ^{32}P-labeled, separated by denaturing PAGE and subdivided into several fractions. Each fraction was then separately subjected to 3' adaptor and 5' adaptor ligation as described previously.

The radiolabeled RNA resembled the band pattern obtained during the first RNA IP approach (compare Fig. 27 and Fig. 29 A). As shown in Fig. 29 B, this pattern changed upon alkaline phosphatase treatment, indicating degradation and/or fragmentation due to the procedure. The treated RNA was further subdivided into 18 fractions, based upon band intensity and size and used for the subsequent adaptor ligation reactions.

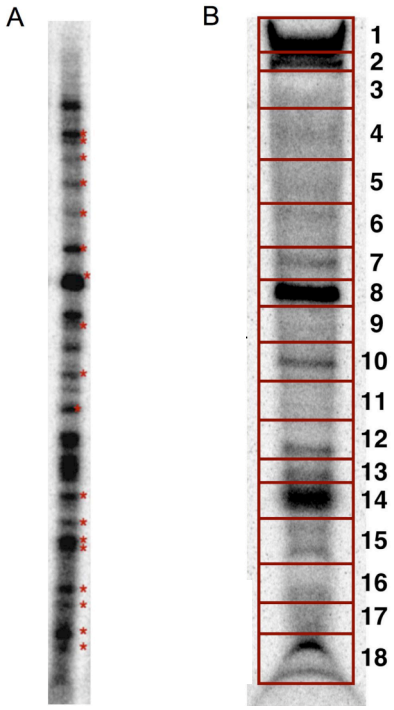


Fig. 29: Fractionation of RNA used in the second RNA cloning approach. (A) Recovered RNA of the modified RNA-IP procedure was ^{32}pCp labeled and separated by an 8% denaturing PAGE. The RNA was visualized by phosphoimaging. Red asterisks indicate bands previously identified as unique for FLAG-XSeb4R RNA-IP (see also Fig. 26). **(B)** The ^{32}P labeled RNA was subjected to alkaline phosphatase treatment and separated by an 8% denaturing PAGE. The 18 red boxes indicate allocated fractions for adaptor ligation, depending on size and band intensity.

Only products of the first 8 fractions were further analyzed, as the expected fragment length of the other fractions was shorter than 80 nucleotides. Modification of the experimental procedure resulted in a total of 3232 clones of which 141 were analyzed, yielded in 145 insert-sequences (a few plasmids harbored multiple inserts). At least three clones of each fraction were analyzed, with more clones of fractions representing long fragments or defined bands (complete table with corresponding fraction and sequences are listed in the appendix).

Most obtained sequences (91.2%) could not be annotated, even though analyzed clones contained longer RNA fragments as in the previous experiment. However, 16 clones did not contain an insert and for 7 clones the sequence reaction failed. Similar to the first RNA IP, ribosomal RNA was identified (10 clones), but was likely to be a contamination and thus not further investigated. In addition, *VegT* was found in the screen, but further sequence analysis indicated this to be a contamination (Tab. 4, labeled in blue). Several other clones could be assigned to a genomic scaffold of *Xenopus tropicalis* utilizing the ENSEMBL BLAST tool, among them a 556 bp long EST-clone which did not harbor an complete open reading frame (BJ035693). No open reading frame could be assigned to the resulting 5 kb sequence of the scaffold and was therefore not further investigated. A second 180 bp long EST clone (AU245504) did not harbor the sequence information of a full-length clone and was therefore not further analyzed.

Due to degradation and fragmentation during the cloning procedure the isolated fragments do not necessarily harbor a site for XSeb4R interaction. As no open reading frame could be linked to the identified clones, none of them were further evaluated for direct XSeb4R binding.

Table 4: XSeb4R targets identified by modified RNA-IP and RNA-cloning

clones 2	individual clones	occurance	annotation	size (bp)	Scaffold JGI
BR 001	9	10	unknown	88 to 369	2356 / 5664
BR 002	9	10	28s ribosomal RNA	62 to 250	4706 / 6387
BR 003	2	10	VegT	288 and 293	12
BR 004	2	3	XL EST (AU245504)	104 and 229	-
BR 005	2	2	unknown	223 and 326	-
BR 006	1	11	unknown	326	-
BR 007	1	2	unknown	353	-
BR 008	1	2	unknown	273	-
BR 009	1	2	unknown	116	-
BR 010	1	2	unknown	103	-
BR 011	1	2	unknown	82	-
BR 012	1	1	unknown	327	-
BR 013	1	1	unknown	326	-
BR 014	1	1	unknown	311	-
BR 015	1	1	unknown	244	-
BR 016	1	1	unknown	238	-
BR 017	1	1	unknown	238	-
BR 018	1	1	unknown	229	-
BR 019	1	1	unknown	214	-
BR 020	1	1	unknown	208	-
BR 021	1	1	unknown	206	-
BR 022	1	1	unknown	205	-
BR 023	1	1	unknown	201	-
BR 024	1	1	unknown	197	-
BR 025	1	1	unknown	192	-
BR 026	1	1	unknown	188	-
BR 027	1	1	unknown	175	-
BR 028	1	1	unknown	165	-
BR 029	1	1	unknown	161	-
BR 030	1	1	unknown	143	-
BR 031	1	1	unknown	142	-
BR 032	1	1	unknown	141	-
BR 033	1	1	unknown	137	-
BR 034	1	1	unknown	134	-
BR 035	1	1	unknown	130	-
BR 036	1	1	unknown	127	-
BR 037	1	1	unknown	126	-
BR 038	1	1	unknown	124	-
BR 039	1	1	unknown	120	-
BR 040	1	1	unknown	117	-

3.9 *Xl G14* is an additional XSeb4R target transcript

The isolated 85 bp fragment of the first RNA-IP aligned within the coding sequence of *Xl G14* and revealed 94% sequence identity. Sequence analysis of a *Xl G14* EST-clone predicted a 118 bp 5' untranslated region (UTR), a 2580 bp coding sequence and a 613 base-pair 3' UTR (Fig. 30).

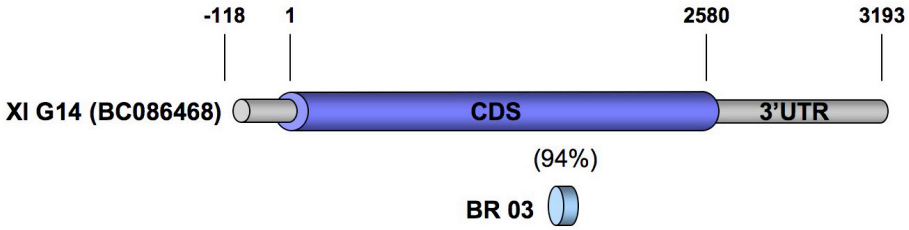
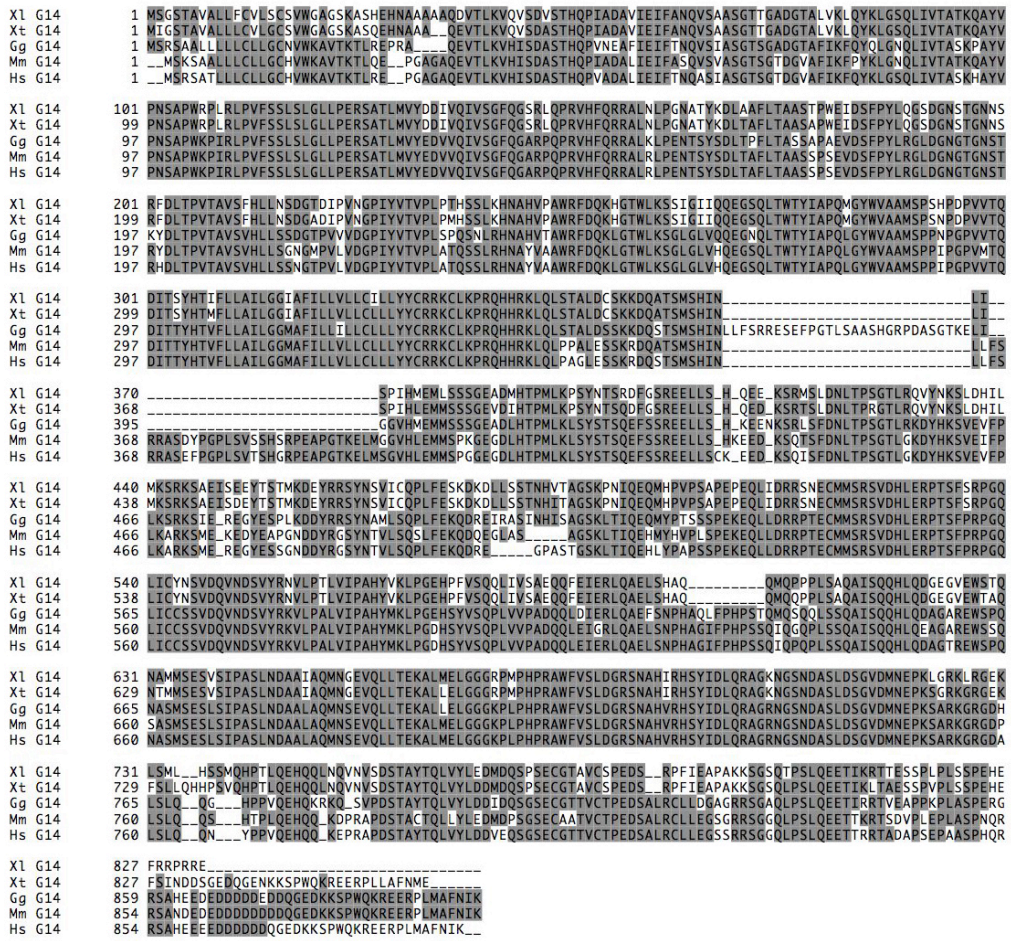


Fig. 30: Schematic alignment of *Xl G14* (BC086468) and BR 03. The 5' UTR is indicated in gray (-118 to 1), the coding sequence (CDS) is indicated in dark blue (1 to 2580) and the 3' UTR is indicated in gray (2580 to 3193). BR 03 represents the in the RNA IP isolated fragment (light blue) and is located in the *Xl G14* coding sequence (1708 to 1792). The fragment was 94% identical to the corresponding region in the *Xl G14* coding sequence. Nucleotides are indicated in respect to the start ATG.

The predicted protein sequence of G14 is conserved within vertebrates (Fig. 31 A). However, no homologues could be identified in invertebrates, plants or yeast. Protein sequence comparison revealed Xl G14 was most closely related to the *Xenopus tropicalis* homologue (94.1%), while the chick, mouse and human related proteins were more distant (66.6-68.8%) (Fig. 31 A and B). However, G14 appeared to be highly conserved between mammals (91.9%). Despite several homolog amino acid stretches, no conserved sequence motifs could be identified.

A



B

% identity	*Xenopus laevis*	*Xenopus tropicalis*	*Gallus gallus*	*Mus musculus*	*Homo sapiens*
Xenopus laevis	-	94.1	68.8	66.6	66.6
Xenopus tropicalis	94.1	-	69.8	67.7	67.7
Gallus gallus	68.8	69.8	-	84.5	87.0
Mus musculus	66.6	67.7	84.5	-	91.9
Homo sapiens	66.6	67.7	87.0	91.9	-

Fig. 31: G14 is conserved in vertebrates. A) Protein sequence alignment of *Xenopus laevis*, *Xenopus tropicalis*, chick, mouse and human G14. **B)** Protein sequence comparison of *Xenopus laevis*, *Xenopus tropicalis*, chick, mouse and human G14 indicated in percent identity utilizing Align (MacMolly Terra Align, version 3.10). Xl: *Xenopus laevis*, Xt: *Xenopus tropicalis*, Gg: *Gallus gallus*, Mm: *Mus musculus*, Hs: *Homo sapiens*.

Xl G14 expression pattern was determined by RT-PCR analysis and whole mount *in situ* hybridization, to determine if it was coexpressed with XSeb4R during embryogenesis.

The RT-PCR analysis, shown in Fig. 32 A, revealed *Xl G14* to be maternally expressed. Through early cleavage and early tailbud stages, transcript levels were maintained at relative constant levels, but decreased at late tailbud stages. Maternal *Xl G14* mRNA was localized to the animal hemisphere of the embryo, as shown by whole mount *in situ* hybridization (Fig. 32 B, stage 3). This signal decreased and disappeared at gastrulation (Fig. 32 B, stage 10). The first transcripts detected after gastrulation, were weakly expressed within the neural plate at early neurula stages (stage 14). At mid-neurula stage, *Xl G14* was also expressed in the neural crest (Fig. 32 B, stage 17). In the late neurula, *Xl G14* was detected in the eye, brain, neural tube and posterior somites (Fig. 32 B, stage 21). Transversal sections of a tailbud-stage embryo revealed *Xl G14* transcripts in the eye, enriched in the dorsal half of the midbrain and in the intermediate zone of the hindbrain, the otic vesicle and the branchial arches (Fig. 32 B, S1 and S2). The transversal section S3 showed *Xl G14* staining in the ventricular and intermediate zone of the dorsal neural tube. Neurons of the ventricular zone represent the neural precursor population. As they become post-mitotic and initiate differentiation, the neurons migrate into the intermediate zone, while the terminally differentiated neurons are located in the outer marginal zone. At stage 32, *Xl G14* mRNA was present in derivates of the central nervous system, lymphatic system and the tail tip. In stage 40 embryos, *Xl G14* was detectable in the branchial arches, brain and weakly in the neural tube.

The XSeb4R RNA-IP was performed with embryos harvested at open neural plate stages (stage 14 to 17), where *Xl G14* and *XSeb4R* are expressed in partially overlapping patterns, further pointing towards *Xl G14* as an XSeb4R-target (Fig. 32 B, Boy *et al*., 2004).

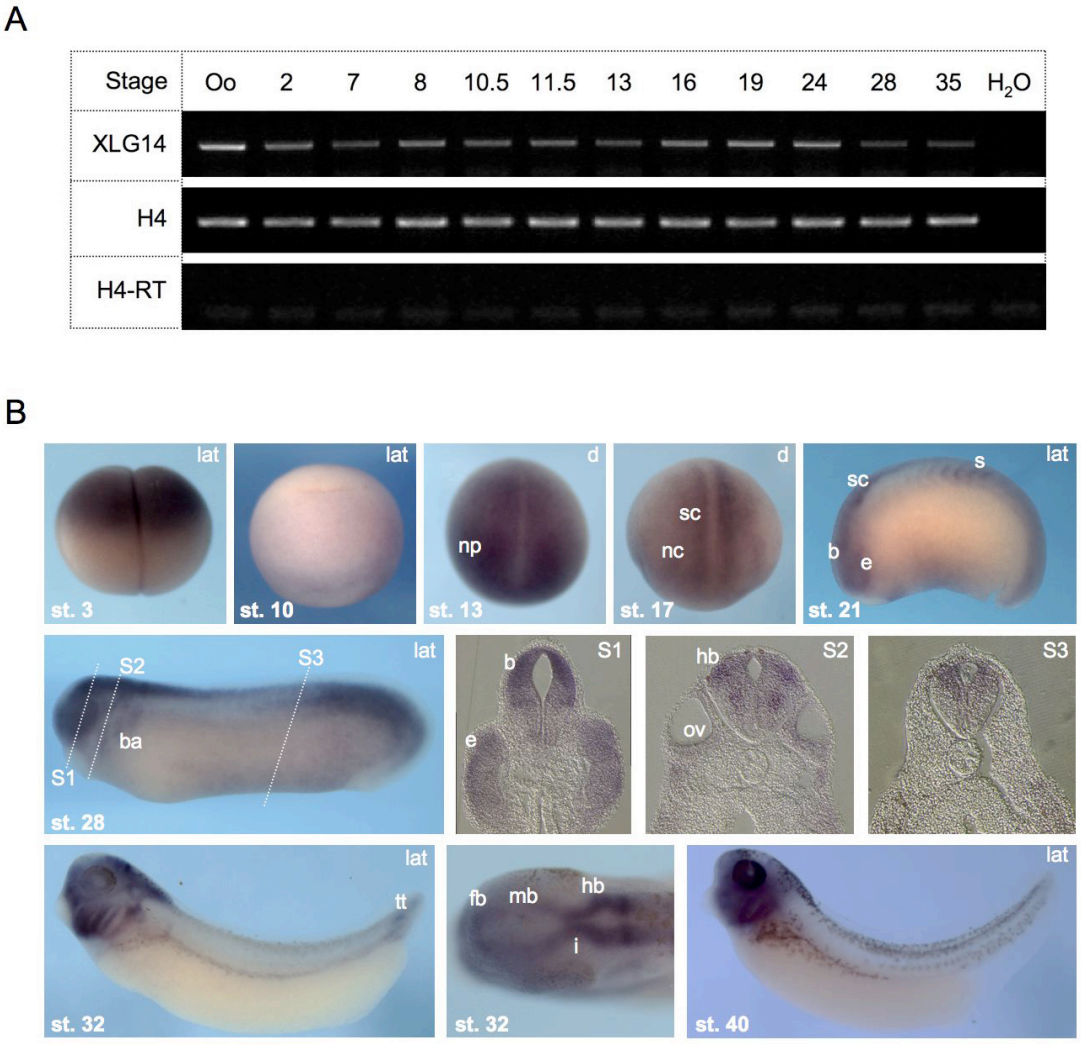


Fig. 32: *Xl G14* is expressed throughout early development and in the territories of primary neurogenesis. (A) Temporal expression of *Xl G14* in *Xenopus* development, determined by RT-PCR. *Histone H4* serves as loading control and *Histone H4*-RT as control for genomic contamination. **(B)** Spatial expression of *Xl G14* in *Xenopus* development, employing whole mount *in situ* hybridization. Oo: oocyte; H4-RT: control for genomic contamination (PCR for Histone H4 on the extracted RNA); lat: lateral view; an: animal view; d: dorsal view; np: neural plate; nc: neurla crest; e: eye; b: brain; s: somites; nt; neural tube; ov; otic vesicle; hb: hind brain; ba; branchial arch; tt: rail rip; fb; forebrain; mb: midbrain; i: isthmus.

XSeb4R activates translation through the 3'UTR of *VegT* and *Xngnr-1*, while the isolated *Xl G14* RNA fragment is part of the coding sequence. The majority of the analyzed transcripts revealed a relative short sequence, suggesting towards fragmentation and/or partially degradation during the RNA recovery and early steps of the cloning procedure. Therefore, it was conceivable that the precipitated *Xl G14* fragment did not necessarily represent the direct XSeb4R binding site but a fragment of the full-length transcript.

To determine of XSeb4R could interact with the *Xl G14* 3'UTR, a UV-crosslinking assay was performed. XSeb4R was found to bind specifically to the *Xl G14* 3'UTR, as binding was competed by unlabeled *Xl G14* 3'UTR but not with *LacZ* mRNA (Fig. 33).

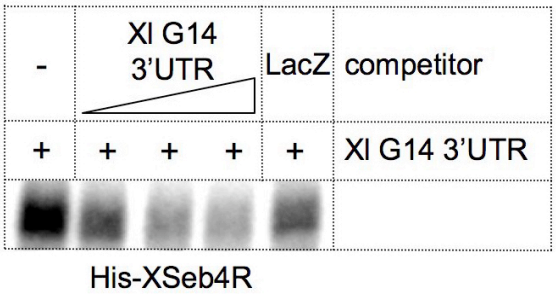


Fig. 33: XSeb4R interacted with the *Xl G14* 3'UTR *in vitro*. Bacterially purified His-XSeb4R protein was tested for binding in an *in vitro* UV-crosslinking assay to ^{32}P-labeled full-length *Xl G14* 3'UTR mRNA. Competition was performed with increasing amounts (100, 500 and 1000 pg) of unlabeled *Xl G14* RNA. *LacZ* RNA (1000 pg) served as negative control. Binding was determined by SDS-PAGE and subsequent phosphoimager analysis.

To investigate if XSeb4R facilitates translational activation via interaction with the *Xl G14* 3'UTR, the oocyte translation assay was performed. As shown in Fig. 34, The full-length *Xl G14* 3' UTR was fused behind the firefly *luciferase* ORF and coinjected together with *XSeb4R* into oocytes, resulting in a moderate (1.5-fold) translational activation, comparable to activation obtained by the full-length *Xngnr-1* 3'UTR (Fig. 25). XSeb4RΔRRM failed to activate translation of the reporter construct.

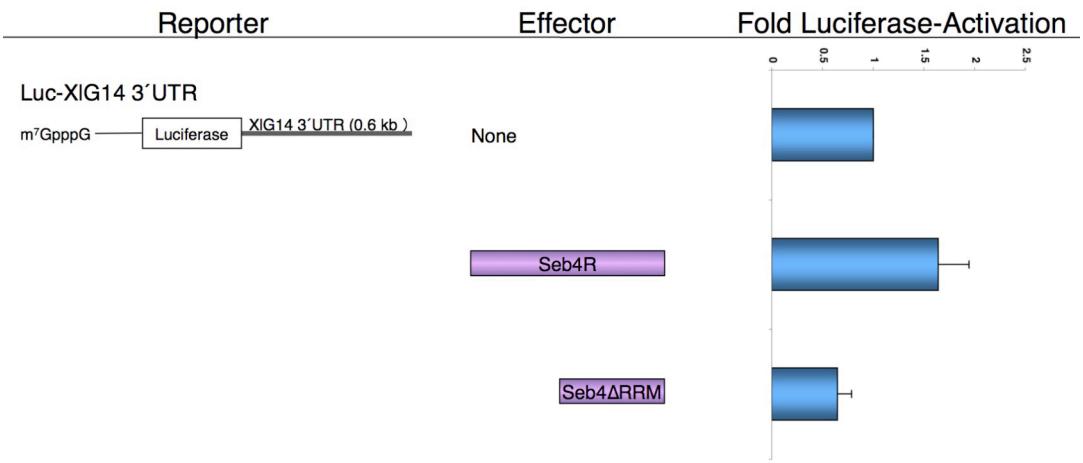


Fig. 34: XSeb4R activated moderate translation through the *Xl G14* 3'UTR. Stage V-VI oocytes were injected with 25 ng effector mRNAs and incubated for 5 to 6 hours. Thereafter, 100 pg of the firefly *Xl G14* 3'UTR-Luc reporter mRNA was injected together with 3.5 pg *Renilla luciferase* mRNA. Oocytes were further incubated overnight, harvested in two pools with 15 oocytes and cell lysates were evaluated for luciferase activity. All firefly Luc-Xl G14 values were normalized to *Renilla* luciferase and shown is the fold-activation relative to the Xl G14 3'UTR reporter activity alone.

The expression pattern of *Xl G14* implicated a function in neural development. Therefore, *Xl G14* was overexpressed in *Xenopus* embryos. Concentrations above 100 pg of *Xl G14* were embryonic lethal. Interestingly, embryos injected with a high amount *Xl G14* died between stage 11.5 and 12, the transition from gastrulation to neurulation. Embryos, injected with lower concentrations were grown until stage 38 but the embryos displayed no morphological abnormalities compared to uninjected controls (not shown). A function of *Xl G14* in neurogenesis was further determined by the expression of molecular markers performing whole mount *in situ* hybridization analysis. The *Xl G14* gain-of-function revealed no effect upon the neural precursor cell population located within the neural plate (Fig. 35, staining for *Sox3*). Similarly, neither expression of the neural determination-factor *Xngnr-1*, nor *N-tubulin*, which demarcates terminal differentiated neurons, was altered.

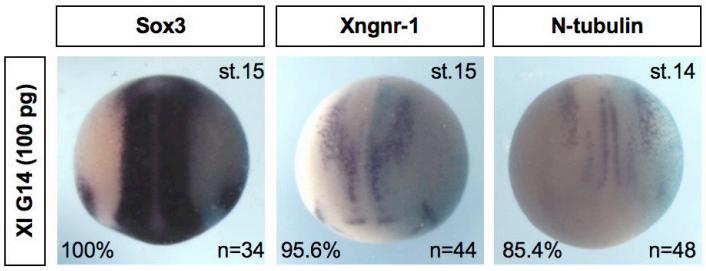


Fig. 35: *Xl G14* over-expression did not alter expression of neural markers. 100 pg *Xl G14* mRNA was injected into one blastomere of the two-cell stage, together with 75 pg *LacZ* mRNA and harvested at stage 14 to 15. Expression of markers was determined by whole mount *in situ* hybridization. Embryos are shown in a dorsal view with the posterior up. The injected site is orientated to the right, indicated by the light blue LacZ staining. Investigated markers are indicated in the box above the displayed embryos. n: number of analyzed embryos, x%: percent of embryos representing the displayed marker-phenotype.

The absence of a phenotype or an effect on the evaluated molecular markers could be explained by the mRNA concentration used in the experiment. It is possible, that a higher dose would result in alterations in morphology and/or markers for neural development. But as mentioned, a higher dose of *Xl G14* was lethal at the onset of neurulation. Different approaches avoiding this side effect would be experiments in the animal cap system and/or testing a hormone inducible construct (Xl G14-GR). Further insights into Xl G14 function could also be gained by loss-of-function experiments utilizing morpholino oligonucleotides.

4. Discussion

4.1 The RNA binding protein XSeb4R functions as a translational activator

4.1.1 XSeb4R binds to RNA

XSeb4R was isolated based on its expression at early neurula stages of development, in a pattern indicative for a role in primary neurogenesis (Boy *et al*., 2004). Previous functional analysis has indeed revealed a proneural function for XSeb4R in the open neural plate and also in the context of retinogenesis, but the underlying molecular mechanism remained elusive.

XSeb4R harbors an RNA recognition motif, which has been characterized in a wide variety of RNPs and is one of the best-described protein-RNA interaction domains (Query *et al*., 1989; Lutz-Freyermuth *et al*., 1990; Hall *et al*., 1992; Kühn *et al*., 1996; Kielkopf *et al*., 2001; Hinman *et al*., 2008). The RRM has been previously reported to further promote binding to DNA and protein-protein interaction (DeAngelo *et al*., 1995; Samuels *et al*., 1998; Selenko *et al*., 2003; ElAntak *et al*., 2007). Affinity chromatography using different RNA homopolymeres showed that XSeb4R binds specifically to RNA and importantly, does not interact with ssDNA or dsDNA. The RRM motif of RDM1, for example, has been shown to bind to both, ssDNA and dsDNA, implicating this factor in transcriptional regulation instead of RNA metabolism (Hamimes *et al*., 2005). XSeb4R avidly binds to polyG but not to the other employed polymers. Similar binding preferences have been reported for many other RRM-type RBPs, with several exceptions (Bagga *et al*., 1998; Newberry *et al*., 1999; Hamimes *et al*., 2006). The PolyA binding protein (four RRM motifs) binds preferentially to polyA tails of transcripts, while Elav/HuR proteins (three RRM motifs) bind to A/U rich regions (Standart *et al*., 1981; Grossi de Sa *et al*., 1988; Bernstein *et al*., 1989; Peng *et al*., 1998; Sladic *et al*., 2004). In many binding activities the secondary structure of transcripts play an important role for RNP target recognition, as described for the stem-loop binding protein, the

testis specific protein RBMY or the *Drosophila* SR protein B52, implicating that the presence of polyG stretches within the 3'UTR of a potential target mRNA might not be sufficient for XSeb4R binding (Shi *et al*., 1997; Dominski *et al*., 1999; Dominski *et al*., 2007; Skrisovska *et al*., 2007). However, the motif and/or structure essential for XSeb4R binding to target transcripts remains to be determined.

4.1.2 XSeb4R functions as a translational activator

RNA binding proteins, which establish protein-RNA interaction via RRM motifs, have been shown to function in all steps of RNA metabolism and activity (Fedoroff, 2002; Colegrove-Otero *et al*., 2005; Sanchez-Diaz *et al*., 2006; Lin *et al*., 2007; Hinman *et al*., 2008). The *Caenorhabditis elegans* homologue of XSeb4R, Sup-12, has been implicated in alternative splicing of target RNAs while the human homologue RNPC1 has been demonstrated to promote transcript stability, suggesting that Seb4R function may not be conserved between species (Anyanful *et al.,* 2004; Shu *et al.,* 2006; Kuroyanagi *et al.,* 2007). To determine XSeb4R function in RNA metabolism, first a possible function for XSeb4R as a translational regulator was tested. The MS2-tethering assay identified XSeb4R as a translational activator. Formation of the closed-loop structure has been suggested to play a crucial role for efficient translational initiation (Jacobson, 1996; Tarun *et al*., 1996; Le *et al*., 1997; Kozak, 2007a; Kozak, 2007b; Amrani *et al*., 2008). The polyA binding protein, a potent translational initiator, has been shown to interact with the cap through interaction of RRM1 and RRM2 bound to the polyA-tract with eIF4G and hereby close the RNA-loop (Tarun *et al*., 1995; Jacobson, 1996; Tarun *et al*., 1996; Tarun *et al*., 1997; Deo *et al*., 1999; Gray *et al*., 2000; Varan *et al*., 2001). The failure of MS2-XSeb4RΔC to activate translation suggests a motif in the carboxy-terminus, responsible for interaction with other proteins. Furthermore, the activation of the *PV-IRES-Luc-MS2* reporter suggests the activity of XSeb4R is dependent on cap-associated proteins. Similarly to MS2-XSeb4R, MS2-PAB1P also activated translation via this *IRES-MS2* reporter construct (Gorgoni *et al*., 2005). The enhanced luciferase activity can be explained by direct binding of eIF4G by PAB1P and the PV-IRES, thus, initiating formation of the closed-loop. PAB1P has been shown to interact with additional factors, such as the PAB-

interacting protein (Paip) (Gray *et al*., 2000). Moreover, Gray *et al*. suggested that PABP might interact with additional known or unknown factors to modulate translation. XSeb4R could be such an interaction partner, thus activating translation indirectly via recruitment of PABP. The *Luc-MS2* reporter does not harbor a polyA-tract but its translation is robustly activated by MS2-XSeb4R. Though, if XSeb4R specifically enhances the activity of the cytoplasmic polyadenylation element binding protein (CPEB) contained in *Xenopus* oocytes, this could result in a prolonged polyA-tail and recruitment of PABP, as the firefly *luciferase* coding sequence harbors four potential CPE-binding sites (Hake *et al*., 1994; Mendez *et al*., 2001; Charleswoth *et al*., 2004; Richter, 2007; Rouhana *et al*., 2007) Radford *et al*., 2008; Standart *et al*., 2008). However, this mechanism is unlikely, as MS2-XSeb4R failed to induce translation via the *CSFV-IRES-MS2-Luc* reporter, harboring the same *luciferase* gene. Thus, the results suggest XSeb4R is more likely to interact with the cap-associated complex. Translational initiation often correlates also with increases in polyA-tract length (Richter, 1987, Rosenthal *et al*., 1987; Preiss *et al*., 1998). If XSeb4R activated translation depends on polyA-tail length, however, remains to be determined, as the employed reporter constructs do not harbor any polyA-tract.

Surprisingly, MS2-XSeb4R and MS2-PAB1P activated translation of the Luc-MS2 reporter, harboring a cap analog, even though the absolute luciferase activity was significantly reduced. An explanation for this observation is provided by the cytoplasmic methyltransferase contained in *Xenopus* oocytes, which could modify the cap analog and thus, facilitating association of cap-binding proteins (Furuichi *et al*., 1977; White *et al*., 1985). To identify potential XSeb4R interaction partners, yeast-two-hybrid screens, using a library of proteins enriched in proteins expressed in the central nervous system and a library containing the known initiation factors, were performed (the latter by N. K. Gray), which did not result in the identification of a potential interaction partner (data not shown). Immunoprecipitation and mass spectrometry of XSeb4R-protein complexes would provide an alternative method to identify interaction partners. Taken together, the results implicate an interaction of XSeb4R with the cap complex (Fig. 36). Whether this interaction is direct or indirect via PABP and/or an alternative, unidentified factor remains to be determined.

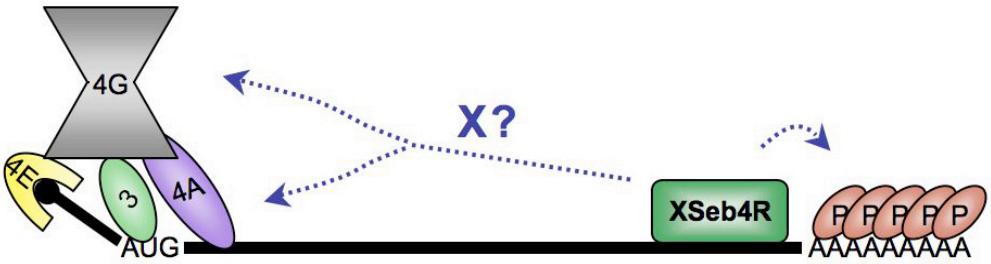


Fig. 36: XSeb4R activates translation. XSeb4R (dark green) binds to the RNA and interacts (blue lines) with cap-associated proteins (eIF4G, gray; eIF3, light green; eIF4A, purple; eIF4E, yellow) either directly, through an unknown factor "X" or via PABP (brown).

4.2 XSeb4R binds to the 3'UTR and activates translation of target transcripts

4.2.1 XSeb4R activates translation of VegT

To date, only a few sequence-specific RNA binding proteins involved in translational regulation have been characterized and their target transcripts identified. *XSeb4R* and *VegT* share partially overlapping expression patterns (Lustig *et al*., 1996; Stennard *et al*., 1996; Zhang *et al*., 1996; Horb *et al*., 1997; Boy *et al*., 2004). Moreover, XSeb4R activated *VegT* expression as well as marker genes representative for the three germ layers in the animal cap assay, most of them VegT targets (Fig. 18, Souopgui *et al*., 2008). Importantly, *VegT* mRNA can be detected in all three germ layers of blastula stage embryos, with low but significant levels in the ectoderm, while XSeb4R can be detected in comparable amount in all parts of the embryo, further suggesting *VegT* as an endogenous XSeb4R target (Clements *et al*., 1999; Boy *et al*., 2004; Cao *et al*., 2007; Souopgui *et al*., 2008).

The UV-crosslink experiments of XSeb4R with the *VegT* 3'UTR, together with the results of the RNA-IP and the observed translational activated obtained by this interaction, confirmed *VegT* as an XSeb4R target transcript. Interestingly, the *VegT* mRNA is translational silent in the oocyte and VegT protein is not detected until egg-maturation (Stennard *et al*., 1999). In this context, translation repression

by cytoplasmic polyadenylation has been suggested, as it has been shown for other maternally provided transcripts, such as *c-mos* and several cyclins (McGrew *et al*., 1989; Sheets *et al*., 1994; Wickens *et al*., 1997; Stennard *et al*., 1999). As XSeb4R is also maternally expressed, it is possible that XSeb4R initiates *VegT* translation early in *Xenopus* development (Lustig *et al*., 1996; Stennard *et al*., 1996; Zhang *et al*., 1996; Horb *et al*., 1997; Boy *et al*., 2004). However, the presence of XSeb4R protein in the oocyte could not be confirmed, as two distinct XSeb4R-specific antibodies failed to detect endogenous XSeb4R in oocytes and embryos, while they both detected overexpressed XSeb4R (Boy *et al*., 2004 and data not shown). Similarly, a VegT/Apod-specific antibody failed to detect endogenous VegT proteins, which could confirm an increase of VegT by XSeb4R (Stennard *et al*., 1999; not shown).

Several transport and anchor proteins, like the Prrp, Vera and Elav have been shown to bind to specific sequences within the *VegT* 3'UTR, responsible for *VegT* localization to the vegetal cortex of the *Xenopus* oocyte (Zhao *et al*., 2001; Kwon *et al*., 2002; Arthur *et al*., submitted). Delineation of the VegT 3'UTR not only suggests multiple XSeb4R binding sites, but further implicates the presence of factors inhibiting XSeb4R mediated translational activation. The *VegT* 3'UTR fragments, which were activated by XSeb4R (F2 and F3), also harbor motifs, shown to be required for *VegT* localization in the oocyte as well as A/U-rich elements (Kwon *et al*., 2002). Therefore, it is possible that XSeb4R could compete or interact with one of these factors. Elav/HuR, has been described to stabilize p21 mRNA, which has also been shown for the human XSeb4R homolog RNPC1, suggesting an interaction of these proteins (Wang *et al*., 2000; Shu *et al*., 2006).

XSeb4R also activates VegT translation during embryogenesis (Fig. 23), which is supported by XSeb4R knockdown experiments that resembled the phenotype of VegT depleted embryos with reduced expression of mesodermal and endodermal marker genes (Zhang *et al*., 1998; Heasman *et al*., 2001; Souopgui *et al*., 2008). Additionally, XSeb4R overexpression in animal caps induces VegT and Apod expression (Fig. 18; Souopgui *et al*., 2008). Here, Elav/HuR and XSeb4R could also cooperate, as both factors share overlapping expression patterns in the embryo (Good, 1995; Perron *et al*., 1999; Boy *et al*., 2004). Elav/HuR proteins facilitate multiple functions of the RNA metabolism, which are highly context dependent (Jain *et al*., 1997; Antic *et al*., 1999; Brennan *et al*., 2000; Brennan *et*

al., 2001; Kullmann *et al*., 2002; Barreau *et al*., 2005; Hinman *et al*., 2008; Pascale *et al*., 2008). An interaction of XSeb4R and Elav later in development could provide additional and/or different functions as observed in the oocyte due to the presence of other, unidentified factors. While XSeb4R fails to alter transcript stability in the oocyte, *VegT* mRNA appears to be stabilized in the embryo, by XSeb4R (Fig. 11; Fig. 18; Fig. 22; Souopgui *et al*., 2008). Interestingly, the *VegT* transcript levels are not only constant but increase over time, which requires *de novo* RNA synthesis. As XSeb4R was not found to bind to DNA, *VegT* transcription must be activated by a different mechanism. Previous studies have argued for a positive feedback loop of VegT (Clements *et al*., 2001; Fujii *et al*., 2008). Recent findings, however, did indicate that VegT is not activating its own transcription but rather that the zygotic splice variant, Apod, underlies this autoregulation (Souopgui *et al*., 2008).

Taken together, the obtained results strongly suggest that XSeb4R stimulates translation of *VegT* mRNA in the oocyte and additionally stabilizes the transcript during embryogenesis (Fig. 37), while putative cofactor(s) remain to be identified.

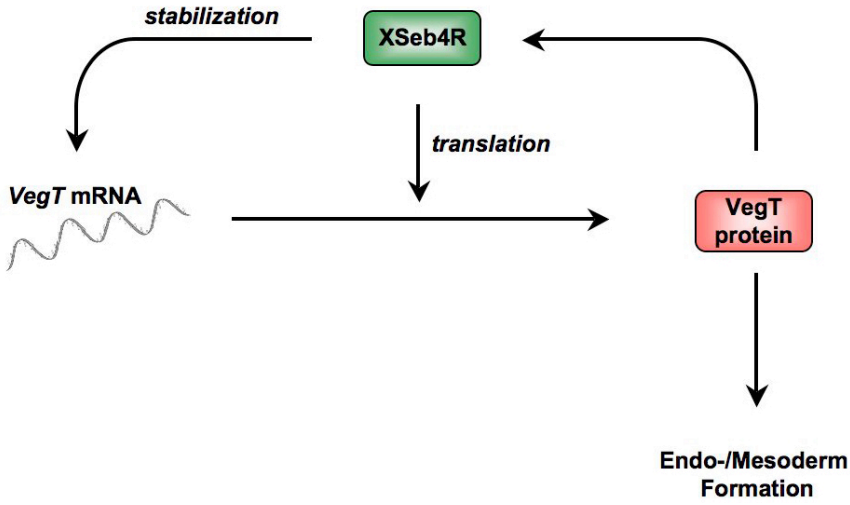


Fig. 37: XSeb4R activates translation of VegT and stabilizes *VegT* mRNA in embryos. XSeb4R (green) binds to the *VegT* 3'UTR, stabilizes the transcript (in embryos) and initiates VegT translation. VegT protein (red) activates transcription of molecules responsible for mesoderm and endoderm formation, as well as XSeb4R transcription (J. Souopgui, data not shown).

4.2.2 XSeb4R activates translation of Xngnr-1

In addition to the mesodermal expression, XSeb4R transcripts are present in the territories of primary neurogenesis and XSeb4R has been described to promote neuronal differentiation (Boy *et al*., 2004). Furthermore, XSeb4R was found to be sufficient to promote a neuronal cell fate in the animal cap system by induction of the neural determination factor *Xngnr-1* (Fig. 18; Souopgui *et al*., 2008). However, the mechanism promoting this increase is unclear. In whole embryos, the neuroectoderm is induced by factors secreted from the underlying mesoderm (Sasai *et al*., 1996; Zimmerman *et al*., 1996; DeRobertis *et al*., 2004). VegT has been shown to induce expression of mesodermal markers, thus transcriptional activation of *Xngnr-1* could be indirect via XSeb4R activation of VegT (Xanthos *et al*., 2001; Zhang *et al*., 1998; Sinner *et al*., 2006). On the other hand, an autoregulatory feedback loop for Xngnr-1 has been suggested, which would suggest a direct association of XSeb4R with the *Xngnr-1* transcript (Dubois *et al*., 1998). Binding of XSeb4R to the *Xngnr-1* 3'UTR argue for a direct activation, which is further confirmed by the translational activation assay, however, the observed effect was only moderate. Delineation of the *Xngnr-1* 3'UTR indicates multiple XSeb4R binding sites and moreover, reveals that binding of XSeb4R is not sufficient to induce translation but requires the RNA-dependent association of one or multiple additional factors or that XSeb4R function can be inhibited by binding of other proteins. The *Xngnr-1* 3'UTR has been much less studied in the context of RNA metabolism as the *VegT* 3'UTR, but it is likely that also the *Xngnr-1* transcript harbors multiple binding sites for several factors involved in translational regulation and transcript stability. Also in the context of *Xngnr-1* regulation, Elav proteins would represent potential XSeb4R interaction partners. Expression and functions of Elav proteins in neural tissues has been previously reported and the *Xngnr-1* 3'UTR contains A/U-rich elements; even though, binding of Elav has not been demonstarted (Perron *et al*., 1995; Perron *et al*., 1999; Amato *et al*., 2005). Similar to VegT in embryos, Elav interaction with XSeb4R could not only affect translation, but also transcript stability, as implicated by the XSeb4R animal cap assay (Fig. 18). This would argue for a general mechanism of XSeb4R function in RNA metabolism (Fig. 37 and Fig. 38), possibly in combination with Elav proteins.

RNPs of the Musashi-family would represent a class of factors, which could also interact with XSeb4R during neurogenesis. While mammalian musashi proteins are suggested to repress translation of their target transcripts, the *Xenopus* homolog, Xnrp1, has been implicated in nucleo-cytoplasmic transport, arguing against an interaction with XSeb4R (Good *et al*., 1993). Furthermore, Xnrp1 target transcripts have not been identified. A summary of the results is shown in Fig. 38.

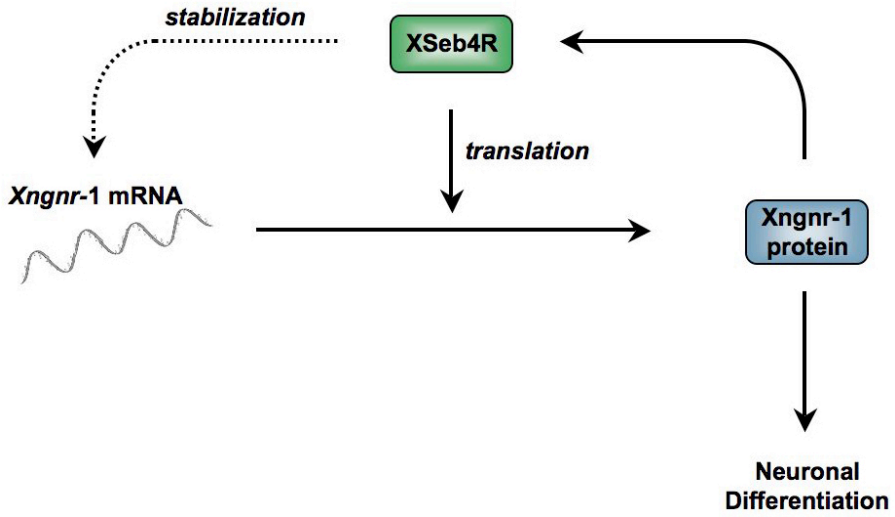


Fig. 38: XSebR activates translation of Xngnr-1. XSeb4R (green) binds to the *Xngnr-1* 3'UTR and activates translation. Xngnr-1 protein (blue) activates transcription of molecules responsible for neuronal differentiation, as well as XSeb4R transcription (Boy *et al*., 2004).

4.3 Identification of additional XSeb4R targets by RNA-IP

As *VegT* and *Xngnr-1* only share partially overlapping expression patterns with *XSeb4R*, it is likely that there are additional XSeb4R targets. Thus, an approach to identify target transcripts was performed, employing modified RNA immunoprecipitation protocols previously described to clone RNA fragments (Niranjanakumari *et al*., 2002; Gilbert *et al*., 2004; Peritz *et al*., 2006). Several identified to were annotated to two *Xenopus tropicalis* genomic scaffolds (JGI 2356/5664); one of these clones matched an expressed sequence tag (EST) in the NCBI database (BJ035693). However, further analysis revealed unspecific binding to XSeb4R (RT-PCR analysis, not shown). As multiple prominent bands of the

Flag-XSeb4R were also precipitated in the Flag-XSeb4RΔRRM and Flag-GFP RNA-IP, this particular clone is likely to represent a fragment present in both samples. This is most likely also the case for ribosomal RNA, which was identified as a XSeb4R target in both RNA-IP experiments. Interaction of XSeb4R with ribosomes was not further investigated, even though XSeb4R is an RNA binding protein involved in translational activation. Ribosomal RNA is the most abundant RNA in the cell and was therefore most likely to be a contamination. Moreover, MS2-XSeb4R failed to enhance translation via the *MS2-CSFV-IRES*, further suggesting that XSeb4R does not directly interacts with ribosomes. Most of the other sequences remain to be annotated, as the genome of *Xenopus laevis* and that of the closely related *Xenopus tropicalis* are both not completely sequenced. Furthermore, XSeb4R is thought to activate translation through binding to the 3'UTR, which is often not represented in the available database information. Even though many EST clones are available in the databases, most of them consist of only partial sequences (Blackshear *et al*., 2001). While improvement of the database would aid the identification of additional putative XSeb4R target transcripts, a phage screen, utilizing the cloned fragments as a probe, would provide an alternative approach.

Nevertheless, an additional potential target, *Xl G14*, could be identified and confirmed by expression pattern analysis and UV-crosslinking assays. Moreover, XSeb4R was found to activate translation via the *Xl G14* 3'UTR at comparable levels to the *Xngnr-1* full-length 3'UTR. Whether XSeb4R also affects *Xl G14* mRNA stability remains to be determined. Delineation of the XSeb4R binding region within *Xl G14* 3'UTR and comparison with that of the identified domains within the *VegT* and *Xngnr-1* 3'UTR may yield a common XSeb4R target motif. However, XSeb4R most likely recognizes a complex secondary structure and mapping of the recognition elements would require different experiments, such as RNase protection assays (Bindereif *et al*., 1986; Penalva *et al*., 2004).

While *Xl G14* transcripts were detected within the area of primary neurogenesis, gain-of-function experiments did not reveal a role in primary neurogenesis. Protein sequence comparison revealed G14 to be conserved within vertebrates but the protein has not yet been characterized in any organism. Mammalian G14 proteins harbor a predicted signal peptide and a transmembrane domain; however, these

domains were not conserved in *Xenopus* G14. Thus, the function of Xl G14 in development remains elusive.

4.4 Summary

While developmental processes have been extensively studied in the context of transcriptional regulation, much less is known about regulation via specific translational regulation and transcript stability. XSeb4R represents a novel sequence-specific RNA binding protein that activates translation of its target transcripts, amongst these important regulators of embryogenesis, such as *VegT* and *Xngnr-1*. Translational activation is mediated by binding to the 3'UTR of target RNAs and most likely occurs through cap-associated proteins. Whether this interaction is direct or indirect, remains to be determined. The RNA-IP provided an additional XSeb4R target, *Xl G14*. Identification of more potential XSeb4R targets may provide further insights of a XSeb4R recognition motif. Furthermore, XSeb4R may also exert a function in stabilizing its targets in the context of embryogenesis, which would correlate with the suggested function of the human homologue RNPC1 and implicating a conserved role of Seb4R in RNA metabolism within vertebrates. Functional analyses provide further evidence of an essential role of XSeb4R in the context of germ layer formation and primary neurogenesis (Boy *et al*., 2004; Souopgui *et al*., 2008). Taken together, these findings stress the significance of gene expression via specific translational regulators and the requirement of further investigation of this mechanism to unravel the complex mechanisms of embryonic development.

5. Bibliography

Abdelmohsen K., Lal A., Kim H. H. and Gorospe M. (2007) Posttranscriptional orchestration of an anti-apoptotic program by HuR. Cell Cycle 6: 1288–1292.

Agius E, Oelgeschlager M, Wessely O, Kemp C, De Robertis EM. (2007) Endodermal Nodal-related signals and mesoderm induction in *Xenopus*. Development. 127(6):1173-83.

Akamatsu, W., H. Fujihara, T. Mitsuhashi, M. Yano, S. Shibata, Y. Hayakawa, H. J. Okano, S. Sakakibara, H. Takano, T. Takano, T. Takahashi, T. Noda, and H. Okano. (2005) The RNA-binding protein HuD regulates neuronal cell identity and maturation. Proc. Natl. Acad. Sci. USA 102:4625-4630.

Amrani N, Ghosh S, Mangus DA, Jacobson A. (2008) Translation factors promote the formation of two states of the closed-loop mRNP. Nature. 26; 453(7199):1276-80.

Amrein, H., Gorman, M. and Nöthiger, R. (1988) The sex-determining gene tra-2 of Drosophila encodes a putative RNA binding protein. Cell 55, pp. 1025–1035.

Anderson KD, Morin MA, Beckel-Mitchener A, Mobarak CD, Neve RL, Furneaux HM, Burry RM, Perrone-Bizzozero NI. (2000) Overexpression of HuD, but not its truncated form HuD I + II, promotes GAP-43 expression and neurite outgrowth in PC12 cells in the absence of nerve growth factor. J Neurochem 75: 1103-1114.

Anderson, K. D., J. Sengupta, M. Morin, R. L. Neve, C. F. Valenzuela, and N. I. Perrone-Bizzozero. (2001) Overexpression of HuD accelerates neurite outgrowth and increases GAP-43 mRNA expression in cortical neurons and retinoic acid-induced embryonic stem cells in vitro. Exp. Neurol. 168:250-258.

Antic D, Lu N, Keene JD. (1999) ELAV tumor antigen, Hel-N1, increases translation of neurofilament M mRNA and induces formation of neurites in human teratocarcinoma cells. Genes Dev 13: 449-461.

Anyanful A., Ono K., Johnsen R.C., Ly H., Jensen V., Baillie D.L., Ono S. (2004) The RNA-binding protein SUP-12 controls muscle-specific splicing of the ADF/cofilin pre-mRNA in C. elegans. J. Cell Biol. 167, 639–647.

Arthur P.K., Koch S., Tarbashevich K., Jahn O., Claussen M., Pieler T. (submitted) Participation of *Xenopus* Elr-type proteins in vegetal mRNA localization during oogenesis.

Atlas, R., L. Behar, E. Elliott, and I. Ginzburg. (2004) The insulin-like growth factor mRNA binding-protein IMP-1 and the Ras-regulatory protein G3BP associate with tau mRNA and HuD protein in differentiated P19 neuronal cells. J. Neurochem. 89:613–626.

Auweter S.D., Oberstrass F.C. and Allain F.H. (2006) Sequence-specific binding of single-stranded RNA: is there a code for recognition? Nucleic Acids Res. 34, 4943–4959.

Baer W.B. and Kornberg R.D. (1983) The protein responsible for the repeating structure of cytoplasmic poly(A)-ribonucleoprotein. *J. Cell. Biol.* 96, 717–721.

Bagga P.S., Arhin G.K. and Wilusz J. (1998) DSEF-1 is a member of the hnRNP H family of RNA-binding proteins and stimulates pre-mRNA cleavage and polyadenylation in vitro. Nucleic Acids Res. 26, 5343–5350.

Baker B.S. (1989) Sex in flies: the splice of life. Nature 340, pp. 521–524.

Baker J.C., Beddington R.S. and Harland R.M. (1999) Wnt signaling in *Xenopus* embryos inhibits bmp4 expression and activates neural development. *Genes Dev* 13, 3149-59.

Bandziulis, R. J., Swanson, M. S., Dreyfuss, G. (1989) RNA-binding proteins as developmental regulators. Genes Dev 3,431-437.

Barreau C., Paillard L., Osborne H.B. (2005) AU-rich elements and associated factors: are there unifying principles? Nucleic Acids Res. 3;33 (22): 7138-50.

Bell L.R., Maine E.M., Schedl P. and Cline T.W. (1988) Sex-lethal, a Drosophila sex determination switch gene, exhibits sex-specific RNA splicing and sequence similarity to RNA binding proteins, Cell 55 pp. 1037–1046.

Bellefroid E.J., Bouguignon C., Holleman T., Ma Q., Anderson D.J., Kintner C. and Pieler T. (1996) X-MyT1, a *Xenopus* C2HC-type Zinc Finger Protein with a regulatory function in neuronal differentiation. Cell, 87, 1191-1202.

Bellefroid E.J., Kobbe A., Gruss P., Pieler T., Gurdon J.B. and Papalopulu N. (1998). Xiro3 encodes a *Xenopus* homolog of the Drosophila Iroquois genes and functions in neural specification. *Embo J* 17, 191-203.

Belsham G.J., McInerney G.M., Ross-Smith N. (2000) Foot-and-mouth disease virus 3C protease induces cleavage of translation initiation factors eIF4A and eIF4G within infected cells. J Virol. 74 (1): 272-80.

Benne R. and Hershey J.W.B. (1978) The mechanism of action of protein synthesis initiation factors from rabbit reticulocytes. J. Biol. Chem. 253: 3078–3087.

Bernstein P. and Ross J. (1989) Poly(A), poly(A) binding protein and the regulation of mRNA stability. Trends Biochem. Sci. 14, pp. 373–377.

Bevilacqua A, Ceriani MC, Capaccioli S and Nicolin A. (2003) Post-transcriptional regulation of gene expression by degradation of messenger RNAs. *Journal of* Cellular Physiology 195: 356–372.

Bindereif A., Green M.R. (1986) Ribonucleoprotein complex formation during pre-mRNA splicing in vitro. Mol Cell Biol. 6 (7): 2582-92.

Blackshear P.J., Lai W.S., Thorn J.M., Kennington E.A., Staffa N.G., Moore D.T., Bouffard G.G., Beckstrom-Sternberg S.M., Touchman J.W., Bonaldo M.F., Soares M.B. (2001) The NIEHS *Xenopus* maternal EST project: interim analysis of the first 13,879 ESTs from unfertilized eggs. Gene. 4; 267 (1): 71-87.

Bollig F., Winzen R., Gaestel M., Kostka S., Resch K. and Holtmann H. (2003) Affinity purification of ARE-binding proteins identifies poly(A)-binding protein 1 as a potential substrate in MK2-induced mRNA stabilization. Biochem Biophys Res Commun 301, pp. 665–670.

Bolognani F., Qiu S., Tanner D.C., Paik J., Perrone-Bizzozero N.I., Weeber E.J. (2007) Associative and spatial learning and memory deficits in transgenic mice overexpressing the RNA-binding protein HuD, Neurobiol. Learn. Mem. 87, pp. 635–643.

Bolognani F. and Perrone-Bizzozero N.I. (2008) RNA-protein interactions and control of mRNA stability in neurons, J. Neurosci. Res. 86, pp. 481–489.

Boy, S., Souopgui, J., Amato, M. A., Wegnez, M., Pieler, T., and Perron, M. (2004) XSEB4R, a novel RNA-binding protein involved in retinal cell differentiation downstream of bHLH proneural genes. *Development* 131, 851-62.

Brennan SM. (1991) Sequence requirements for embryonic transcriptional activation of a gastrula-specific actin gene in *Xenopus laevis*. Mol Reprod Dev., 30(4):293-303.

Brennan C.M., Gallouzi I.E., Steitz J.A. (2000) Protein ligands to HuR modulate its interaction with target mRNAs in vivo. J Cell Biol. 2; 151 (1): 1-14.

Brennan C.M., Steitz J.A. (2001) HuR and mRNA stability. Cell Mol Life Sci. 58 (2): 266-77.

Brewer, G. (1991) An A+U-rich element RNA-binding factor regulates c-myc mRNA stability in vitro. Mol. Cell. Biol. 11:2460-2466.

Brewer G. (2002) Messenger RNA decay during aging and development, Ageing Res. Rev. 1, pp. 607–625.

Brewster, R., Ruiz i Altaba, A. (1998) Gli/Zic factors pattern the neural plate by defining domains of cell differentiation. Nature, 393, 579-583.

Brune, C., Munchel S. E., Fischer N., Podtelejnikov A.V. and Weis K. (2005) Yeast poly(A)-binding protein Pab1 shuttles between the nucleus and the cytoplasm and functions in mRNA export. RNA 11:517-531.

Bullock W.O., Fernandez J.M., Short J.M. (1987) XL1-Blue: A high efficiency plasmid transformation recA Escherichia coli strain with betagalactosidase selection. Biotechniques 5(4): 376–379.

Burd, C.G. and Dreyfuss, G. (1994) Conserved structures and diversity of functions of RNA-binding proteins. Science 265: 615–621.

Cao Y., Siegel D., Donow C., Knöchel S., Yuan L., Knöchel W. (2007) POU-V factors antagonize maternal VegT activity and beta-Catenin signaling in *Xenopus* embryos. EMBO J. 20; 26 (12): 2942-54.

Caput D., Beutler B., Hartog K., Thayer R., Brown-Shimer S., and Cerami A. (1986) Identification of a common nucleotide sequence in the 39-untranslated regions of mRNA molecules specifying inflammatory mediators..Proc. Natl. Acad. Sci. USA 83:1670–1674.

Cha, B.-J. and Gard, D.L. (1999) XMAP230 is requires for the organization of cortical microtubules and patterning of the dorsoventral axis in fertilized *Xenopus* eggs. Dev. Biol. 205, pp. 275–286.

Chang, P., Perez-Mongiovi, D. and Houliston, E. (1999) Organisation of *Xenopus* oocyte and egg cortices. *Microsc. Res. Tech.* 44, 415-429.

Charlesworth A., Cox L.L. and MacNicol, A.M. (2004) Cytoplasmic polyadenylation element (CPE)- and CPE-binding protein (CPEB)-independent mechanisms regulate early class maternal mRNA translational activation in *Xenopus* oocytes. J. Biol. Chem. 279: 17650–17659.

Charlesworth A., Wilczynska A., Thampi P., Cox L.L. and MacNicol A.M. (2006) Musashi regulates the temporal order of mRNA translation during *Xenopus* oocyte maturation. EMBO J. 25, 2792-2801.

Chen, C.Y. and Shyu, A.B. (1995) AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem. Sci. 20, pp. 465–470.

Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJ, Stoecklin G, Moroni C, Mann M, Karin M. (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. Cell. 107:451–464.

Chen P.Y., Manninga H., Slanchev K., Chien M., Russo J.J., Ju J., Sheridan R., John B., Marks D.S., Gaidatzis D., Sander C., Zavolan M., Tuschl T. (2005) The developmental miRNA profiles of zebrafish as determined by small RNA cloning. Genes Dev. 1; 19 (11): 1288-93.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995) Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the Drosophila neurogenic gene Delta. *Nature* 375, 761-6.

Chitnis, A., and Kintner, C. (1996) Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in *Xenopus* embryos. *Development* 122, 2295-301.

Chung SM, Eckrich M, Perrone-Bizzozero NI, Kohn DT, Furneaux HM. (1997) The Elav-like proteins bind to a conserved regulatory element in the 3 untranslated region of GAP-43 mRNA. J Biol Chem 272: 6593-6598.

Clements, D., Friday, R.V., and Woodland, H.R. (1999) Mode of action of VegT in mesoderm and endoderm formation. Development 126: 4903–4911.

Colegrove-Otero L.J., Minshall N., Standart N. (2005) RNA-binding proteins in early development. Crit Rev Biochem Mol Biol. 40 (1): 21-73.

Coller J.M., Gray N.K., Wickens M.P. (1998) mRNA stabilization by poly(A) binding protein is independent of poly(A) and requires translation. Genes Dev. 15; 12 (20): 3226-35.

Collier B., Gorgoni B., Loveridge C., Cooke H.J., Gray N.K. (2005) The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells. EMBO J. 20; 24 (14): 2656-66.

Cosson, B., Couturier, A., Chabelskaya, S., Kiktev, D., Inge-Vechtomov, S., Philippe, M., Zhouravleva, G. (2002) Poly(A)-binding protein acts in translation termination via eukaryotic release factor 3 interaction and does not influence [PSI(+)] propagation. Mol. Cell. Biol.; 22:3301–3315.

Czaplinski K. and Singer R.H. (2006) Pathways for mRNA localization in the cytoplasm, Trends Biochem. Sci. 31, pp. 687–693.

Dale L., Matthews G., Tabe L., and Colman A. (1989) Developmental expression of the protein product of Vg1, a localised maternal mRNA in the frog *Xenopus laevis*. EMBO J. 8:1057-1065.

Dale L. and Jones C. M. (1999) BMP signalling in early *Xenopus* development. Bioassays, 21, 751-760.

Dalmau J., Furneaux H.M., Cordon-Cardo C., Posner J.B. (1992) The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues. Am J Pathol. 141:881–886.

DeAngelo D.J., DeFalco J., Rybacki L. and Childs G. (1995) The embryonic enhancer-binding protein SSAP contains a novel DNA-binding domain which has homology to several RNA-binding proteins. Mol. Cell Biol 15, 1254-1264.

de la Calle-Mustienes E., Glavic A., Modolell J. and Gomez-Skarmeta J. L.
(2002) Xiro homeoproteins coordinate cell cycle exit and primary neuron formation by upregulating neuronal-fate repressors and downregulating the cell-cycle inhibitor XGadd45-gamma. *Mech Dev* 119, 69-80.

Delaune E., Lemaire P., and Kodjabachian L. (2005) Neural induction in *Xenopus* requires early FGF signalling in addition to BMP inhibition. *Development* 132, 299-310.

DeJong E.S., Marzluff W.F., Nikonowicz E.P. (2002) NMR structure and dynamics of the RNA-binding site for the histone mRNA stem-loop binding protein. RNA 8(1): 83–96.

de Moor C.H., Meijer H. and Lissenden S. (2005) Mechanisms of translational control by the 3' UTR in development and differentiation. Semin. Cell Dev. Biol. 16: 49–58.

Deo R.C., Bonanno J.B., Sonenberg N. and Burley S.K. (1999) Recognition of polyadenylate RNA by the poly(A)-binding protein. Cell 98, pp. 835–845.

DeRobertis E.M., Larrain J., Oelgeschläger M., Wessely O. (2000) The establishment of Spemann's organizer and patterning of the vertebrate embryo. Nat Rev Genet. 1:171–81.

DeRobertis E.M., and Kuroda H. (2004) Dorsal-ventral patterning and neural induction in *Xenopus* embryos. *Annu Rev Cell Dev Biol* 20, 285-308

Derry M.C., Yanagiya A., Martineau Y., Sonenberg N. (2006) Regulation of poly(A)-binding protein through PABP-interacting proteins. Cold Spring Harb Symp Quant Biol. 71:537-43.

Ding J., Hayashi M.K., Zhang Y., Manche L., Krainer A.R., Xu R.M. (1999) Crystal structure of the two-RRM domain of hnRNP A1 (UP1) complexed with single-stranded telomeric DNA. Genes Dev 13: 1102–1115

Dominguez I. and Green J.B. (2001) Missing links in GSK3 regulation. *Dev. Biol.* 235, 303-313.

Dominsky Z. and Marzluff W.F. (1999) Formation of the 3' end of histone mRNA. Gene 239, pp. 1–14.

Dominski Z., Marzluff W.F. (2007) Formation of the 3' end of histone mRNA: getting closer to the end. Gene 396:373–390.

Doyle K., Zhang Y., Baer R. and Bina M. (1994) Distinguishable patterns of protein-DNA interactions involving complexes of basic helix-loop-helix proteins. J. Biol. Chem. 269:12099-12105

Dreyfuss G., Swanson M.S., Pinol-Roma S. (1988) Heterogeneous nuclear ribonucleoprotein particles and the pathway of mRNA formation. Trends Biochem. Sci.; 13:86–91.

Dubnau J., Struhl G. (1996) RNA recognition and translational regulation by a homeodomain protein. Nature 379: 694– 99

Dunn E.F., Hammell C.M., Hodge C.A. and Cole C.N. (2005) Yeast poly(A)-binding protein, Pab1, and PAN, a poly(A) nuclease complex recruited by Pab1, connect mRNA biogenesis to export. *Genes & Dev.* 19: 90–103.

Dubois L., Bally-Cuif L., Crozatier M., Moreau J., Paquereau L., Vincent A. (1998) XCoe2, a transcription factor of the Col/Olf-1/EBF family involved in the specification of primary neurons in *Xenopus*. Curr Biol 8: 199–209

ElAntak L., Tzakos A.G., Locker N. and Lukavsky P.J. (2007) Structure of eIF3b RNA recognition motif and its interaction with eIF3j: structural insights into the recruitment of eIF3b to the 40 S ribosomal subunit, J. Biol. Chem. 282, pp. 8165–8174.

Elinson R.P. and Rowning B. (1988) A transient array of parallel microtubules in frog eggs: potential tracks for a cytoplasmic rotation that specifies the dorso-ventral axis. Dev. Biol. 128, pp. 185–197.

Emanuilov I., Sabatini D.D., Lake J.A., and Freienstein C. (1978) Localization of eukaryotic initiation factor 3 on native small ribosomal subunits. Proc. Natl. Acad. Sci. 75: 1389–1393.

Fedoroff N.V. (2002) RNA-binding proteins in plants: the tip of an iceberg? Curr Opin Plant Biol. 5 (5): 452-9.

Fetka I., Radeghieri A. and Bouwmeester T. (2000) Expression of the RNA recognition motif-containing protein SEB-4 during *Xenopus* embryonic development. Mech. Dev. 94,283 -286.

Ford L.P., Bagga P.S., Wilusz J. (1997) The poly(A) tail inhibits the assembly of a 3'-to-5' exonuclease in an in vitro RNA stability system. Mol Cell Biol. 17:398–406.

Fortini M.E. and Artavanis-Tsakonas S. (1994) The suppressor of hairless protein participates in notch receptor signaling. *Cell* 79, 273-82.

Furuichi Y., LaFiandra A., and Shatkin A. (1977) 5'-Terminal structures and mRNA stability. Nature 266: 235-239

Garneau N.L., Wilusz J. and Wilusz C.J. (2007) The highways and byways of mRNA decay. Nat. Rev. Mol. Cell Biol. 8: 113–126.

Gebauer F. and Hentze M.W. (2004) Molecular mechanisms of translational control. Nat. Rev. Mol. Cell. Biol., 5, 827–835.

Gerhart J., Danilchik M., Doniach T., Roberts S., Rowning B., Stewart R. (1989) Cortical rotation of the *Xenopus* egg: consequences for the anteroposterior pattern of embryonic dorsal development. Development. 107 Suppl:37-51.

Gherzi R., Lee K.Y., Briata P., Wegmuller D., Moroni C., Karin M., Chen C.Y.. (2004) A KH domain RNA binding protein, KSRP, promotes ARE-directed mRNA turnover by recruiting the degradation machinery. Mol. Cell. 14:571–583.

Gilbert W., Guthrie C. (2004) The Glc7p nuclear phosphatase promotes mRNA export by facilitating association of Mex67p with mRNA. Mol Cell. 30; 13 (2): 201-12.

Gingras A.C., Gygi S.P., Raught B., Polakiewicz R.D., Abraham R.T., Hoekstra M.F., Aebersold R. and Sonenberg N. (1999) Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev. 13, 1422–1437

Gomez-Skarmeta J.L., Glavic A., de la Calle-Mustienes E., Modolell J. and Mayor R. (1998) Xiro, a *Xenopus* homolog of the Drosophila Iroquois complex genes, controls development at the neural plate. *Embo J* 17, 181-90.

Good P.J., Rebbert M.L. and Dawid I. B. (1993) Three new members of the RNP protein family in *Xenopus*. Nucleic Acids Res. 21,999 -1006.

Goralski T.J., Edstrom J.E. and Baker B.S. (1989) The sex determination locus transformer2 of Drosophila encodes a polypeptide with similarity to RNA binding proteins. Cell. 56, pp. 1011–1018.

Gorgoni B., Andrews S., Schaller A., Schumperli D., Gray N.K., and Muller B. (2005) The stem-loop binding protein stimulates histone translation at an early step in the initiation pathway. RNA. 11:1030-1042.

Gorospe M. (2002) Regulation of mRNA turnover by cellular stress. In: Bradshaw, R., Dennis, E. (Eds.), Handbook of Cell Signaling. Academic Press, San Diego.

Gray N.K., Coller J.M., Dickson K.S., Wickens M. (2000) Multiple portions of poly(A)-binding protein stimulate translation in vivo. EMBO J. 1 (17): 4723-33.

Greenberg J.R. (1972) High stability of messenger RNA in growing cultured cells. Nature. 10;240(5376):102-4.

Grossi de Sa M., Standart N., Martins de Sa C., Akhayat O., Huesca M. and Scherrer K. (1988) The poly(A)-binding protein facilitates *in vitro* translation of poly(A)-rich mRNA. *Eur. J. Biochem.* 176, 521–526.

Guddat W., Bakken A.H., Pieler T. (1990) Protein mediated nuclear export of RNA: rRNA containing small RNPs in *Xenopus* oocytes. Cell. 60:619–628.

Hake L.E., Richter J.D. (1994) CPEB is a specificity factor that mediates cytoplasmic polyadenylation during *Xenopus* oocyte maturation. Cell 79: 617– 27

Hall K.B., Stump W.T. (1992) Interaction of N-terminal domain of U1A protein with an RNA stem/loop. Nucleic Acids Res. 20:4283–4290.

Hamimes S., Arakawa H., Stasiak A.Z., Kierzek A.M., Hirano S., Yang Y.G., Takata M., Stasiak A., Buerstedde J.M., Van Dyck E. (2005) RDM1, a novel RNA recognition motif (RRM)-containing protein involved in the cell response to cisplatin in vertebrates. J. Biol. Chem. 280:9225–9235.

Hamimes S., Bourgeon D., Stasiak A.Z., Stasiak A., Van Dyck E. (2006) Nucleic acid-binding properties of the RRM-containing protein RDM1. Biochem. Biophys. Res. Commun. 344:87–94.

Hardcastle Z. and Papalopulu N. (2000) Distinct effects of XBF-1 in regulating the cell cycle inhibitor p27(XIC1) and imparting a neural fate. *Development* 127, 1303-14.

Harland R.M. (1991) In situ hybridization: An improved whole-mount method for *Xenopus* embryos. Methods Cell Biol. 36: 685–695

Harland R.M. and Gerhart J. (1997) Formation and function of Spemann's Organizer. *Annu. Rev. Cell Dev. Biol.* 13, 611–667.

Heasman J. (1997) Patterning of the *Xenopus* gastrula. *Development* 124, 4179–4191.

Heasman J., Wessely O., Langland R., Craig E.J., and Kessler D.S. (2001) Vegetal localization of maternal mRNAs is disrupted by VegT depletion. Dev. Biol. 240: 377–386.

Heeg-Truesdell E. and LaBonne C. (2006) Neural induction in *Xenopus* requires inhibition of Wnt-[beta]-catenin signaling. *Developmental Biology* 1;298(1):71-86.

Hinman M.N., Lou H. (2008) Diverse molecular functions of Hu proteins. Cell Mol Life Sci. 26.

Hollemann T., Pieler T. (1999) Xpitx-1: a homeobox gene expressed during pituitary and cement gland formation of *Xenopus* embryos. Mech Dev. 88 (2): 249-52

Holowacz T. and Elinson R.P. (1993) Cortical cytoplasm, which induces dorsal axis formation in *Xenopus*, is inactivated by UV irradiation of the oocyte. *Development.* 119, 277-285.

Horb M.E. and Thomsen G.H. (1997) A vegetally localized T-box transcription factor in *Xenopus* eggs specifies mesoderm and endoderm and is essential for embryonic mesoderm formation. Development. 124, pp. 1689–1698.

Horvay K., Claussen M., Katzer M., Landgrebe J., Pieler T. (2006) *Xenopus* Dead end mRNA is a localized maternal determinant that serves a conserved function in germ cell development. Dev Biol. 291:1–11.

Hoshino S., Imai M., Mizutani M., Kikuchi Y., Hanaoka F., Ui M. and Katada T. (1998) Molecular cloning of a novel member of the eukaryotic polypeptide chain-releasing factors (eRF). Its identification as eRF3 interacting with eRF1. J Biol Chem. 273, 22254-22259.

Houliston E. and Elinson R.P. (1992) Microtubules and cytoplasmic reorganization in the frog egg. Curr. Top. Dev. Biol. 26, pp. 53–70.

Houston D.W. and King M.L. (2000) A critical role for xdazl, a germ plasm-localized RNA, in the differentiation of primordial germ cells in *Xenopus*, Development. 127, pp. 447–456.

Huelsken J. and Behrens J. (2002) The Wnt signalling pathway. *J. Cell Sci.* 115, 3977-3978.

Imai T., Tokunaga A., Yoshida T., Hashimoto M., Mikoshiba K., Weinmaster G., Nakafuku M., Okano H. (2001) The neural RNA-binding protein Musashi1 translationally regulates mammalian numb gene expression by interacting with its mRNA. Mol Cell Biol 21: 3888–3900.

Imataka H., Gradi A. and Sonenberg N. (1998) A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A) dependent translation. EMBO J. 17:7480-7489.

Inoue K., Hoshijima K., Sakamoto H. and Shimura Y. (1990) Binding of the Drosophila Sex-lethal gene product to the alternative splice set of transformer primary transcript. Nature 344, 461–463.

Jacobson, A. (1996) Poly(A) metabolism and translation: the closed-loop model, p. 451-480. In J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Jacobson A. (2004) Regulation of mRNA decay: decapping goes solo. Mol Cell. 2;15(1):1-2.

Jain R.G., Andrews L.G., McGowan K.M., Pekala P.H. and Keene J.D. (1997) Ectopic expression of Hel-N1, an RNA-binding protein, increases glucose transporter (GLUT 1) expression in 3T3-L1 adipocytes. Mol Cell Biol, 17, 954–962.

Jakob U., Lilie H., Meyer I., Buchner J. (1995) Transient interaction of Hsp90 with early unfolding intermediates of citrate synthase. Implications for heat shock in vivo. J Biol Chem. 270(13):7288-94.

Joseph E.M., Melton D.A. (1998) Mutant Vg1 ligands disrupt endoderm and mesoderm formation in *Xenopus* embryos. *Development.* 125:2677-2685.

Johnston S.D., Lew J.E., Berman J. (1999) Gbp1p, a protein with RNA recognition motifs, binds single-stranded telomeric DNA and changes its binding specificity upon dimerization. Mol Cell Biol. 19:923–933.

Kadesch T. (2004) Notch signaling: the demise of elegant simplicity, Curr. Opin. Genet. Dev. 14, pp. 506–512.

Kageura H. (1997) Activation of dorsal development by contact between the cortical dorsal development and the equatorial core cytoplasm in eggs of *Xenopus laevis*. *Development* 124, 1543-1551.

Kenan D.J., Query C.C., Keene J.D. (1991) RNA recognition: towards identifying determinants of specificity. Trends Biochem Sci 16:2 14-220.

Kasashima K., Terashima K., Yamamoto K., Sakashita E. and Sakamoto H. (1999) Cytoplasmic localization is required for the mammalian ELAV-like protein HuD to induce neuronal differentiation. Genes Cells 4:667-683

Keller R., Shih J., Sater A. (1992) The cellular basis of the convergence and extension of the *Xenopus* neural plate. Dev Dyn. 193(3):199-217.

Keller R., Davidson L.A. and Shook D.R. (2003) How we are shaped: the biomechanics of gastrulation, Differentiation 71, pp. 171–205.

Keller R., Shook D., Skoglund P. (2008) The forces that shape embryos: physical aspects of convergent extension by cell intercalation. Phys Biol. 10; 5(1): 15007.

Kessler S.H., Sachs A.B. (1998) RNA recognition motif 2 of yeast Pab1p is required for its functional interaction with eukaryotic translation initiation factor 4G. Mol Cell Biol. 18:51–57.

Keyoung H.M., Roy N.S., Benraiss A., Louissaint Jr. A., Suzuki A., Hashimoto M., Rashbaum W.K., Okano H., Goldman S.A. (2001) High-yield selection and extraction of two promoter-defined phenotypes of neural stem cells from the fetal human brain. Nat Biotechnol 19: 843-850.

Khanam T., Muddashetty R.S., Kahvejian A., Sonenberg N., Brosius J. (2006) Poly(A)-binding protein binds to A-rich sequences via RNA-binding domains 1+2 and 3+4. RNA Biol. 2006 (4): 170-7.

Kielkopf C.L., Rodionova N.A., Green M.R. and Burley S.K. (2001) A novel peptide recognition mode revealed by the X-ray structure of a core U2AF35/U2AF65 heterodimer. Cell 106, pp. 595–605.

Kikkawa M., Takano K. and Shinagawa A. (1996) Location and behavior of dorsal determinants during first cell cycle in *Xenopus* eggs. *Development* 122, 3687-3696.

Kimelman D. (2006) Mesoderm induction: from caps to chips, Nat. Rev. Genet. 7, pp. 360–372.

King M.L., Messitt T.J., Mowry K.L. (2005) Putting RNAs in the right place at the right time: RNA localization in the frog oocyte. Biol Cell. 97(1):19-33.

Kintner C.R., and Melton D.A. (1987) Expression of *Xenopus* N-CAM RNA in ectoderm is an early response to neural induction. *Development* 99, 311-25.

Kishi M., Mizuseki K., Sasai N., Yamazaki H., Shiota K., Nakanishi S.,
Sasai Y. (2000) Requirement of Sox2-mediated signaling for differentiation of early *Xenopus* neuroectoderm. Development, 127, 791-800.

Klisch T.J., Souopgui J., Juergens K., Rust B., Pieler T., and Henningfeld K.A. (2006) Mxi1 is essential for neurogenesis in *Xenopus* and acts by bridging the pan-neural and proneural genes. Dev. Biol. 292:470-485.

Kloc M., Wilk K., Vargas D., Shirato Y., Bilinski S. and Etkin L. D. (2005) Potential structural role of non-coding and coding RNAs in the organization of the cytoskeleton at the vegetal cortex of *Xenopus* oocytes. Development 132,3445 - 3457.

Klymkowsky M.W. (1999) Plakophilin, armadillo repeats, and nuclear localization. Microsc Res Tech. 1; 45 (1): 43-54.

Knecht A.K., Good P.J., Dawid I.B., Harland R.M. (1995) Dorsal-ventral patterning and differentiation of noggin-induced neural tissue in the absence of mesoderm. Development. 121 (6): 1927-35.

Körner C.G., Wahle E. (1997) Poly(A) tail shortening by a mammalian poly(A)-specific 3'-exoribonuclease. J Biol Chem. 18;272(16):10448–10456.

Körner C.G., Wormington M., Muckenthaler M., Schneider S., Dehlin E., Wahle E. (1998) The deadenylating nuclease (DAN) is involved in poly(A) tail removal during the meiotic maturation of *Xenopus* oocytes. EMBO J. 17:5427–5437.

Kozak M. (2007a) Lessons (not) learned from mistakes about translation. Gene. 403(1-2): 194-203.

Kozak M. (2007b) Some thoughts about translational regulation: forward and backward glances. J Cell Biochem, 102: 280-290

Kühn U., Pieler T. (1996) *Xenopus* poly(A) binding protein: functional domains in RNA binding and protein-protein interaction. J Mol Biol. 256:20–30.

Kullmann M., Göpfert U., Siewe B., Hengst L. (2002) ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5'UTR. Genes Dev. 1; 16 (23): 3087-99.

Kuroda H., Wessely O., and DeRobertis E.M. (2004) Neural induction in *Xenopus*: requirement for ectodermal and endomesodermal signals via Chordin, Noggin, beta-Catenin, and Cerberus. *PLoS Biol* 2, E92.

Kuroyanagi H., Ohno G., Mitani S. and Hagiwara M. (2007) Fox-1 family and SUP-12 coordinately regulate tissue-specific alternative splicing in vivo. Mol. Cell. Biol. 27: 8612–8621.

Kwon S., Abramson T., Munro T.P., John C.M., Kohrmann M. and Schnapp B.J. (2002) UUCAC and Vera dependent localization of VegT RNA in *Xenopus* oocytes, Curr. Biol. 12, pp. 558–564.

Lai W.S., Carballo E., Strum J.R., Kennington E.A., Phillips R.S. and Blackshear P.J, (1999) Evidence that tristetraprolin binds to A+U-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. Mol. Cell. Biol. 19, pp. 4311–4323.

Lai W.S., Kennington E.A. and Blackshear P.J. (2003) Tristetraprolin and its family members can promote the cell-free deadenylation of AU-rich element-containing mRNAs by poly(A) ribonuclease. *Mol. Cell. Biol.* 23: 3798–3812.

Lane M.C. and Sheets M.D. (2006) Heading in a new direction: implications of the revised fate map for understanding *Xenopus laevis* development, Dev. Biol. 296, pp. 12–28.

Launay C., Fromentoux V., Shi D.L. and Boucaut J. C. (1996) A truncated FGF receptor blocks neural induction by endogenous *Xenopus* inducers. *Development* 122, 869-80.

Le H., Tanguay R.L., Balasta M.L., Wei C.C., Browning K.S., Metz A.M., Goss D.J. and Gallie D.R. (1997) Translation initiation factors eIF-iso4G and eIF-4B interact with the poly(A)-binding protein and increase its RNA binding activity. J. Biol. Chem., 272: 16247–16255.

Lee J.E., Hollenberg S.M., Snider L., Turner D.L., Lipnick N., Weintraub H. (1995) Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix–loop–helix protein. Science 268: 836–844

Lewis R.A., Mowry K.L. (2007) Ribonucleoprotein remodeling during RNA localization. Differentiation. 75 (6): 507-18.

Lin H., Yutzey K.E., Konieczny S.F. (1991) Muscle-specific expression of the troponin I gene requires interactions between helix-loop-helix muscle regulatory factors and ubiquitous transcription factors. Mol Cell Biol. 11(1):267–280.

Lin A.C. and Holt C.E. (2007) Local translation and directional steering in axons. EMBO J. 26: 3729–3736.

Ling J., Morley S.J., Pain V.M., Marzluff W.F. and Gallie D.R. (2002) The histone 3′ terminal stem-loop-binding protein enhances translation through a functional and physical interaction with eucaryotic initiation factor 4G (eIF4G) and eIF3. Mol. Cell. Biol. 22:7853-7867.

Liu H. and Kiledjian M. (2006) Decapping the message: A beginning or an end. Biochem. Soc. Trans. 34: 35–38.

Louvi A. and Artavanis-Tsakonas S. (2006) Notch signalling in vertebrate neural development. *Nat Rev Neurosci* 7, 93-102.

Lunde B.M., Moore C., Varani G. (2007) RNA-binding proteins: modular design for efficient function. Nat. Rev. Mol. Cell Biol. 8:479–490.

Lustig K.D., Kroll K.L., Sun E.E. and Kirchner M.W. (1996) Expression cloning of a *Xenopus*T-related gene (Xombi) involved in mesodermal patterning and blastopore lip formation. Development 122: 4001–4012.

Lutz-Freyermuth C., Keene J.D. (1989) The U1 RNA-binding site of the U1 small nuclear ribonucleoprotein (snRNP)-associated A protein suggests a similarity with U2 snRNPs. Mol Cell Biol 9:2975–2982.

Lutz-Freyermuth C., Query C.C., Keene J.D. (1990) Quantitative determination that one of two potential RNA-binding domains of the A protein component of the U1 small nuclear ribonucleoprotein complex binds with high affinity to stem–loop II of U1 RNA. Proc. Natl. Acad. Sci. 87:6393–6397.

Ma Q., Kintner C., Anderson D.J. (1996) Identification of neurogenin, a vertebrate neuronal differentiation gene. Cell, 87, 43-52.

Ma W.J., Chung S. and Furneaux H. (1997) The ELAV-like proteins bind to A+U-rich elements and to the poly(A) tail of mRNA. Nucleic Acids Res. 25, pp. 3564–3569.

Mahone M., Saffman E.E., Lasko P.F. (1995) Localized Bicaudal-C RNA encodes a protein containing a KH domain, the RNA binding motif of FMR1. EMBO J. 14:2043–2055.

Mangus D.A., Evans M.C. and Jacobson A. (2003) Poly(A)-binding proteins: Multifunctional scaffolds for the post-transcriptional control of gene expression. Genome Biol. 4: 223.1-223.14.

Manohar C.F., Short M.L., Nguyen A., Nguyen N.N., Chagnovich D., Yang Q., Cohn S.L. (2002) HuD, a neuronal-specific RNA-binding protein, increases the in vivo stability of MYCN RNA. J. Biol. Chem., 277: 1967-1973.

Marzluff W.F. and Duronio R.J. (2002) Histone mRNA expression: multiple levels of cell cycle regulation and important developmental consequences, Curr. Opin. Cell Biol. 14, pp. 692–699.

McGrew L.L., Dworkin-Rastl E., Dworkin M.B., Richter J.D. (1989) Poly (A) elongation during *Xenopus* oocyte maturation is required for translational recruitment and is mediated by a short sequence element. Genes Dev. 3: 803–15.

Melton D.A. (1987) Translocation of a localized maternal mRNA to the vegetal pole of *Xenopus* oocytes. Nature 328,80 -82.

Mendez R., Richter J.D. (2001) Translational control by CPEB: A means to the end. Nat Rev Mol Cell Biol 2:521–529.

Meyer S., Temme C. and Wahle E. (2004) Messenger RNA turnover in eukaryotes: Pathways and enzymes. Crit. Rev. Biochem. Mol. Biol. 39: 197–216.

Miller J.R., Rowning B.A., Larabell C.A., Yang-Snyder J.A., Bates R. L. and Moon R.T. (1999) Establishment of the dorsal-ventral axis in *Xenopus* embryos coincides with the dorsal enrichment of dishevelled that is dependent on cortical rotation. *J. Cell Biol.* 146, 427-437.

Minakhina S., Steward R. (2005) Axes formation and RNA localization. Curr Opin Genet Dev. 15 (4): 416-21.

Mizuseki K., Kishi M., Matsui M., Nakanishi S. and Sasai, Y. (1998a) *Xenopus* Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. *Development* 125, 579-87.

Mizuseki K., Kishi M., Shiota K., Nakanishi S. and Sasai Y. (1998b) SoxD: an essential mediator of induction of anterior neural tissues in *Xenopus* embryos. *Neuron* 21, 77-85.

Mobarak C.D., Andrson K.D., Morin M., Beckel-Mitchener A., Rogers S.L., Furneaux H.M., King P. and Perrone-Bizzozero N.I. (2000) The RNA-binding protein HuD is required for GAP-43 mRNA stability, GAP-43 gene expression, and PKC-dependent neurite outgrowth in PC12 cells. Mol. Biol. Cell 11:3191-3203.

Moody S.A., Miller V., Spanos A. and Frankfurter A. (1996) Developmental expression of a neuron-specific beta-tubulin in frog (*Xenopus laevis*): a marker for growing axons during the embryonic period. *J Comp Neurol* 364, 219-30.

Moon R.T. and Kimelman D. (1998) From cortical rotation to organizer gene expression: toward a molecular explanation of axis specification in *Xenopus*. *BioEssays* 20, 536–545.

Moran-Jones K., Wayman L., Kennedy D.D., Reddel R.R., Sara S., Snee M.J., Smith R. (2005) hnRNP A2, a potential ssDNA/RNA molecular adapter at the telomere Nucleic Acids Res. 33: 486–496.

Mullin C., Duning K., Barnekow A., Richter D., Kremerskothen J., Mohr E. (2004) Interaction of rat poly(A)-binding protein with poly(A)-and non-poly(A) sequences is preferentially mediated by RNA recognition motifs 3+4. FEBS Lett 576:437-41.

Murre C., McCaw P.S., Vaessin H., Caudy M., Jan L.Y. (1989a) Interactions between heterologous helix-loop-helix proteins generate complexes that specifically to a common DNA sequence. Cell, 58, 537-544.

Nakata K., Nagai T., Aruga J. and Mikoshiba K. (1997) *Xenopus* Zic3, a primary regulator both in neural and neural crest development. *Proc Natl Acad Sci U S A* 94, 11980-5.

Newberry E.P., Latifi T. and Towler D.A. (1999) The RRM domain of MINT, a novel Msx2 binding protein, recognizes and regulates the rat osteocalcin promoter. Biochemistry 38, pp. 10678–10690.

Nieuwkoop P.D., Faber J. (1956) Normal Tables of *Xenopus laevis*, North–Holland, Amsterdam

Niessing D., Blanke S., Jackle H. (2002) Bicoid associates with the 5′-cap-bound complex of caudal mRNA and represses translation. Genes Dev. 16:2576–82.

Niranjanakumari S., Lasda E., Brazas R., Garcia-Blanco M.A. (2002) Reversible cross-linking combined with immunoprecipitation to study RNA-protein interactions in vivo. Methods. 26 (2): 182-90.

Ohnuma S., Philpott A., Wang K., Holt C.E. and Harris W.A. (1999) p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in *Xenopus* retina. *Cell* 99, 499-510.

Okano H., Imai T., Okabe M. (2002) Musashi: a translational regulator of cell fate. J Cell Sci 115: 1355–1359

Okano H., Kawahara H., Toriya M., Nakao K., Shibata S., Imai T. (2005) Function of RNA-binding protein Musashi-1 in stem cells. Exp Cell Res 306: 349–356

Oleynikov Y. and Singer R.H. (1998) RNA localization: different zipcodes, same postman? Trends Cell Biol. 8, pp. 381–383.

Oschwald R., Richter K. and Grunz H. (1991) Localization of a nervous systemspecific class II beta-tubulin gene in *Xenopus laevis* embryos by whole-mount in situ hybridisation. Int. J Dev Biol, 35, 399-405.

Otero L.J., Ashe M.P. and Sachs A.B. (1999) The yeast poly(A)-binding protein Pab1p stimulates in vitro poly(A)-dependent and cap-dependent translation by distinct mechanisms. EMBO J. 18, pp. 3153–3163.

Pascale A., Gusev P.A., Amadio M., Dottorini T., Govoni S., Alkon D.L., Quattrone A. (2004) Increase of the RNA-binding protein HuD and posttranscriptional up-regulation of the GAP-43 gene during spatial memory. Proc Natl Acad Sci U S A, 101:1217-1222.

Pascale A., Amadio M., Quattrone A. (2008) Defining a neuron: neuronal ELAV proteins. Cell Mol Life Sci. 65 (1): 128-40.

Penalva L.O., Keene J.D. (2004) Biotinylated tags for recovery and characterization of ribonucleoprotein complexes. Biotechniques. 37 (4): 604, 606, 608-10.

Peng S.S.Y., Chen C.Y.A., Xu N.H. and Shyu A.B. (1998) RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. EMBO J. 17: 3461-3470

Pera E.M., Ikeda A., Eivers E. and DeRobertis E.M. (2003) Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. *Genes Dev* 17, 3023-8.

Peritz T., Zeng F., Kannanayakal T.J., Kilk K., Eiríksdóttir E., Langel U., Eberwine J. (2006) Immunoprecipitation of mRNA-protein complexes. Nat Protoc. 1 (2): 577-80.

Pestova T.V., Shatsky I.N., Fletcher S.P., Jackson R.J., Hellen C.U. (1998) A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. Genes Dev. 1; 12 (1): 67-83.

Pfeffer S., Lagos-Quintana M. and Tuschl T. (2003) Cloning of small RNA molecules. In *Current protocols in molecular biology* (eds. F.M. Ausubel *et al.*), pp. 26.4.1–26.4.16. John Wiley and Sons, New York.

Picard J., Wegnez M. (1979) Isolation of a 7S particle from *Xenopus laevis* oocytes: A 5S RNA-protein complex. Proc. Natl. Acad. Sci. 76:241–245.

Piccioni F., Zappavigna V. and Verrotti A.C. (2005) Translational regulation during oogenesis and early development: the cappoly(A) tail relationship. C. R. Biol. 328: 863–881.

Powell L.M., Zur Lage P.I., Prentice D.R., Senthinathan B., Jarman A.P. (2004) The proneural proteins Atonal and Scute regulate neural target genes through different E-box binding sites. Mol Cell Biol. 2004 Nov;24(21):9517-26.

Pozzoli O., Bosetti A., Croci L., Consalez G.G., Vetter M.L. (2001) Xebf3 is a regulator of neuronal differentiation during primary neurogenesis in *Xenopus*. Dev Biol 233: 495–512

Pratt W.B., Toft D.O. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev. 18 (3): 306-60.

Preiss T., Muckenthaler M. and Hentze M.W. (1998) Poly(A)-tail-promoted translation in yeast: implications for translational control. RNA 4, pp. 1321–1331.

Prevot D., Darlix J.L. and Ohlmann T. (2003) Conducting the initiation of protein synthesis: the role of eIF4G, Biol. Cell 95, pp. 141–156.

Quattrone A., Pascale A., Nogues X., Zhao W., Gusev P., Pacini A. and Alkon D.L. (2001) Posttranscriptional regulation of gene expression in learning by the neuronal ELAV-like mRNA-stabilizing proteins. Proc. Natl. Acad. Sci. USA 98, pp. 11668–11673.

Query C.C., Bently R.C., Keene J.D. (1989) A common RNA recognition motif identified within the defined U1 RNA binding domain of the 70K U1-snRNP protein. Cell. 7; 57(1): 89-101.

Radford H.E., Meijer H.A., de Moor C.H. (2008) Translational control by cytoplasmic polyadenylation in *Xenopus* oocytes. Biochim Biophys Acta. 1779(4):217-29.

Reverte C.G., Yuan L., Keady B.T., Lacza C., Attfield K.R., Mahon G.M., Freeman B., Whitehead I.P. and Hake L.E. (2003) XGef is a CPEB-interacting protein involved in *Xenopus* oocyte maturation, Dev. Biol. 255, p. 383.

Richard-Parpaillon L., Heligon C., Chesnel F., Boujard D. and Philpott A. (2002) The IGF pathway regulates head formation by inhibiting Wnt signaling in *Xenopus*. *Dev Biol* 244, 407-17.

Richter J.D. (1987) Molecular mechanisms of translational control during the early development of *Xenopus leavis.* In *Translational regulation of gene expression* (ed. J. Han), pp. 111 -139. Plenum Publishing, New York.

Richter K., Good P.J. and Dawid I.B. (1990) A developmentally regulated, nervous system-specific gene in *Xenopus* encodes a putative RNAbinding protein. *New Biol* 2, 556-65.

Richter J.D. (2007) CPEB: a life in translation. Trends Biochem Sci 32: 279–285.

Rivera-Pomar R., Niessing D., Schmidt-Ott U., Gehring W.J., Jäckle H. (1996) RNA binding and translational suppression in bicoid. Nature 379: 746– 49.
Roberts L.O., Boxall A.J., Lewis L.J., Belsham G.J., Kass G.E. (2000) Caspases are not involved in the cleavage of translation initiation factor eIF4GI during picornavirus infection. J Gen Virol. 81 (Pt 7): 1703-7.
Rogers G.W. Jr., Komar A.A., Merrick W.C. (2002) eIF4A: the godfather of the DEAD box helicases. Prog Nucleic Acid Res Mol Biol. 2002;72:307-31.

Rouhana L., Wickens M. (2007) Autoregulation of GLD-2 cytoplasmic poly(A) polymerase. RNA. 13(2):188-99.

Rosenthal E. and Wilt F. (1987) Selective messenger RNA translation in marine invertebrate oocytes, eggs and zygotes. In *translational regulation of gene expression* (ed. J. Han), pp. 111-139. Plenum Publishing, New York.

Ross J. (1995) mRNA stability in mammalian cells. Microbiol. Rev. 59:423-450.

Rossi C.C., Hernandez-Lagunas L., Zhang C., Choi I.F., Kwok L., Klymkowsky M., Artinger K.B.. (2008) Rohon-Beard sensory neurons are induced by BMP4 expressing non-neural ectoderm in *Xenopus laevis*. Dev Biol. 15;314(2):351-61.

Rowning B.A., Wells J., Wu M., Gerhart J.C., Moon R.T., Larabell C.A. (1997) Microtubule-mediated transport of organelles and localization of β-catenin to the future dorsal side of *Xenopus* eggs. *Proc. Natl Acad. Sci. USA* 94, 1224–1229.

Ryan K., Garrett N., Mitchell A. and Gurdon J.B. (1996) Eomesodermin, a key early gene in *Xenopus* mesoderm differentiation. Cell 87, pp. 989–1000.

Sachs A.B., Bond M.W. and Kornberg R.D. (1986) A single gene from yeast for both nuclear and cytoplasmic polyadenylate-binding proteins: domain structure and expression. Cell 45:827–835.

Sachs A.B., Davis R.W. and Kornberg R.D. (1987) A single domain of the yeast poly(A) binding protein is necessary and sufficient for RNA binding and cell viability. *Mol. Cell. Biol.* 7, 3268–3276.

Sachs A.B. and Davis R.W. (1989) The poly(A) binding protein is required for poly(A) shortening and 60S ribosomal subunit-dependent translation initiation. Cell 58:857–867.

Sachs A.B. and Varani G. (2000) Eukaryotic translation initiation: there are (at least) two sides to every story. Nat. Struct. Biol. 7, pp. 356–361.

Saffman E.E., Styhler S., Rother K., Li W., Richard S., Lasko P. (1998) Premature translation of oskar in oocytes lacking the RNA-binding protein Bicaudal-C. Mol. Cell. Biol. 18:4855–4862.

Sakai M. (1996) The vegetal determinants required for the Spemann organizer move equatorially during the first cell cycle. *Development* 122, 2207-2214.

Sakakibara S., Imai T., Hamaguchi K., Okabe M., Aruga J., Kakajima Y., Yasutomi D., Nagata T., Kurihara Y., Uesugi S. (1996) Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell. Dev. Biol. 176, pp. 230–242.

Sakakibara, S. and Okano H. (1997) Expression of neural RNA-binding proteins in the postnatal CNS: Implications of their roles in neuronal and glial cell development. *Journal of Neuroscience* 17:8300–12.

Sakakibara S., Nakamura Y., Satoh H. and Okano H. (2001) RNA-binding protein Musashi2: developmentally regulated expression in neural precursor cells and subpopulations of neurons in mammalian CNS, J. Neurosci. 21, pp. 8091–8107.

Sakakibara S., Nakamura Y., Yoshida T., Shibata S., Koike M., Takano H., Ueda S., Uchiyama Y., Noda T. and Okano H. (2002) RNA-binding protein Musashi family: roles for CNS stem cells and a subpopulation of ependymal cells revealed by targeted disruption and antisense ablation, Proc. Natl. Acad. Sci. U. S. A. 99, pp. 15194–15199.

Samuels M., Deshpande G., Schedl P. (1998) Activities of the Sex-lethal protein in RNA binding and protein:protein interactions. Nucleic Acids Res. 26:2625–2637.

Sánchez R. and Marzluff.W.F. (2002) The stem-loop binding protein is required for efficient translation of histone mRNA in vivo and in vitro. Mol. Cell. Biol. 22:7093-7104.

Sánchez R. and Marzluff.W.F. (2004) The oligo(A) tail on histone mRNA plays an active role in translational silencing of histone mRNA during *Xenopus* oogenesis. Mol. Cell. Biol. 24:2513-2525.

Sanchez-Diaz P., Penalva L.O. (2006) Post-transcription meets post-genomic: the saga of RNA binding proteins in a new era. RNA Biol. 3 (3): 101-9.

Sasai Y., Lu B., Steinbeisser H., Geissert D., Gont L.K., DeRobertis E.M. (1994) *Xenopus* chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. Cell. 2;79(5):779-90.

Sasai Y., Lu B., Piccolo S. and DeRobertis E.M. (1996) Endoderm induction by the organizer-secreted factors chordin and noggin in *Xenopus* animal caps. *Embo J* 15, 4547-55.

Sasai Y. (1998) Identifying the missing links: genes that connect neural induction and primary neurogenesis in vertebrate embryos. Neuron. 21(3):455-8.

Scharf S.R. and Gerhart J.C. (1983) Axis determination in eggs of *Xenopus laevis*: a critical period before first cleavage, identified by the common effects of cold, pressure, and ultraviolet irradiation. *Dev. Biol.* 99, 75-87.

Scheibel T., Neuhofen S., Weikl T., Mayr C., Reinstein J., Vogel P.D., Buchner J. (1997) ATP-binding properties of human Hsp90. J Biol Chem. 25; 272 (30): 18608-13.

Scherly D., Boelens W., van Venrooij W.J., Dathan N.A., Hamm J., Mattaj I.W. (1989) Identification of the RNA binding segment of human U1 A protein and definition of its binding site on U1 snRNA. EMBO J. 8:4163–4170.

Schnapp B.J., Arn E.A., Deshler J.O. and Highett M.I. (1997) RNA localization in *Xenopus* oocyte, Semin. Cell Dev. Biol. 8, pp. 529–540.

Schroeter E.H., Kisslinger J.A. and Kopan R. (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* 393, 382-6.

Selenko P., Gregorovic G., Sprangers R., Stier G., Rhani Z., Kramer A. and Sattler M. (2003) Structural basis for the molecular recognition between human splicing factors U2AF65 and SF1/mBBP, Mol. Cell 11, pp. 965–976.

Seo S., Richardson G.A. and Kroll K.L. (2005) The SWI/SNF chromatin remodeling protein Brg1 is required for vertebrate neurogenesis and mediates transactivation of Ngn and NeuroD. *Development* 132, 105-15.

Shaw G.,Kamen R. (1986) A conserved AU sequence from the 3 untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46: 659-667.

Sheets M.D., Fox C.A., Hunt T., Vande Woude G., Wickens M. (1994) The 3'-untranslated regions of *c-mos* and *cyclin* mRNAs stimulate translation by regulating cytoplasmic polyadenylation. Genes Dev. 8(8):926-38.

Shi H., Hoffman B.E. and Lis J.T. (1997) A specific RNA hairpin loop structure binds the recognition motifs of the Drosophila SR protein B52. Mol. Cell. Biol. 17: 2649-2657

Shivdasani R.A. (2002) Molecular regulation of vertebrate early endoderm development. Dev Biol 249: 191-203.

Shu L., Yan W. and Chen X. (2006) RNPC1, an RNA-binding protein and a target of the p53 family, is required for maintaining the stability of the basal and stress-induced p21 transcript. *Genes* & *Dev.* 20: 2961–2972.

Siddiqui N., Mangus D.A., Chang T.C., Palermino J.M., Shyu A.B. and Gehring K. (2007) Poly(A) nuclease interacts with the C-terminal domain of polyadenylate-binding protein domain from poly(A)-binding protein. J. Biol. Chem. 282: 25067–25075.

Simon E., Camier S. and Seraphin B. (2006) New insights into the control of mRNA decapping. Trends Biochem. Sci. 31: 241–243.

Sinner D., Kirilenko P., Rankin S., Wei E., Howard L., Kofron M., Heasman J., Woodland H.R. and Zorn A.M. (2006) Global analysis of the transcriptional network controlling *Xenopus* endoderm formation. Development 133: 1955–1966.

Sladic R., Lagnado C., Bagley C., Goodall G. (2004) Human PABP binds AU-rich RNA via RNA-binding domains 3 and 4. Eur J Biochem 271: 450–457.

Slepenkov S.V., Korneeva N.L., Rhoads R.E. (2008) Kinetic Mechanism for Assembly of the m7GpppG{middle dot}eIF4E{middle dot}eIF4G Complex. J Biol Chem. 12; 283 (37): 25227-37.

Skrisovska L., Bourgeois C.F., Stefl R., Grellscheid S.N., Kister L., Wenter P., Elliott D.J., Stevenin J., Allain F.H. (2007) The testis-specific human protein RBMY recognizes RNA through a novel mode of interaction. EMBO Rep 8: 372–379

Smith J.C. (1989) Induction and early amphibian development. Curr Opin Cell Biol. 1(6):1061-70.

Smith W.C. and Harland R.M. (1992) Expression cloning of noggin, a new dorsalizing factor localized in the Spemann organizer in *Xenopus* embryos. Cell 70, 829–840.

Sokol S.Y., Klingensmith J., Perrimon N. and Itoh K. (1995) Dorsalizing and neuralizing properties of *Xdsh*, a maternally expressed *Xenopus* homolog of *dishevelled*. *Development* 121, 1637-1647.

Song H.W., Cauffman K., Chan A.P., Zhou Y., King M.L., Etkin L.D. and Kloc M. (2007) Hermes RNA binding protein targets RNAs encoding proteins involved in meiotic maturation, early cleavage, and germline development, Differentiation. 75(6):519-28.

Souopgui J., Sölter M. and Pieler T. (2002) XPak3 promotes cell cycle withdrawal during primary neurogenesis in *Xenopus laevis*. *Embo J* 21, 6429-39.

Souopgui J., Rust B., Vanhomwegen J., Heasman J., Henningfeld K.A., Bellefroid E., Pieler T. (2008) The RNA-binding protein XSeb4R: a positive regulator of VegT mRNA stability and translation that is required for germ layer formation in *Xenopus*. Genes Dev. 1;22(17):2347-52.

Spemann H. (1924) Vererbung und Entwicklungsmechanik. Naturwissenchaften. 12:65–79.

Spemann H. (1938) Embryonic Development and Induction. Yale University Press; New Haven, CT.

Standart N., Vincent A. and Scherrer K. (1981) The polyribosomal poly(A)-binding protein is highly conserved in vertebrate species, FEBS Lett. 135, pp. 56–60.

Standart N., Minshall N. (2008) Translational control in early development: CPEB, P-bodies and germinal granules. Biochem Soc Trans. 36(Pt 4):671-6.

Stefl R., Skrisovska L., Xu M., Emeson R.B. and Allain F.H. (2005) Resonance assignments of the double-stranded RNA-binding of adenosine deaminase acting on RNA 2 (ADAR2). J. Biomol. NMR 31: 71–72.

Stennard F., Carnac G. and Gurdon J.B. (1996) The *Xenopus*T-box gene, Antipodean, encodes a vegetally localised maternal mRNA and can trigger mesoderm formation. Development 122: 4179–4188.

Stennard F., Zorn A.M., Ryan K., Garrrett N. and Gurdon J.B. (1999) Differential expression of VegT and Antipodean protein isoforms in *Xenopus*. Mech. Dev. 86: 87–98.

Swanson M.S., Nakagawa T.Y., LeVan K., Dreyfuss G. (1987) Primary structure of human nuclear ribonucleoprotein particle C proteins: conservation of sequence and domain structures in heterogeneous nuclear RNA, mRNA, and pre-rRNA-binding proteins. Mol. Cell Biol. 7:1731–1739.

Tannahill D., and Melton D.A. (1989) Localized synthesis of the Vg1 protein during early *Xenopus* development. Development 106:775-785.

Tao Q., Yokota C., Puck H., Kofron M., Birsoy B., Yan D., Asashima M., Wylie C.C., Lin X., Heasman J. (2005) Maternal Wnt11 activates the canonical Wnt signalling pathway required for axis formation in *Xenopus* embryos. Cell 120, 857–871.

Tarun S.Z. and Sachs A.B. (1996) Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G. EMBO J., 15: 7168–7177.

Tarun S.Z. Jr., Wells S.E., Deardorff J.A., Sachs A.B. (1997) Translation initiation factor eIF4G mediates in vitro poly(A) tail-dependent translation. Proc Natl Acad Sci U S A. 94:9046–51.

Theunissen O., Rudt F. and Pieler T. (1998) Structural determinants in 5S RNA and TFIIIA for 7S RNP formation, Eur J Biochem 258, pp. 758–767.

Tourriere H., Chebli K. and Tazi J. (2002) mRNA degradation machines in eukaryotic cells, Biochimie 84, pp. 821–837.

Townley-Tilson W.H., Pendergrass S.A., Marzluff W.F. and Whitfield M.L. (2006) Genome-wide analysis of mRNAs bound to the histone stem–loop binding protein. RNA 12: 1853–1867.

Trachsel H. and Staehelin T. (1979) Initiation of mammalian protein synthesis. The multiple functions of the initiation factor eIF-3. Biochim. Biophys. Acta 565: 305–314.

Twiss J.L., van Minnen J. (2006) New insights into neuronal regeneration: the role of axonal protein synthesis in pathfinding and axonal extension. J Neurotrauma 23: 295–308

Uchida N., Hoshino S., Imataka H., Sonenberg N., Katada T. (2002) A novel role of the mammalian GSPT/eRF3 associating with poly(A)-binding protein in cap/poly(A)-dependent translation. J. Biol. Chem. 277:50286–50292.

van der Giessen K. and Gallouzi I.E. (2007) Involvement of transportin 2-mediated HuR import in muscle cell differentiation. Mol. Biol. Cell. 18:2619–2629.

Varani G. (2001) Delivering messages from the 3′ end. Proc. Natl. Acad. Sci. U. S. A. 98, pp. 4288–4289.
Vernon A.E., Devine C. and Philpott A. (2003) The cdk inhibitor p27Xic1 is required for differentiation of primary neurons in *Xenopus*. *Development* 130, 85-92.

Voeltz G.K., Ongkasuwan J., Standart N., Steitz J.A. (2001) A novel embryonic poly(A) binding protein, ePAB, regulates mRNA deadenylation in *Xenopus* egg extracts. Genes Dev 15: 774–788

Vogt W. (1929) Gestaltungsanalyse am Amphibienkeim mit örtlicher Vitalfärbung II. Gastrulation und Mesodermbildung bei Urodelen und Anuren., W. Roux Arch f Entw Mech 120, pp. 384–706.

Wang Z.F., Whitfield M.L., Ingledue T.C. 3rd, Dominski Z., Marzluff W.F. (1996) The protein that binds the 3' end of histone mRNA: a novel RNA-binding protein required for histone pre-mRNAs processing Genes Dev, . 10, 3028–3040

Wang Z.F., Ingledue T.C., Dominski Z., Sanchez R., and Marzluff W.F. (1999) Two *Xenopus* proteins that bind the 3′ end of histone mRNA: implications for translational control of histone synthesis during oogenesis. Mol. Cell. Biol. 19:835-845.

Wang S. and Barres B.A. (2000a) Up a notch: instructing gliogenesis. *Neuron* 27, 197-200.

Wang S., Furneaux H., Cheng H., Caldwell M.C., Hutter D., Liu Y., Holbrook N.J. and Gorospe M. (2000b) HuR regulates p21 mRNA stabilization by UV light. Mol. Cell. Biol. 20, pp. 760–769.

Weaver C. and Kimelman D. (2004) Move it or lose it: axis specification in *Xenopus*. *Development* 131, 3491-9.

Weidinger G., Stebler J., Slanchev K., Dumstrei K., Wise C., Lovell-Badge R., Thisse C., Thisse B. and Raz E. (2003) dead end, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. Curr. Biol. 13, pp. 1429–1434.

Welch E.M., Wang W., Peltz S.W. (2000) Translation termination: it's not the end of the story. In N Sonenberg, JWB Hershey, MB Mathews, eds, Translational Control of Gene Expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 467–486.

White M.M., Mayne K.M., Lester H.A. and Davidson N. (1985) Mouse-Torpedo hybrid acetylcholine receptors: functional homology does not equal sequence homology. Proc. Natl. Acad. Sci. USA 82:4852-4856.

Wickens M., Anderson P., Jackson R.J. (1997) Life and death in the cytoplasm: messages from the 3' end. Curr Opin Genet Dev. 7(2):220-32.

Wilson G.M., Sutphen K., Moutafis M., Sinha S. and Brewer G. (2001) Structural remodeling of an A+U-rich RNA element by cation or AUF1 binding. J. Biol. Chem. 276, pp. 38400–38409.
Wormington M., Searfoss A.M., Hurney C.A. (1996) Overexpression of poly(A) binding protein prevents maturation-specific deadenylation and translational inactivation in *Xenopus* oocytes. EMBO J. 15:900–909.

Wylie C., Kofron M., Payne C., Anderson R., Hosobuchi M., Joseph E., Heasman J. (1996) Maternal β-Catenin establishes a 'dorsal signal' in early *Xenopus* embryos. *Development* 122, 2987–2996.

Xanthos J.B., Kofron M., Wylie C., and Heasman J. (2001) Maternal VegT is the initiator of a molecular network specifying endoderm in *Xenopus laevis*. Development 128: 167–180.

Xanthos J.B., Kofron M., Tao Q., Schaible K., Wylie C., and Heasman J. (2002) The roles of the three signaling pathways in the formation and function of the Spemann Organizer. Development 129: 4027–4043.

Yasuo H. and Lemaire P. (2001) Generation of the germ layers along the animal-vegetal axis in *Xenopus laevis*.Int. J. Dev. Biol. 45,229 -235.

Yoon K. and Gaiano N. (2005) Notch signaling in the mammalian central nervous system: insights from mouse mutants. *Nat Neurosci* 8, 709-15.

Zanier K., Luyten I., Crombie C., Muller B., Schumperli D., Linge J.P., Nilges M. and Sattler M. (2002) Structure of the histone mRNA hairpin required for cell cycle regulation of histone gene expression. RNA 8: 29–46.

Zhang J. and King M.L. (1996) *Xenopus* VegT RNA is localized to the vegetal cortex during oogenesis and encodes a novel T-box transcription factor involved in mesodermal patterning. Development 122: 4119–4129.

Zhang J., Houston D.W., King M.L., Payne C., Wylie C., and Heasman J. (1998) The role of maternal VegT in establishing the primary germ layers in *Xenopus* embryos. Cell 94: 515–524.

Zhao W.M., Jiang C., Kroll T.T. and Huber P.W. (2001) A proline-rich protein binds to the localization element of *Xenopus Vg1* mRNA and to ligands involved in actin polymerization. EMBO J. 20, pp. 2315–2325

Zheng L.X., Dominski Z., Yang X., Elms P., Raska C.S., Borchers C.H. and Marzluff W.F. (2003) Phosphorylation of SLBP on two threonines triggers degradation of SLBP, the sole cell-cycle regulated factor required for regulation of histone mRNA processing, at the end of S phase. Mol. Cell. Biol. 23:1590-1601.

Zimmerman L.B., De Jesus-Escobar J.M. and Harland R.M. (1996) The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4, Cell 86, pp. 599–606.

6. Appendix

6.1 Potential XSeb4R-targets identified by RNA-IP and RNA-cloning

Table 5 represents a complete list of the putative XSeb4R-target fragments obtained by RNA-IP. The corresponding fraction is indicated by the first number in the column “clone number” (fraction.clone). Shown are fragment length without the fused adaptors. Insert consisting solely of adaptors are represented with an insert length of “0”. All fragments were cloned in the pGEM-Teasy vector (Promega) and sequenced using the SP6 primer. Complete sequences are listed according to their fraction.

Table 5: Complete list of targets obtained by the RNA-IP

clone	clone number	insert	lenght w/o adaptors 1	lenght w/o adaptors 2	lenght w/o adaptors 3	lenght w/o adaptors 4
BR 01	2.8	28S rRNA	159 nt			
BR 02	19.1	28S rRNA	85 nt			
BR 03	19.2	TGas110b17	85 nt			
BR 04	23.6	18S rRNA	46 nt			
BR 05	7.1	28S rRNA	43 nt			
BR 06	3.7	2 insers	43 nt	0		
BR 07	7.6	2 inserts	35 nt	20 nt		
BR 08	22.2	28S rRNA	31 nt			
BR 09	3.8	2 inserts	18nt	0		
BR 10	1.6	unknown	17 nt			
BR 11	4.6	2 inserts	16 nt	15 nt		
BR 12	2.11	2 inserts	17 nt	7 nt		
BR 13	6.3	2 inserts	16 nt	0		
BR 14	3.11	unknown	14 nt			
BR 15	8.5	unknown	14 nt			
BR 17	9.3	unknown	13 nt			
BR 18	7.7	2 inserts	9 nt	2 nt		
BR 19	5.2	4 inserts	8 nt	5 nt	2 nt	0
BR 20	5.5	2 inserts	5 nt	5 nt		
BR 21	2.3	3 inserts	5 nt	0	0	
BR 22	1.9	2 inserts	4 nt	3 nt		
BR 23	3.6	2 inserts	2 nt	4 nt		
BR 24	8.8	2 inserts	2 nt	2 nt		
BR 25	16.3	2 inserts	2 nt	2 nt		
BR 26	4.8	2 inserts	2 nt	1 nt		
BR 27	2.5	2 inserts	2 nt	0		
BR 28	4.2	2 inserts	2 nt	0		
BR 29	8.1	2 inserts	2 nt	0		
BR 30	1.7	2 inserts	2 nt	0		
BR 31	19.7	2 inserts	2 nt	0		
BR 32	19.8	2 inserts	2 nt	0		
BR 33	26.8	2 insers	2 nt	0		
BR 34	4.11	unknown	2 nt			
BR 35	5.1	2 inserts	0	0		
BR 36	1.4	2 inserts	0	0		
BR 37	1.3	empty vector				
BR 38	1.8	empty vector				
BR 39	3.1	empty vector				
BR 40	6.4	empty vector				
BR 41	7.2	empty vector				
BR 42	6.6	-				
BR 43	6.12	-				
BR 44	3.2	-				
BR 45	6.8	-				
BR 46	7.1	-				
BR 47	8.1	-				
BR 48	21.5	-				
BR 49	22.6	-				
BR 50	23.5	-				
BR 51	26.4	-				
BR 52	VegT (+)	~ 500				
BR 53	H_2O	6 nt				

Clone 1.6 (BR 10)
5’-GTTGATGGANATTGCCN-3’

Clone 1.7 (BR 29)
5’-GG-3’

Clone 1.9 (BR 22)
1. 5’-CTCG-3’
2. 5’-CTC-3’

Clone 2.3 (BR 21)
5’-CTCGA-3’

Clone 2.5 (BR 27)
5’-GG-3’

Clone 2.8 (BR 01)
5’-CGACTTTTAGCGGTGGATCACTCGGCTCGTGCGTCGATGAAGAACGCAGCTAGCT
GCGAGAATTAGTGTGAATTGCAGGACACATTGATCATCGACACTTCGAACGCACCTTG
CGGCCCCGGGTTCCTCCCGGGGCCACGCCTGTCTGAGGGTCGCTC-3’

Clone 2.11 (BR 12)
1. 5’-GGACCACCATTCGAG-3’
2. 5’-CTCTGTN-3’

Clone 3.6 (BR 23)
1. 5’-TCAG-3’
2. 5’-GG-3’

Clone 3.7 (BR 06)
5’-TGGGTTGAGNGAGGAATTCCGTACCNCNAATTCNNGGTCAAA-3’

Clone 3.8 (BR 09)
5’-CTCAAATTCGCGGTTAAA-3’

Clone 3.11 (BR 14)
5’-CGGAAGGATCTTGT-3’

Clone 4.2 (BR 28)
5’-GG-3’

Clone 4.6 (BR 11)
1. 5’-AGGCCCACGTACGTACA-3’
2. 5’-GGACCACGATTCGAG-3’

Clone 4.8 (BR 26)
1. 5’-GG-3’
2. 5’-G-3’

Clone 4.11 (BR 34)
5’-GG-3’

Clone 5.2 (BR 19)
1. 5’-CGAACCGA-3’
2. 5’-CTCTA-3’
3. 5’-GG-3’

Clone 5.5 (BR 20)
1. 5’-TCGAG-3’
2. 5’-TCGAG-3’

Clone 6.3 (BR 13)
5’-CCTTCTCCCAAGCAAG-3’

Clone 7.1 (BR 05)
5’-AGGGCGAAGCCAGNAGGAAACTCTGGTGGAGGTCCGTAGCGG-3’

Clone 7.6 (BR 07)
1. 5’-ANAAGCTCCCCACCNTGGGAGGGNTCGGGGATTGC-3’
2. 5’-GGTGTAATTGGNAN-3’

Clone 7.7 (BR 18)
1. 5’-GGGTCACGG-3’
2. 5’-CC-3’

Clone 8.1 (BR 29)
5’-AC-3’

Clone 8.5 (BR 15)
5’-ATGATGTTCTCGGG-3’

Clone 8.8 (BR 24)
1. 5’-GA-3’
2. 5’-GG-3’

Clone 9.3 (BR 17)
5’-CGGGCACGTACCC-3’

Clone 19.7 (BR 31)
5’-CC-3’

Clone 19.8 (BR 32)
5’-AC-3’

Clone 16.3 (BR 25)
1. 5’-GG-3’
2. 5’-CC-3’

Clone 19.1 (BR 02)
5’-GAGCGACCCTCAGACAGGCGTGGCCCCGGGGAGGAACCCGGGGCCGCAAGGTG
CGTTCGAAGTGTCGATGATCAATNTNTNCTNA-3’

Clone 19.2 (BR 03)
5’-TTGCCTGGAGAACATCCCTTTGTCAGCCAGCAGTTGATTGTCTCTGCTGAACAGCA
GTTTGATATCGAGAGACTTCAGGCGGGAC-3’

Clone 22.2 (BR 08)
5’-CTGGGTATAGGGGCGAAAGACTAATCGAACC-3’

Clone 23.6 (BR 04)
5’-GATGCGTTCGAAGTGTCGATGATCAATGTGCCCCTTCGACGCAGTG-3’

Clone 26.8 pGEM-Teasy (BR 33)
5’-CC-3’

Clone VegT (BR 52)
5’-GGGCCATCTAAAGCAAAGCTACAGCTTCCAGTATTGCCTCTAGNATGAGAGCGCTA
CTTCTGCAATAGCAGGGTCACCAAAGGCTGCCTAACTCTGCATATTTTCACATTAGCAT
AACCTCTGGCAATGATTGGGACAATTGACTCCAAAAGTGGGGTAAACATGTAACAAAT
ATTAATCCTTGCATTCTGTGATTAGTTAAATAAACATGGGATGGTTTGTTTGCTCTTTGT
TCTACAGATTCAGTAGCAGCTCAGCNGTNAAAGGGTTTGTGTAATTGCTTGTAAAGCT
AGCTTTAACCGCGAATTCGAGAATCCCGCGGCCATGGCGGCCGGGAGCATGCGACG
TCGGGCCCNATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACA
ACGTCGTGACTGGGAAAACCCTGGCGTTNCCCANCTTAATCGCCTTGCNNCACATCC
NCCTTTCNCCNGCTGGNGTNATANCNAANAGGCCCGCNCCNATCGNNCTTNCNANCN
NTTGCNCNNCC-3’

Clone H_2O (BR 53)
5’-GCTACC-3’

6.2 Potential XSeb4R-targets identified by modified RNA-IP

Table 6 represents a complete list of putative XSeb4R-target fragments obtained by the modified RNA-IP protocol. The corresponding fraction is indicated by the first number in the column “clone number”. Shown are fragment length without the fused adaptors. All fragments were cloned in the pGEM-Teasy vector (Promega) and sequenced using the SP6 primer. Clones containing the as contamination identified VegT-fragment are indicated in blue. Complete sequences are listed according to their fraction.

Table 6: Complete list of targets obtained by the RNA-IP a

BR scaffold	clones	occurance	gene	region	accesion no	clone number	size (bp)	Scaffold JGI
BR 001	9	10	unknown	-	- (3C2=BJ35693)	3C2 (4A6), 3C13, 7C3, 3B15, 7A2, 3C11, 8B4, 8D3, 7B4	369 (368), 347, 278, 247, 145, 137, 124, 118, 88	2356 / 5664
BR 002	9	10	28s ribosomal RNA		X59734	5A25, 6A1, 6C3, 1B15, 1C17, 3B11 (7C1), 6C5a, 5A16, 6C5b	250, 214, 173, 141, 117, 99, 94, 79, 62	4706 / 6387
BR 003*	2	10	VegT	3'UTR	U59483	3B14 (3B25, 3C3, 4A16, 6B8, 7D1, 5A20), 4A10 (4A26, 8A4)	293, 288	12
BR 004	2	3	XL EST	-	AU 245504	3B28, 1B19 (5D3)	229, 104	-
BR 005	2	2	unknown	-	-	3C12, 3B9	326, 223	-
BR 006	1	11	unknown	-	-	1A25 (3C12, 3C21, 4A1, 4A11, 4A15, 4A17, 4A18, 4A20, 4A22, 4A27)	326	-
BR 007	1	2	unknown	-	-	3C17 (4A12)	353	-
BR 008	1	2	unknown	-	-	3B1, 7D3	273	-
BR 009	1	2	unknown	-	-	6C2a (8D1)	116	-
BR 010	1	2	unknown	-	-	1A26 (4C5)	103 (102)	-
BR 011	1	2	unknown	-	-	3C9 (3D4)	82 (79)	-
BR 012	1	1	unknown	-	-	3C19	327	-
BR 013	1	1	unknown	-	-	3B7	326	-
BR 014	1	1	unknown	-	-	3C16	311	-
BR 015	1	1	unknown	-	-	3B18	244	-
BR 016	1	1	unknown	-	-	3B6	238	-
BR 017	1	1	unknown	-	-	3B19	238	-
BR 018	1	1	unknown	-	-	5C2	229	-
BR 019	1	1	unknown	-	-	6B1	214	-
BR 020	1	1	unknown	-	-	1A3	208	-
BR 021	1	1	unknown	-	-	6B7	206	-
BR 022	1	1	unknown	-	-	3B10	205	-
BR 023	1	1	unknown	-	-	5A23	201	-
BR 024	1	1	unknown	-	-	5A8	197	-
BR 025	1	1	unknown	-	-	3A15	192	-
BR 026	1	1	unknown	-	-	2A1	188	-
BR 027	1	1	unknown	-	-	5A13	175	-
BR 028	1	1	unknown	-	-	1A12	165	-
BR 029	1	1	unknown	-	-	3A6	161	-
BR 030	1	1	unknown	-	-	3B4	143	-
BR 031	1	1	unknown	-	-	1C20	142	-
BR 032	1	1	unknown	-	-	1C1	141	-
BR 033	1	1	unknown	-	-	7A5	137	-
BR 034	1	1	unknown	-	-	4A28	134	-
BR 035	1	1	unknown	-	-	1B22	130	-
BR 036	1	1	unknown	-	-	8C1	127	-
BR 037	1	1	unknown	-	-	2B8	126	-
BR 038	1	1	unknown	-	-	1B10	124	-
BR 039	1	1	unknown	-	-	5D2	120	-
BR 040	1	1	unknown	-	-	6C2b	117	-

Table 7: Complete list of targets obtained by the RNA-IP b

BR scaffold	clones	occurance	gene	region	accesion no	clone number	size (bp)	Scaffold JGI
BR 041	1	1	unknown	-	-	1C3	115	-
BR 042	1	1	unknown	-	-	8B1	115	-
BR 043	1	1	unknown	-	-	1B11	113	-
BR 044	1	1	unknown	-	-	3A2	113	-
BR 045	1	1	unknown	-	-	4B1	112	-
BR 046	1	1	unknown	-	-	4A21	111	-
BR 047	1	1	unknown	-	-	1B4	109	-
BR 048	1	1	unknown	-	-	1A24	106	-
BR 049	1	1	unknown	-	-	1B12	102	-
BR 050	1	1	unknown	-	-	1C21	102	-
BR 051	1	1	unknown	-	-	3A10	101	-
BR 052	1	1	unknown	-	-	4D2	100	-
BR 053	1	1	unknown	-	-	3B12	100	-
BR 054	1	1	unknown	-	-	1B17	97	-
BR 055	1	1	unknown	-	-	1C16	97	-
BR 056	1	1	unknown	-	-	1C28	91	-
BR 057	1	1	unknown	-	-	1A13	90	-
BR 058	1	1	unknown	-	-	6D2	90	-
BR 059	1	1	unknown	-	-	1B9	89	-
BR 060	1	1	unknown	-	-	1B25	89	-
BR 061	1	1	unknown	-	-	1B26	87	-
BR 062	1	1	unknown	-	-	1B24	83	-
BR 063	1	1	unknown	-	-	7B2	82	-
BR 064	1	1	unknown	-	-	1C13	81	-
BR 065	1	1	unknown	-	-	6C4	80	-
BR 066	1	1	unknown	-	-	4C7	78	-
BR 067	1	1	unknown	-	-	4D7	78	-
BR 068	1	1	unknown	-	-	6D8b	78	-
BR 069	1	1	unknown	-	-	1C12	74	-
BR 070	1	1	unknown	-	-	3B27	70	-
BR 071	1	1	unknown	-	-	2C2	64	-
BR 072	1	1	unknown	-	-	4B5b	64	-
BR 073	1	1	unknown	-	-	1B23	61	-
BR 074	1	1	unknown	-	-	1C24	61	-
BR 075	1	1	unknown	-	-	1B16	58	-
BR 076	1	1	unknown	-	-	1B7	54	-
BR 077	1	1	unknown	-	-	6D8a	20	-
BR 078	1	1	unknown	-	-	4B5a	16	-

Clone 1A3
5’-CAAGTTTGGCNGNCTTTTTTTTCATCTGTNGCAAGAGCATTTATCACACAAGCATAT
ATTGTAAAGCTTGCNCGACAGTTAGGTCATTGTCNCATCCAAATGCAGGCTTTNTTTAA
TCTCTTCCATCACAATCAAACTGNGGGTTNCCACCGCCGCTGGNNNTTGCTNTAAGAT
ATTGCCNANTAACTTGNGGNAAGAACTCNTCTCT-3’

Clone 1A12
5’-CNNNTNAGTGAANAGGGCCTGTCTNNTCTGTGAAGCTCTTTAAGTACAGGGTAAGA
AACNGGNNGAGCTAATAGCCTGTTGCTAATGACGGTACCTGAANAATAANCACCGGN
TAACATACGTANCNANNGCCGCGNTGATACNTATGGTGCNANCNNTGATCNT-3’

Clone 1A13
5’-CCAACATCCGTTATGTGCGTCCGATTCTGCTTGATAGCGAAGTCACCGTTGTGATT
GAGACGGCAGACTGCAAATCTGCGTCATTACAGC-3’

Clone 1A24
5’-CCTCAGTCATACCCATACTTTCTTAAAACTCTTGTTTTTCATCGTCATCCAGCTGGGC
GATTTCAGATTCGATTTGGTTGCATAGCGCGACCACCAACGCATCATC-3’

Clone 1A25
5’-TGAATTGGGTAGCCATCCCAGTATTTTTAGTCCGCTTTGGTATGGGCTATCGTTTGG
CATGGGTGCGGTAGCAGGATTGATTTCTGATGAGTTTAGTCTGGGGTTTGTCGCAGA
GACAGAAACCCAAGTCAGCGAGCATTTGCACAGCCATCTTGGTAAGCTGCCTGCACA
AGATAAACGCAGTGCTGAGATTTTAAGCCAAATGGATAAAGAAGAATTACATCACCGT
GATAAAGCAGTCGAATCAGGCGCCAGCCCATTGCCACTACCCATCAAAACTACGATG
CGTTTTTTGGCTAACTGCATGAAAACGGTGGCGTATCATA-3’

Clone 1A26
5’-AGATTGTGAAATGCCTTTGAGTGCCAATAAATGTGACGCGCGAGAATCGGGATTTTT
AAGCGTATTATAGGCTTCATTAATAATAGCAGACGCTTGCTCAGGC-3’

Clone 1B4
5’-TGTGGATGCCGGTAGCGAAGACGATGCGTTAGCAATCATCAAACTGATTGATAGCT
TTAACCTAGCCTATTTACATATCTCTGAGCCAGACTGGGCAGGTGGAAAACCC-3’

Clone 1B7
5’-AGGCGAGGCCGAAAGGCGTAGTCGATGGGAAATCGGTTAATATTCCGATACTTG-3’

Clone 1B9
5’-GGCAATGATACCGCCAAACGCAGATTCTGGGTCAGTGGCAAAGGCTTTTTGATATG
CATGGGTTAAGTCGGCATCCACGGCCACACCAT-3’

Clone 1B10
5’-GAAAATTCAGTTCAAGAAAACAACTCAGTTCAAGAAAACTCAGTCAAATGATTCATT
GACCCATTCAACCATAAAGGTTAAATAAAAATTGAACTATCACTTAGCAAAGTCTATAG
CCATCCCG-3’

Clone 1B11
5’-CTATTAACCCATTTTTTCCGTCAAATATTTCCAATAGCGTTTTGGTAAATACTGAATAT
GCAGCTTGCGATTAACAAAATATTAACGCTTACAATTTCCTGATGCGGNATTTT-3’

Clone 1B12
5’-GATTGTGAAATGCCTTTGAGTGCCAATAAATGTGACGCGCGAGAATCGGGATTTTTA
AGCGTATTATAGGCTTCATTAATAATAGCAGACGCTTGCTCAGGC-3’

Clone 1B15
5’-AGGGCGAAGCCAGAGGAAACTCTGGTGGGGGTCCGTAGCGGTCCTGACGTGCAA
ATCGGTCGTCCGACCTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCTG
GTTCCCTCCGAAGTTTCCCTCAGGATAGCA-3’

Clone 1B16
5’-GTTCAATGTAAAGCTGTAGTAAAGGTTCACGGGGTCTTTCCGTCTAGCCGCGGGTA
CA-3’

Clone 1B17
5’-CGCGCGGGCCGGTCCCGCCTGCGGGAACCTGTTCATTAACTTCGCCCTCAAAGGA
TGTTTAGGGGGCGAACGTCATGGCCCGGCCCGAATTCCCCAG-3’

Clone 1B19
5’-CAAGTATCGGAATATTAACCGATTTCCCATCGACTACGCCTTTCGGCCTCGCCTTAG
GGGTCGACTCACCCAGCCCCGATTAACGTTGGACTGGAACCCTTGGT-3’

Clone 1B22
5’-ACGGAGTTCCTCACTAAACTTGTTCAAAGTAGGAAAATAATTTTTGTTGGTCTTTTTC
GCTAATACCTAAACCGGTATCTTTTACCGTTATTTTTAACCAATCTTTGCTGACAGTCAT
ATTTTGGCTGGT-3’

Clone 1B23
5’-ACTCGACTTCATCAGAGGTAGAGACTTTATAAATAGTCTGTCCGTCAGCACCTTTCT
CTTC-3’

Clone 1B24
5’-AGTTTACGGAATTCTTACAAAAGCGTATTCTTCGATATCATCTGTCATATCAAGTAGC
TCAGTCAGACGCACGTTAATTTCCT-3’

Clone 1B25
5’-ATATAAAGATAACATATGGGATCCTAAAGGATCGTAACGATGGATATATCGATAAAT
CATATATTGGCGCACCAANCATCACTAAAGAT-3’

Clone 1B26
5’-TGAATCTCGTGAAACACTTTACCGAAGCGTTGCTGCTTTTCATTAGGCAAGGTGGTT
TTACTCGCAAGTTTTAGTTGGTATAGTGGC-3’

Clone 1C1
5’-GCCATTCATCACCGATAACACGGTAATGAGCACGGCAACGCCAAGCGTCAAACCCA
CCATTGAAATCAAAGAAATAAAAGAAATAAATCGGTTACTTCGCTCAGCACGGGTATAC
CGTAATCCTGAAAACAAAGCCAACGG-3’

Clone 1C3
5’-CTATCCGACACGATACCGCCTGTGGCTTCGGCAATTGCTTGGATTTTTGGGTCGGT
ATAATAGTGAGCAATCAACGTGGCATTATTCTGTTTTAACAAATCGGCAATGCGCTGT
A-3’

Clone 1C12
5’-TCAAGAATACCGACAATTTCACTGGAAATATGCTGGACATGTGAAAAGCGCTCGATA
TCCATTAAGTTACGTAC-3’

Clone 1C13
5’-TGACCCGCAAGTTTCCTTTGTTTTTGGGGTTCAGCAGGTCTCTAACACGCTCACAAT
ATATTTCCATATAGCTCACCTCTA-3’

Clone 1C16
5’-GCATGTCTTGCCAAGATCTTGACGAAAATTCACCCAATCCAATAGGGATTAGCGACA
CCGAATTAGAAACTAGAGTTAAACAGACCATTGACCAGGC-3’

Clone 1C17
5’-AGGACCGCTACGGACCTCCACCAGAGTTTCCTCTGGCTTCGCCCTGCCCAGGCAT
AGTTCACCATCTTTCGGGTCCTATCGCGCGCGCTCACGCTCCACCTCCCCGACGGGG
CGGGC-3’

Clone 1C20
5’-GCTGTGGTATGGGATGAAGCCGAAAATCGTCTGCATGCCCAAAAAGCGCTGATGG
AATTTTTGTTAACCGATAAAATCAAACTCGCCTAGTCTATGTGGTCTGGGCGTTATCTT
TGCGCTCTACGCTGCCATTATTACTATC-3’

Clone 1C21
5’-TCGGCTACGCCAATAGCTTTATCCGTTGGTTTGCCGAAGAAGGCAAACGCATCTAT
GGCGACATCATCCCTAGCACCAACAAAGACCTACGTTACGTGGTGT-3’

Clone 1C24
5’-GGACATGGGCATCGGCTTCGCACGCGCCAAGGTCGGCGACCGCTACATGCTGGAA
GTGATG-3’

Clone 1C24
5’-AGGGNGGTAGACGCGATANTGTACAGCNCTCNGAGCGTGANNGTANTATNCANGG
NTNGNTCGACCANNNCATTCACTACGGTCATCAGGG-3’

Clone 2A1
5’-AGTGACGCGCATGAATGGATGAACGAGATTCCCACTGTCCCTACCTACTATCTAGC
GAAACCACAGCCAAGGGAACGGGCTTGGCGGAATCAGCGGGGAAAGAAGACCCTGT
TGAGCTTGACTCTAGTCTGCAACTGTGAAGAGACATGAGAGGTGTAGGATAAGTGGG
AGGCCCCCGCGCTCGTCGC-3’

Clone 2B8
5’-TAAAGCCTTTGTCATCCATAACACCGATATCACCTGTTTTAAAATAGCCTGTCTTACT
AAAAGCTTTGAGCGTTTCAGTAGGTGCATTGTGGTAGCCTTTCATGACTTGAGGGCCT
TTGACGGCAA-3’

Clone 2C2
5’-GGTCGGTGAGGCTTTCTACAGATTTTAAGGCTTGATACAGACGCGTGAGGCTGGTT
TGGGCTTC-3’

Clone 3A2
5’-ATTTTGCATATCGGTGATCATTTACAAGATCTGGTCAACAACTACGGCAATTGGATT
TATGCGATTTTGTTTGCCATTGTGTTCTGCGAGACCGGTCTGGTGGTGTTGCCATT-3’

Clone 3A6
5’-TTCCCGCAGCCGACCTGATTGCCGGCTACCGCCAGGTGATCGCGCGCGCCCACG
CGCACGGCATCACCGTGATCGGCGCCACCTTGCCGCCGATGGAAGGCTTCGTCTACT
ACACGGCGGCGCGCGAAGCGGTGCCGCGAGCGGCAAACAGCTGGATCCGC-3’

Clone 3A10
5’-GGTGGTCGACAGCAGCAAAAAAACAATCAAAAACAGCAAGCAATCAATCATTGGGG
TAAGATTGATTTCTAATGGTTCGACCGTGGGTTTACGAAATCGCA-3’

Clone 3A15
5’-AGGGTGGTTGGATTGATACCTGTTTGGAAATCTAAGTTAACAGATTTTGGGGGGGN
GATAGTAATTTCATTCGTGCCGACACCCGCTGCACCAAGTGCAGGAAGATTTTCCGTC
ATAGCGACATCNTGGTCGATTTCAGAGCGATAGGTTAACGCGGCTTTGAGGGCAATTT
CAGGTTTTAAATAAGCAATC-3’

Clone 3B1
5’-AAAGTGATTGAGCTATGGGCACAGACTGGTGCAGAAGTGGTAACCATGCCAGCGG
ATTATCATGATAAGGTGCTCGCGCGTACCAGTCATTTACCCCACTTGCTAGCCTACAA
TCTAGTTGCTCAACTAGCCAAGCACAACGACAATATGGATATTTTTCGTTTCGCAGCG
GGTGGCTTTCGGGATTTTACCCGTATCGCCGCCAGCGACCCGACCATGTGGCATGAT
ATTTTTTATGCCAATAAAACCGCGCTCCTCGATGCCATTGATGAA-3’

Clone 3B4
5’-CCATCCATGACTTCAATCATCCAGTGGGCATAATGTTCCCAATCGGTGGCAGCATG
GAATATGCCGCCTTTTTTAAGCACCCGTTCCACCGCCCGCATGCGATCGGNNCTGAC
AAAACGGCGNTTGNNATGGNGCTTTTTTTG-3’

Clone 3B6
5’-TGCTTTATCACGGTGATGTAATTCTTCTTTATCCATTTGGCTTAAAATCTCAGCACTG
CGTTTATCTTGTGCAGGCAGCTTACCAAGATGGCTGTGCAAATGCTCGCTGACTTGGG
TTTCTGTCTCTGCGACAAACCCCAGACTAAACTCATCAGAAATCAATCCTGCTACCGC
ACCCATGCCAATCGATAGCCCATACCAAAGCGGACTAAAAATACTGGGATGGCTACCC
AATTCA-3’

Clone 3B7
5’-TATGATACGCCACCGTTTTCATGCAGTTAGCCAAAAAACGCATCGTAGTTTTGATGG
GTAGTGGCAATGGGCTGGCGCCTGATTCGACTGCTTTATCACGGTGATGTAATTCTTC
TTTATCCATTTGGCTTAAAATCTCAGCACTGCGTTTATCTTGTGCAGGCAGCTTACCAA
NATGGCTGTGCAAATGCTCGCTGACTTGGGTTTCTGTCTCTGCGACAAACCCCANACT
AAACTCATCACAAATCAATCCTGCTACCGCACCCATGCCAAACNATANNTTTTATTTNA
GCGGACTAAAAATNNTGGGATGGCTACCCCATTCA-3’

Clone 3B9
5’-TGAATTGGGTAGCCATCCCAGTATTTTTAGTCCCCTTTNNTATGGGCTATGGGNTGN
CATGNATGCNGTAGCAGGATTGATTTCTGATGAGTTTAGTCTGGGGTTTGTCGCANAG
ACAGAAACCCAAGTCAGCGAGCATTTGCACAGCCATCTTGGTAAGCTGCCTGCACAA
GATATACGCAGTGCTGAGATTTTAAGCCAAATGGATAAANAAAAATTACAT-3’

Clone 3B10
5’-GCATGTAAATAATCGACTAGTAAAGTTACATAATGTTGGTCTGCATCCATCAAATAAT
CACCTAAACGATTCCTAATTGCATCAGCTAAGTCAAAGTGACTAATAAATTTAAAATGC
TCTGGTAATGTTTCAGGTTTATGAATACCTAAAATAATATAATGCCCGTTTCTCTCTCCT
CGCCTTAACTTGCCATAATTCACATAAC-3’

Clone 3B11
5’-CCAAAAAGTCAGAAGGATCGTGAGGCCCCGCTTTCACGGTCTGTATTCATACCGAA
AATCAAGATCAAGCGAGCTTTTCCCCCCGGAACCCAAAGACTT-3’

Clone 3B12
5’-ANATGTGTGGTTTGGTCAATTCTCGCCCGCTGGGTCGGTGAGGCTTTNTACATTAT
CTTNAAGGCTTGATGCATGACNCNTGANGCTGGTTNGGGCTTC-3’

Clone 3B14
5’-TTTAGCTTTACAAGCAATTACACAAACCCTTTAACTGCTGAGCTGCTACTGAATCTGT
AGAACAAAGAGCAAACAAACCATCCCATGTTTATTTAACTAATCACAGAATGCAAGGAT
TAATATTTGTTACATGTTTACCCCACTTTTGGAGTCAATTGTCCCAATCATTGCCAGAG
GTTATGCTAATGTGAAAATATGCAGAGTTAGGCAGCCTTTGGTGACCCTGCTATTGCA
GAAGTAGCGCTCTCATCTAGAGGCAATACTGGAAGCTGTAGCTTTGCTTTAGAGGCC
C-3’

Clone 3B15
5’-AGTCAAGCCAAACGAGCAATTAGTATTGGTTAGCTACACATATCACTATGCTCCCAC
ACCCAACCTATCAACGTCGTAGTCTACAACGGCTCTTTAGGGAAATCTAATCTTAAGGT
GGGCTTCCCGCTTAGATGCTTTCAGCGGTTATCCCATCCGAACATAGCTACTCGGCAA
TGCGACTGGCGTCACAACCGAAACACCAGAGGTTCGTCCACTCTGGTCCTCTCGTAC
TAGGAGCAGATCCTCT-3’

Clone 3B18
5’-ACCAGCCAAAATATGACTGTCAGCAAAGATTGGTTAAAAATAACGGTAAAAGATACC
GGTTTAGGTATTAGCGAAAAAGACCAACAAAAATTATTTTCCTACTTTGAACAAGCCAA
TGATTCCATTAGTCGGCAGTTTGGCGGTACAGGACTTGGTTTAGCTATTTCCAATAGTT
TTTCACATTTATTGGGTGGCTTTATTCATTTAGAAAGTCAATTTGGTCAAGGCAGTGAA
TTCCAACTAT-3’

Clone 3B19
5’-CTTTTAGCAGGCTTGAATAATAGTTTAGACATAAGCTATTTCATTGGCTGCGNGNNT
CATCAAAATAGTGATAATTTTTAGGAATGCTATGTTTCGACCGTCGGCTTTGTTTTTAG
GATTACGGTATACCCGTGCTGAGCGAAGTAACCGATTTATTTCTTTTATTTCTTTGATTT
CAATGGTGGGTTTGACGCTTGGCGTTGCCNCGCTCATTACCCTGNTATCGGTGATGA
ATGGC-3’

Clone 3B25
5’-GGGCCATTAAAGCAAAGCTACAGCTTCCAGTATTGCCTCTAGATGAGAGCGCTACT
TCTGCAATAGCAGGGTCACCAAAGGCTGCCTAACTCTGCATATTTTCACATTAGCATAA
CCTCTGGCAATGATTGGGACAATTGACTCCAAAAGTGGGGTAAACATGTAACAAATAT
TAATCCTTGCATTCTGTGATTAGTTAAATAAACATGGGATGGTTTGTTTGCTCTTTGTTC
TACAGATTCAGTAGCAGCTCAGCAGTTAAAGGGCTTGTGTAATTGCTTGTTNAGCTAG
A-3’

Clone 3B27
5’-CTGCTCGCACCATCCCCACCATTTTGAGTAACATATATGCCACCTTGTCCACGAAGT
ACATCTATCGCGC-3’

Clone 3B28
5’-ATTAAGTGGTAGGGGAGCATTGTGTAAGCCTGTGAAGGTGTGTTGTAAAGCATGCT
GGAGGTATCACAAGAGCGAATGCTGACGTGAGTAACGACAAAACGGGTGAAAAGCCC
GTTCGCCGGAAGACCAAGGGTTCCAGTCCAACGTTAATCGGGGCTGGGTGAGTCGAC
CCCTAAGGCGAGGCCGAAAGGCGTAGTCGATGGGAAATCGGTTAATATTCCGATACT
TG-3’

Clone 3C2
5’-ATAGCTGGTTCTCCCCGAAAGCTATTTAGGTAGCGCCTCGGACGAACACCATTGGG
GGTAGAGCACTGTTTCGGCTAGGGGGTCATCTCGACTTACCAAACCGATGCAAACTC
CGAATACCGATGAGTGATATCCGGGAGACAGACGGCGGGTGCTAACGTCCGTCGTCA
AGAGGGAAACAACCCAGACCGCCAGCTAAGGCCCCAAATTCCTAGTTAAGTGGGAAA
CGATGTGGGAAGGCACAGACAGCTAGGAGGTTGGCTTAGAAGCAGCCATCCTTTAAA
GAAAGCGTAATAGCTCACTAGTCGAGTCGGCCTGCGCGGAAGATGTAACGGGGCTCA
AACTAGGAGCCGAAGCTGCGGATTTAAT-3’

Clone 3C3
5’-CTAGCTTTACAAGCAATTACACAAACCCTTTAACTGCTGAGCTGCTACTGAATCTGT
AGAACAAAGAGCAAACAAACCATCCCATGTTTATTTTACTAGTCACAGAATGCAAGGAT
TAATATTTGTTACATGTTTACCCCACTTTTGGAGCCAATTGTCCCAATCATTGCCAGAG
GTTATGCTAATGTGAAAATATGCAGAGTTAGGCAGCCTTTGGTGACCCTGCTATTGCA
GAAGTAGCGCTCTCATCTAGAGGCAATACTGGAAGCTGTAGCCTTGCTTTANATGGCC
C-3’

Clone 3C9
5’-TATCTTCAGGGTCAGTCAGCGTATCCGCTGCTTTTTCAGGGGGGATGAGGTCATTT
TTCCAATCCGAAATCATTTTGATAGC-3’

Clone 3C11
5’-AGGATGATCAGTCACACCGGAACTGAGACACGGTCCGGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGGACAATGGGGGCAGCCCTGATCCAGCCATGCCGCGTGTGTG
AAGAATGCCTTTTGGTTGTAAAGCAC-3’

Clone 3C12
5’-TGAATTGGGTAGCCATCCCAGTATTTTTAGTCCGCTTTGGTATGGGCTATCGTTTGG
CATGGGTGCGGTAGCAGGATTGATTTCTGATGAGTTTAGTCTGGGGTTTGTCGCAGA
GACAGAAACCCAAGTCAGCGAGCATTTGCACAGCCATCTTGGTAAGCTGCCTGCACA
AGATAAACGCAGTGCTGAGATTTTAAGCCAAATGGATAAAGAAGAATTACATCACCGT
GATAAAGCAGTCGAATCAGGCGCCAGCCCATTGCCACTACCCATCAAAACTACGATG
CGTTTTTTGGCTAACTGCATGAAAACGGTGGCGTATCATA-3’

Clone 3C13
5’-ATTAAATCCGCAGCTTCGGCTCCTAGTTTGAGCCCCGTTACACCTTCCGCGCAGGC
CGACTCGGCTAGTGAGCTATTACGCTTTCTTTAAAGGATGGCTGCTTCTAAGCCAACC
TCCTAGCTGTCTGTGCCTTCCCACATCGTTTCCCACTTAACTAGGAATTTGGGGCCTT
AGCTGGCGGTCTGGGTTGTTTCCCTCTTGACGACGGACGTTAGCACCCGCCGTCTGT
CTCCCGGATATCACTCATCGGTATTCGGAGTTTGCATCGGTTTGGTAAGTCGGGATGA
CCCCCTAGCCGAAACAGTGCTCTACCCCCAATGGTGTTCGTCCGAGGCGCTACCTAA
ATA-3’

Clone 3C16
5’-TAGGNGCCCCCATACCNATGTCAGCCTGAGTGGCGACGCAGCTCCACTTAGACCG
CGAGGACNTGGCGATGGNGCATGCCCACNCCCTATACGATTTCTATCTGGACATGTT
GGGGGACGGGGATTCCCCGGGGCCNGGATTTACCCCCCACGACTCCGCCCCCTACT
GCGCTCTGGATACGGCCNACTTCAAGTTTGAGCAGATGTTTACCGATGCCCTTANAAT
TGACGAGTACGGTGGGCCCAATTCNAATCCCGCGGCCATGGCNGCCGGGAGCATGC
NACNTCNGGCCCAATTCNCCCTATAGTGA-3’

Clone 3C17
5’-GACGCTTAGTTAAGATTCTCAGTTAAATATGATACGCCACCGTTTTCATGCAGTTAG
CCAAAAAACGCATCGTAGTTTTGATGGGTAGTGGCAATGGGCTGGCGCCTGATTCGA
CTGCTTTATCACGGTGATGTAATTCTTCTTTATCCATTTGGCTTAAAATCTCAGCACTG
CGTTTATCTTGTGCAGGCAGCTTACCAAGATGGCTGTGCAAATGCTCGCTGACTTGGG
TTTCTGTCTCTGCGACAAACCCCAGACTAAACTCATCAGAAATCAATCCTGCTACCGC
ACCCATGCCAAACGATAGCCCATACCAAAGCGGACTAAAAATACTGGGATGGCTACC
CAATTCA-3’

Clone 3C19
5’-CGAAGGGAAGACCTCTCCCGCGGAAATCGTCGCCAACACCGACAGCCATGCGGCC
AGCATCGTCAACGTCACGGTAGGGGGCGGGTCGACAAAAGTCCGGGTCGCCGACTT
CGCCGCCATCGACGCAGGACGAGCCCATGGCAGGTTCGAGCCAGAAGCTGCCATGA
GCGTCGGTGCCGCACGGCTACCAGCTGTCAGCCACCCCCGCGAGATCCTGCCTGGC
CACCAAGCACTCGTGGAGGTTCTCCTGACCTCGAGCGTGCCTTCAGCGCACGCCAAC
GGGTACGACATCACCATCAAGGATGGGCCGTACACGCATGTCGTGCA-3’

Clone 3C21
5’-TGAATTGGGTAGCCATCCCAGTATTTTTAGTCCGCTTTGGTATGGGCTATCGTTTGG
CATGGGTGCGGTAGCAGGATTGATTTCTGATGAGTTTAGTCTGGGGTTTGTCGCAGA
GACAGAAACCCAAGTCAGCGAGCATTTGCACAGCCATCTTGGTAAGCTGCCTGCACA
AGATAAACGCAGTGCTGAGATTTTAAGCCAAATGGATAAAGAAGAATTACATCACCGT
GATAAAGCAGTCGAATCAGGCGCCAGCCCATTGCCACTACCCATCAAAACTACGATG
CGTTTTTTGGCTAACTGCATGGAAACGGTGGCGTATCATA-3’

Clone 3D4
5’-GCTATCAAAATGATTTCGGATTGGAAAAATGACCTCATCCCCCCTGAAAAAGCAGC
GGATACGCTGACTGACCCTGAAG-3’

Clone 4A1
5’-TATGATACGCCACCGTTTTCATGCAGTTAGCCAAAAAACGCANANNAGTTNCGATG
GGTAGTGGCAATGGGCTGGCGCCTGATTCGACTGCTTTATCACGGTGATGTAATTCTT
CTTTATCCATTTGGCTTAAAATCTCAGCACTGCGTTTATCTTGTGCAGGCAGCTTACCA
AGATGGCTGTGCAAATGCTCGCTGACTTGGGTTTCTGTCTCTGCGACAAACCCCAGAC
TAAACTCATCAGAAATCAATCCTGCTACCGCACCCATGCCAAACGATANNTTTTTTTTN
AGCGGACTAAAAATACTGGGATGGCTACCCAATTCA-3’

Clone 4A6
5’-ATAGCTGGTTCTCCCCGAAAGCTATTTAGGTAGCGCCTCGGAAAACACCACTGGGG
GTAGAGCACTGTTTCGGCTAGGGGGTCATCCCGACTTACCAAACCGATGCAAACTCC
GAATACCGATGAGTTATATCCGGGAGACAGACGGCGGGTGCTAACGTCCGTCGTCAA
GAGGGAAACAACCCAGACCGCCAGCTAAGGCCCCAAATTCCTAGTTAAGTGGGAAAC
GATGTGGGAAGGCACAGACAGCTAGGAGGTTGGCTTAGAAGCAGCCATCCTTTTTTTT
TTNGTAANAGCTCACTANTCTAGTCNGCCTGCGCGGAAGATGTAACGGGGCTCAAAC
TANGAGCCCAAGCTGCNGNATTAAAT-3’

Clone 4A10
5’-ACTAGCTTTACAAGCAATTACACAAACCCTTTAACTGCTGAGCTGCTACTGAATCTG
TAGAACAAAGAGCAAACAAACCATCCCATGTTTATTTAACTAATCACAGAATGCGAGGA
TTAATATTTGTTACATGTTTACCCCACTTTTGGAGTCAATTGTCCCAATCATTGCCAGA
GGTTATGCTAATGTGAAAATATGCAGAGTTAGGCAGCCTTTGGTGACCCTGCTATTGC
AGAAGTAGCGCTCTCATCTAGAGGCAATACTGGAAGCTGTAGCTTTGCTTTAGATGGC
CC-3’

Clone 4A11
5’-TATGATACGCCACCGTTTTCATGCAGTTAGCCAAAAAACGCATCGTAGTTTTGATGG
GTAGTGGCAATGGGCTGGCGCCTGATTCGACTGCTTTATCACGGTGATGTAATTCTTC
TTTATCCATTTGGCTTAAAATCTCAGCACTGCGTTTATCTTGTGCAGGCAGCTTACCAA
GATGGCTGTGCAAATGCTCGCTGACTTGGGTTTCTGTCTCTGCGACAAACCCCAGACT
AAACTCATCAGAAATCAATCCTGCTACCGCACCCATGCCAAACGATAGCCCATACCAA
AGCGGACTAAAAATACTGGGATGGCTACCCAATTCA-3’

Clone 4A12
5’-GACGCTTAGTTAAAGATTCTCAGTTAAATATGATACGCCACCGTTTTCATGCAGTTA
GCCAAAAAACGCATCGTAGTTTTGATGGGTAGTGGCAATGGGCTGGCGCCTGATTCG
ACTGCTTTATCACGGTGATGTAATTCTTCTTTATCCATTTGGCTTAAAATCTCAGCACTG
CGTTTATCTTGTGCAGGCAGCTTACCAAGATGGCTGTGCAAATGCTCGCTGACTTGGG
TTTCTCTCTCTGCGACAAACCCCAGACTAAACTCATCAGAAATCAATCCTGCTCCCGC
ACCCATGCCAAACGATAGCCCATACCAAAGCGGACTAAAATACTGGGATGGCTACCC
AATTCA-3’

Clone 4A15
5’-TGAATTGGGTAGCCATCCCAGTATTTTTAGTCCGCTTTGGTATGGGCTATCGTTTGG
CATGGGTGCGGTAGCAGGATTGATTTCTGATGAGTTTAGTCTGGGGTTTGTCGCAGA
GACAGAAACCCAAGTCAGCGAGCATTTGCACAGCCATCTTGGTAAGCTGCCTGCACA
AGATAAACGCAGTGCTGAGATTTTAAGCCAAATGGATAAAGAAGAATTACATCACCGT
GATAAAGCAGTCGAATCAGGCGCCAGCCCATTGCCACTACCCATCAAAACTACGATG
CGTTTTTTGGCTAACTGCATGAAAACGGTGGCGTATCATA-3’

Clone 4A16
5’-TACAGCTTCCAGTATTGCCTCTAGCATGAGAGCGCTACTTCTGCAATAGCAGGGTC
ACCAAAGGCTGCCTAACTCTGCATATTTTCACATTAGCATAACCTCTGGCAATGATTGG
GACAATTGACTCCAAAAGTGGGGTAAACATGTAACAAATATTAATCCTTGCATTCTGTG
ATTAGTTAAATAAACATGGGATGGTTTGTTTGCTCTTTGTTCTACAGATTCAGTAGCAG
CTCAGCAGTTAAAGGGTTTGTGTAATTGCTTGTAAAGCTAGT-3’

Clone 4A17
5’-TGAATTGGGTAGCCATCCCAGTATTTTTAGTCCGCTTTGGTGTGGGCTATCGTTTGG
CATGGGTGCGGTAGCAGGATTGATTTCTGATGAGTTTAGTCTGGGGTTTGTCGCAGA
GACAGAAACCCAAGTCAGCGAGCATTTGCACAGCCATCTTGGTAAGCTGCCTGCACA
AGATAAACGCAGTGCTGAGATTTTAAGCCAAATGGATAAAGAAGAATTACATCACCGT
GATAAGGCAGTCGAATCAGGCGCCAGCCCATTGCCACTGCCCATCAAAACTACGATG
CGTTTTTTGGCTAACTGCATGAAAACGGTGGCGTATCATA-3’

Clone 4A18
5’-TGAATTGGGTAGCCATCCCAGTATTTTTAGTCCGCTTTGGTATGGGCTATCGTTTGG
CATGGGTGCGGTAGCAGGATTGATTTCTGATGAGTTTGGTCTGGGGTTTGTCGCAGA
GACAGAAACCCAGTCAGCGAGCATTTGCACAGCCATCTTGGTAAGCTGCCTGCACAA
GATAAACGCAGTGCTGAGATTTTAAGCCAAATGGATAAAGAAGAATTACATCACCGTG
ATAAAGCAGTCGAATCAGGCGCCAGCCCATTGCCACTATCCATCAAAACTACGATGCG
TTTTTTGGCTAACTGCATGAAAACGGTGGCGTATCATA-3’

Clone 4A20
5’-TATGATACGCCACCGTTTTCATGCAGTTAGCCAAAAAACGCATCGTAGTTTTGATGG
GTAGTGGCAATGGGCTGGCGCCTGATTCGACTGCTTTATCACGGTGATGTAATTCTTC
TTTATCCATTTGGCTTAAAATCTCAGCACTGCGTTTATCTTGTGCAGGCAGCTTACCAA
GATGGCTGTGCGAATGCTCGCTGACTTGGGTTTCTGCCTCTGCGACAAACCCCAGAC
TAAACTCATCAGAAATCAATCCTGCTACCGCACCCATGCCAAACGGTAGCCCATACCA
AAGCGGACTAAAAATACTGGGATGGCTACCCAATTCA-3’

Clone 4A21
5’-CATGGCACATTTATGCCCTTGTAGTCGCTGGGCTTTTAATTATTTAGTGTTATTTCCC
CGTATTCCAAAAATTGGCTCGCTGATTCCCTCGCCGCTCGTGACTATTGTGAC-3’

Clone 4A22
5’-TGAATTGGGTAGCCATCCCAGTATTTTTAGTCCGCTTTGGTATGGGCTATCGTTTGG
CATGGGTGCGGTAGCAGGATTGATTTCTGATGAGTTTAGTCTGGGGTTTGTCGCAGA
GACAGAAACCCAAGTCAGCGAGCATTTGCACAGCCATCTTGGTAAGCTGCCTGCACA
AGATAAACGCAGTGCTGAGATTTTAAGCCAAATGGATAAAGAAGAATTACATCACCGT
GATAAAGCAGTCGAATCAGGCGCCAGCCCATTGCCACTACCCATCAAAACTACGATG
CGTTTTTTGGCTAACTGCATGAAAACGGTGGCGTATCATA-3’

Clone 4A26
5’-ACTAGCTTTACAAGCAATTACACAAACCCTTTAACTGCTGAGCTGCTACTGAATCTG
TAGAACAAAGAGCAAACAAACCATCCCATGTTTATTTAACTAATCACAGAATGCAAGGA
TTAATATTTGTTACATGTTTACCCCACTTTTGGAGTCAATTGTCCCAATCATTGCCAGA
GGTTATGCTAATGTGAAAATATGCAGAGTTAGGCAGCCTTTGGTGACCCTGCTATTGC
AGAAGTAGCGCTCTCATCTAGTGGCAATACTGGAAGCTGTAGCTTTGCTTTAGATGGC
CC-3’

Clone 4A27
5’-TGAATTAGGTAGCCATCCCAGTATTTTTAGTCCGCTTTGGTATGGGCTATCGTTTGG
CATGGGTGCGGTAGCAGGATTGATTTCTGATGAGTTTAGTCTGGGGTTTGTCGCAGA
GACAGAAACCCAAGTCAGCGAGCATTTGCACAGCCATCTTGGTAAGCTGCCTGCACA
AGATAAACGCAGTGCTGAGATTTTAAGCCAAATGGATAAAGAAGAATTACATCACCGT
GATAAAGCAGTCGAATCAGGCGCCAGCCCATTGCCACTACCCATCAAAACTACGATG
CGTTTTTTGGCTAACTGCATGAAAACGGTGGCGTATCATA-3’

Clone 4A28
5’-AGTGCTTGACCAAGTTATTAGTAAGATATAAATACCGATAAATAACGGAATAAGCAC
TGAATGGATAAAGCGTACTAACACGCCAAATACCAACAGTCCCGTCAATGAGCCCGCC
AAAAATTGCCAAAAATAGG-3’

Clone 4B1
5’-TCCAAATTTGGTCAATAAAAGCTTGTTGGTATTTTTGGAGATAAAGCTATCTACCAAG
TCGGGCGATGTCTTTGAGCAAGTGAGCAAAAAACTGTCAGAAATTCCAGAGATA-3’

Clone 4B5
5’-TAATAGTGCGTCCTTTCTTGTGTTGTTCATACTTGACTGTGATGTCGGTGTGTTCGT
TGATTTC-3’

Clone 4C5
5’-GCCTGAGCAAGCGTCTGCTATTATTAATGAAGCCTATAATACGNTTAAAAATCCCGA
TTCTCGCGCGTCACATTTATTGGCACTCAAAGGCATTTCACAATC-3’

Clone 4C7
5’-CAAATACAGGGCGATTTGCCATTTTAAATTACCTTTTCATAAAAACTTCTGGTTTACA
GTGACGTTATAACCAAACTC-3’

Clone 4D2
5’-GGGTTTTGGCAAATAATCATCCGCGCCTGCTTCAAGTCCTGCGATACGATCGGCAT
CGGAACCTTTAGCGGTCAACATAATAATCGGAATATCGGAGTTT-3’

Clone 5A8
5’-GCAGCACAAGCAAATGGCAAAAATGATGAAAATGTTATCTGACCCATCGGGCATCA
GCAAAATGATGAAAGCGGTGCAAGGGTTGACCAAAGGCATGGGCGGTGGCGGTGGT
CCATTATTTGGACAAAACAACCAAGCAGGCGCAAATACCACGACAGTTAACGGCCAAG
CCAATCCAGTTGCGCCAAAATTTAAAA-3’

Clone 5A13
5’-CATAGTGATAATTTTTAGGAATGCTATGTTTCGACCGTTGGCTTTGTTTTTAGGATTA
CGGTATACCCGTGCTGAGCGAAGTAACNGATTTATTTCTTTTATTTCTTTGATTTCAAT
GGTGGGTTTGACGCTTGGCGTTGCCGTGCTCATTACCGTGTTATCGGTGATGAATGG
C-3’

Clone 5A16
5’-GAGCGACCCTCAGACAGGCGTGGCCCCGGGAGGAACCCGGGGCCGNGAAGGTG
CGTTCGAAGTGTCGATGATCAATGTG-3’

Clone 5A20
5’-GGGCCATTAAAGCAAAGCTATAGCTTCCAGTATTGCCTCTAGATGAGAGCGCTACT
TCTGCAATAGCAGGGTCACCAAAGGCTGCCTAACTCTGCATATTTTCACATTAGCATAA
CCTCTGGCAATGATTGGGACAATTGACTCCAAAAGTGGGGTAAACATGTAACAAATAT
TAATCCTTGCATTCTGTGATTAGTTAAATAAACATGGGATGGTTTGTTTGCTCTTTGTTC
TACAGATTCAGTAGCAGCTCAGCAGTTAAAGGGTTTGTGTAATTGCTCGTAAAGCTAG
C-3’

Clone 5A23
5’-GGTAGCGATAAATCTTGATAAATCAGCTCAAAACGGCGATGGGTCGGCGTTTGTTC
ACGGCGGGCTTGTACCATACGCCAAAGCTGTAGTAGATAATAAATATCTTCTTCTAGC
TTGGATTGCGGGATATTTTCGGCAGCGGTACGGGCAATCAATCCGCCTTGTAGATTGA
CTTGGTGCATCAGCTCGCCAAGCTGTGTT-3’

Clone 5A25
5’-GAAAACCACACAAGAATAGAACCGGAGTCCTATTCACATTATTTCTCTAACTAGAAG
TATACCACGGTCGACGGCGCGCCTGCTTGNAACACTCTAATTTTTTCAAAGNNAACGC
TTCGCAGCCCCCGGGACACTNAGTCAAGAGCATCGGGGAGGCGCCGAGAGGCAGG
GGCTGGGACAGGCGGTANCTCGCTCTCGCGGCGGACCGNCAGCTCGATCCCTNGAT
ACAACTACNAGCTT-3’

Clone 5C2
5’-GATTGGATTTGCGGGGAATGGCGCCGAGCGCTTGTACGCCGANTGGCATTTTGCC
CATATCATCCACATCTCGCACACAGCCATACACAATCACGCTAGCCCAGCCATTATCA
ACCGCACTTTGTGCAATCATATCACCCAGCAAAGCACAGCGCATTGACGCCCCACCG
TCTACCACCAAAACCTTGCCATTGCCTTTCTCATCTTTGCCATTCGTGGCTAACAGCT
C-3’

Clone 5D2
5’-TGCTNTGGAANTTCGTGACAGTNNTTTCGCTGACTTGCTACAAAGACATCGCCCGA
CTTGGATAGATAGCTTTATACATCCAACAAATACACAACAAGCTTNTATATGACCAAAT
TTGGA-3’

Clone 5D3
5’-CAAGTATCGGAATATTAACCGATTTCCCATCGACTACGCCTTTCGGCCTCGCCTTAG
GGGTCGACTCACCCAGCCCCGATTAACGTTGGACTGGAACCCTTGGT-3’

Clone 6B1
5’-GGGAAACAACCCAGTACCGCCAGCCTAANGCCCCAAAGTTCNNAGAAAAAGATGG
GAAACAGAGTCGNGGGAAGGCACAGAACATGTCGTACGTGTAGGATTGGCNNATAAG
CANCCNNCCNTTAAAGAAAGCGNAATAGCNNACTAGCCCGAGTCGGCCAGNNCGNAN
GANGTAACGGGGCTCNAACTAGGAGCCGAAGCTGCNGATTCAATT-3’

Clone 6B7
5’-CCTGCACTAAAGGGAAGACCGCTTCTTTGACACAGCGATAAAAATGTTGGAATCAC
CTCANTGGTAAAGTTTTAGTGTCCACAAGCGTTGTGCCAGACCATACAACGCGCAGCG
ACTTGTGCAAGTGAAGTGCCGATGCATTTTGATNCAAAAGGCACCGTACGTGAGGCA
CGCGGATTGACTTCAAGAATATAAACCACACCATC-3’

Clone 6B8
5’-GGGCCATCTAAAGCAAAGCTACAGCTTCCAGTACTGCCTCTAGATGAGAGCGCTAC
TTCTGCAATAGCAGGGTCACCAAAGGCTGCCTAACTCTGCATATTTTCACATTAGCATA
ACCTCTGGCAATGATTGGGACAATTGACTCCAAAAGTGGGGTGAACATGTAACAAATA
TTAATCCTTGCATTATGTGATTAGTTAAATAAACATGGGATGGTTTGTTTGCTCTTTGTT
CTACAGATTCAGTAGCAGCTCAGCAGTTAAAGGGTTTGTGTAACTGCTTGTTNNGCTA
G-3’

Clone 6C2a
5’-GAGCTGACGATGACCTACTCTCACATGGGCGAACCACACTACCATTGGCGCATTGG
AGTTTCACTTCTGAGTTCGGGAAGGGATCAGGTGGGACCTCCAAGCTATTATCGTCAG
CA-3’

Clone 6C2b
5’-TTTGACAGTAATTGTGCTACATCGAGACAAGCCGCTTGGGTTAATGGTGCGGTTTT
GATGATGGTTGGACAAAAATCACGATTACCCGCCAAATTGTCATTTACTCGCCATCGG
CTG-3’

Clone 6C3
5’-GTGTAAATCTCGCGCCGGGCCGTACCCATATCCGCAGCAGGTANTCAAAGGNGAA
CAGCCTCTGGCATGTTAGAACAATGTAGGTAAGGGAAGTCGGCAAGTCAGATCCGTA
ACTTCGGGATAAGGATTGGCTCTAAGGGCTGGGTCGGTCGGGCTGGGGCGCGAAGC
GGGGC-3’

Clone 6C4
5’-AATCACAGTAGGATTTGCGCACCTTTTTNAGCGTTATTACCTNNAAGGCGAACAACC
ACAGGCACGGTGACGTTGACTTC-3’

Clone 6C5
5’-ATTCAAACGAGAACTTTGAAGGCCGAAGTGGAGAAGGGTTCCATGTGAACAGCAGT
TGAAAC-3’

Clone 6D2
5’-NGNTAAAANTTTGGNTANCAATAAAGATATCGCCNTGATAGGCACAAAAACCCAAAA
AAATTTGCAATAGGGNGGATTTGCCGCTGCCAC-3’

Clone 6D8a
5’-CACCTTGACGGATTTCCATC-3’

Clone 6D8b
5’-TGCCACAGGCTCAAGATAATCTTTAGCTTTTTGACAATCCCATACGCAGGTGCACAG
TCGCATCAAATCGCCATCGCC-3’

Clone 7A2
5’-AGTGGGAAACGATGTGGGAAGGCACAGACAGCTAGGAGGTTGGCTTAGAAGCAGC
CATCCTTTAAAGAAAGCGTAATAGCTCACTAGTCGAGTCGGCCTGCGCGGAAGATGTA
ACGGGGCTCAAACTAGGAGCCGAAGCTGCGGA-3’

Clone 7A5
5’-CAGAGTACCGCTGTCTATGTGAACCCATGAACTGTAACGTTNANGGTGGGTATGGC
AACCACTCTTAAGGCTTCCTGAATCATCGCAGCGTAGCTGGATGCAGGCTTGCACAAC
AAGTGAAAAGAACGCATACCCTT-3’

Clone 7B2
5’-AATCGGGGCTGGGTGAGTCGACCCCTAAGGCGAGGCCGAAAGAGCGTAGTCGAT
GGGAAATCGGTTAATATTCCGATACTTG-3’

Clone 7B4
5’-TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACNAGCGCAACCCTTT
TCCTTATTTGCCAGCGGGTTAAGCCGGGAAGC-3’

Clone 7C1
5’-AAGTCTTTGGGTTCCGGGGGGAAAAGCTCGCTTGATCTTGAANNCAGTACCGAATA
CAGACCGTGAAAGCGGGGCCTCACGATCCTTCTGACTTTTTGG-3’

Clone 7C3
5’-GGGAAACGATGTGGGAAGGCACAGACAGCTAGGAGGTTGGCAAAGAAGCACCCAT
CCTTTAAAGAAAGCGTAATAGCTCACTAGTCGAGTCGGCCTGCGCGGAAGATGTAAC
GGGGCTCAAACTAGGAGCCGAAGCTGCGGATTTAAT-3’

Clone 7D1
5’-GCTAGCTTTACAAGCAATTACACAAACCCTTTAACTGCAGAGNTGCTACTGAATCTG
TAGAACAAAGAGCAAACAAACCATCCCATGTTTATTTAACTAATCACAGAATGCAAGGA
TTAATATTTGTTACATGTTTACCCCACTTTTGGAGTCAATTGTCCCAATCATTGCCAGA
GGTTATGCTAATGTGAAAATATGCAGAGTTAGGCAGCCTTTGGTGACCCTGCTATTGC
AGAAGTAGCGCTCTCATCTAGAGGCAATACTGGAAGCTGTAGCTT-3’

Clone 7D3
5’-TTCATCAATGGCATCGAGGAGCGCGGTTTCATTGGCATAAAAAATATCATGCCACAT
GGTCGGGTCGCTGGCGGCGATACGGGTAAAATCCCGAAAGCCACCCGCTGCGAAAC
GAAAAATATCCATATTGTCGTTGTGCTTGGCTAGTTGAGCAACTAGATTGTAGGCTAG
CAAGTGGGGTAAATGACTGGTACGCGCGAGCACCTTATCATGATAATCCGCTGGCAT
GGTTACCACTTCTGCACCAGTCTGTGCCCATAGCTCAANCACTTT-3’

Clone 8A4
5’-ATCTAAAGCAAAGCTACAGCTTCCAGTATTGCCTCTAGATGANAGCGCTACTTCTGC
AATAGCAGGGTCACCAAAGGCTGCCTAACTCTGCATATTTTCACATTAGCATAACCTCT
GGCAATGATTGGGACAATTGACTCCAAAAGTGGGGTAAACATGTAACAAATATTAATC
CTTGCATTCTGTGATTAGTTAAATAAACATGGGATGGTTTGTTTGCTCTTCGTTCTACA
GATTCAGTAGCAGCTCAGCAGTTAAAGGGTTTGTGTAATTGCTTGTTAAGCTAGG-3’

Clone 8B1
5’-GAAGAATTACATCACCGTGATAAAGCAGTCGAATCAGGCGCNAGCCCATTGCCACT
ACCCATCAAAACTACGATGCGTTTTTTGGCTAACTGCATGAAAACGGTGGCGTATCAT
A-3’

Clone 8B4
5’-AATTAGAATCCGCAGCTTCGGCTCCTAGTTTGAGCCCCGTTANATCTTCCGCGCAG
GCCGACTCGACTAGTGAGCTATTACGCTTTCTTTAAAGGATGGCTGCTTCTAAGCCAA
CCTCCTAGCT-3’

Clone 8C1
5’-GGCACCAATCCACCGCTGCCCGTGCGGCTATTGGACTGCCANCGGTAGGCAAGCA
CGCTGGGGTCAAGTTGTATGGGCTTGGGCAGCTGCGTCTGTAAAAATTGGGTGATTA
AGTGTTCTAAATTAG-3’

Clone 8D1
5’-TGCTGACGATAATAGCTTGGAGGCCCCACCGGANCCCTACCCGAACTCGAACGGG
AAACTCCAACGCGCCAATGGTAGTGTGGTTCGCCCATGTGAGAGCCGGTCATCGTCA
GCTC-3’

Clone 8D3
5’-GGCAGCTTCCTGGCATAATATTGACACTGAGATTCGAAAGCNAGGGNAGCAAACAG
GATTAGATACCCTGGTAGACCACGCCGTAAACGATGTCTACTAGCCGTTGGGGTCCTT
GAGA-3’

Curriculum Vitae

Name:	Rust, Barbara
Date of birth:	25.04.1979
Place of birth:	Duderstadt, Germany
Nationality:	German
Address:	Herzberger Landstraße 11
	37085 Göttingen
	Germany

Education:

24.06.1998	Abitur, Otto-Hahn-Gymnasium, Göttingen, Germany
1998 – 2000	Biology studies, Georg-August University, Göttingen, Germany
19.10.2000	Vordiplom, Georg-August University, Göttingen, Germany
2000 – 2003	Biology studies, Georg-August University, Göttingen, Germany
2003	Diploma thesis, Dept. of Developmental Biochemistry, Georg-August University, Göttingen; PI: Prof. Tomas Pieler
18.12.2003	Diploma (Charakterisierung des Myc-Max-Mad Netzwerkes in der frühen Embryonalentwicklung von *Xenopus laevis*) (Biology)
since 01.2004	PhD thesis, Dept. of Developmental Biochemistry, Georg-August University, Göttingen; PI: Prof. Tomas Pieler

Publications:

Jürgens K., Rust B., Pieler T., Henningfeld K.A. (2005) Isolation and comparative expression analysis of the Myc-regulatory proteins Mad1, Mad3, and Mnt during *Xenopus* development. Dev Dyn. 233 (4): 1554-9.

Klisch T.J., Souopgui J., Jürgens K., Rust B., Pieler T. and Henningfeld K.A. (2006) Mxi1 is essential for neurogenesis in *Xenopus* and acts by bridging the pan-neural and proneural genes. *Dev Biol* 292, 470-85.

Souopgui J., Rust B., Vanhomwegen J., Heasman J., Henningfeld K.A., Bellefroid E., Pieler T. (2008) The RNA-binding protein XSeb4R: a positive regulator of *VegT* mRNA stability and translation that is required for germ layer formation in *Xenopus*. Genes Dev. 1; 22 (17): 2347-52.

Göttingen, 16.09.2008