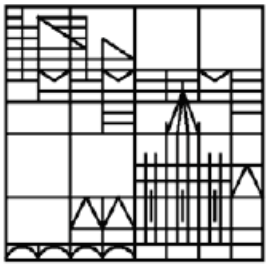


Universität Konstanz

Diploma Thesis

Characterization of gene regulation and neuronal transgene expression of prion protein in zebrafish

Julia M.I. Barth

Konstanz, Germany

April 2007

Supervisors:

Professor Dr. Claudia A.O. Stürmer

Dr. Edward Málaga-Trillo

Dr. Gonzalo Solis

Abstract

The normal function of the prion protein (PrP) is usually neglected because of its fascinating role in neurodegenerative disorders like Creutzfeldt-Jakob disease in human, bovine spongiform encephalopathy (BSE) in cattle and Scrapie in sheep. The cause of transmissible spongiform encephalopathies is the accumulation of an atypical isoform ("prion") of the normal cellular prion protein, a phenomenon until now known only in mammals, and which inevitably leads to death. The prion protein is highly conserved between several vertebrate species, which suggests a common, evolutionary preserved function. Although numerous functions, e.g. signal transduction, cell adhesion and cell recognition, lymphocyte activation and others have been proposed, they do not add up to one common physiological role. However, cellular function and disease likely represent two sides of the same coin and, in order to understand the molecular mechanism of prion diseases, it is essential to dissect also the natural function of PrP. To this aim, the zebrafish (*Danio rerio*) was used in this study as an *in vivo* model. Due to an ancient genome duplication event, zebrafish possess two duplicated prion proteins (PrP-1 und -2), which exhibit an overall similar structure (repetitive domain, hydrophobic stretch and stable globular domain) to the mammalian prion protein. Nevertheless, they show distinctly restricted expression patterns: whereas PrP-1 is expressed ubiquitously and early during zebrafish development, PrP-2 expression has a later peak limited to neuronal structures. Probably the different expression patterns were adopted through a modified regulation. In order to verify this assumption and to generate PrP transgenic zebrafish, the regulatory regions of PrP-1 and -2 were identified in the zebrafish genome, amplified, cloned and functionally characterized. Concordant expression of PrP-1 and -2 in trigeminal ganglion and Rohon-Beard neurons was driven by main, and therefore probably shared regulatory elements. However, finer dissection and testing of the PrP-1 regulatory sequence showed that this sequence is likely to contain a repressor or silencing motif which stops expression during early zebrafish development. This is consistent with the early-restricted expression pattern of endogenous PrP-1.

Furthermore, a method was developed in order to overexpress the prion protein in zebrafish neurons. To this end, specific neuronal enhancers (zCREST), which normally regulate the expression of the *Islet-1* gene, were amplified, cloned upstream of EGFP-tagged prion proteins of several species and overexpressed in zebrafish neurons. Since PrP transgene expression could be observed at 24 hpf but not at 48 hpf it would appear that PrP overexpression is developmentally downregulated. PrP overexpressing neurons like trigeminal ganglion and Rohon-Beard neurons were analyzed using coinjections and immunohistochemistry but no major morphological defects could be identified. Whether the developmental downregulation of PrP involves mechanisms like degradation or apoptosis remains to be clarified.

Kurzfassung

Die normale Funktion des Prionproteins (PrP) findet weniger Beachtung als sein Beitrag zu neurodegenerativen Erkrankungen, wie zum Beispiel der Creutzfeldt-Jakob-Krankheit (CJD) beim Menschen, Bovine Spongiforme Enzephalopathie (BSE) bei Rindern und Scrapie bei Schafen. Ursache dieser übertragbaren Gehirnkrankheiten, welche zwangsläufig zum Tode führen und bisher nur bei Säugetieren bekannt sind, ist vor allem die Akkumulation einer atypisch gefalteten Form („Prion“) des normalen, körpereigenen Prionproteins. Dieses Prionprotein ist bei sämtlichen Wirbeltierklassen hoch konserviert, was auf eine gemeinsame, evolutionär erhaltene Funktion schließen lässt. Funktionen wie Signaltransduktion, Zelladhäsion und Zellerkennung, Lymphozytenaktivierung und andere wurden nachgewiesen, doch eine übereinstimmende physiologische Wirkungsweise konnte bislang nicht identifiziert werden. Zelluläre Funktion und Krankheit sind jedoch eng miteinander verbunden und ein Verständnis der PrP-Funktion ist Vorraussetzung für eine Einsicht in die molekularen Mechanismen der Krankheit. In dieser Arbeit wurde der Zebrabärbling (*Danio rerio*) als *in vivo* Modell eingesetzt. Fische besitzen aufgrund einer evolutionär weit zurückliegenden Genomduplikation zwei Prionproteine (PrP-1 und -2), welche jedoch dieselben charakteristischen Strukturmerkmale (eine Domäne mit repetitivem Motiv, gefolgt von einem hydrophoben Bereich und einer stabilen, globulären Domäne) aufweisen wie das Säugetier-Prionprotein. Allerdings verfügen sie über sehr unterschiedliche Expressionsmuster: Während PrP-1 im Zebrabärbling-Embryo sehr früh und überall exprimiert wird, erreicht PrP-2 sein Expressionsmaximum später und ist auf neuronale Strukturen beschränkt, was auf eine veränderte Genregulation hinweist. Um dies nachzuweisen und um Prionprotein-transgene Zebrabärblinge herstellen zu können, wurden in dieser Arbeit die regulatorischen Bereiche von PrP-1 und -2 im Zebrabärbling-Genom identifiziert, amplifiziert, kloniert und funktionell charakterisiert. Die regulatorischen Elemente von PrP-1 und -2 führten zu einem übereinstimmenden Expressionsmuster beider Gene, welches Expression in trigeminalen Ganglion- und Rohon-Beard-Neuronen aufweist. Allerdings zeigte sich, nachdem der klonierte PrP-1 regulierende Sequenzabschnitt feiner unterteilt und analysiert wurde, dass vermutlich Repressoren zur Abschaltung des Gens während der Zebrabärbling-Entwicklung führen. Dieses Ergebnis würde mit dem frühzeitigen endogenen Expressionsmuster von PrP-1 übereinstimmen.

Des Weiteren wurde eine Methode entwickelt, um Prionproteine in neuronalen Strukturen zu überexprimieren. Hierfür wurden spezifische Enhancer (zCREST), welche normalerweise die Expression des *Islet-1* Gens regulieren, amplifiziert, vor EGFP-fusionierte Prionproteine verschiedener Tierarten kloniert und in Zebrabärbling-Embryos überexprimiert. Da die Expression von PrPs in 24 Stunden alten, jedoch nicht in 48 Stunden alten Zebrabärblingen beobachtet werden konnte, ist es wahrscheinlich,

dass die Überexpression mit der Zeit herunterreguliert wurde. Neuronale Strukturen, wie trigeminale Ganglion- und Rohon-Beard-Neuronen, welche PrP überexprimieren, wurden mittels Koinjektionen und Immunhistochemie auf Veränderungen untersucht, es konnten jedoch keine eindeutigen morphologischen Defekte entdeckt werden. Inwiefern die Herunterregulierung Mechanismen wie Protein Degradation oder Apoptosis involviert, muss noch aufgeklärt werden.

List of contents

List of figures

List of tables

Abbreviations and annotations

°C	Degree centigrade
µg	Microgram, unit of mass
µl	Microliter, unit of volume
3-D	Three dimensional
A	Adenine nucleobase
aa	Amino acid
ac	Anterior commissure
AP-1, -2	Transcription factor Ap-1, -2
APS	Ammonium persulfate
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
C	Cytosine nucleobase
c	Concentration (of a substance in another substance)
$CaCl_2$	Calcium chloride
CCAAT box	Cis-regulatory element with the core DNA sequence 5'-CCAAT-3'
chk	Chicken
CIAP	Calf intestine alkaline phosphatase
cis	Latin: on the same side
CJD	Creutzfeldt-Jakob disease
cm	Centimeter, unit of length
CMV	Cytomegalovirus
CNS	Central nervous system
CO_2	Carbon dioxide
C-terminus	Carboxyl-terminus (-COOH)
d	Diencephalon
dlt	Dorsal longitudinal tract
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides-triphosphate
Dpl	Doppel
dsDNA	Double-stranded Deoxyribonucleic Acid
DsRed	*Discosoma sp.* Red fluorescent protein
DTT	Dithiotreithol
E	Exon
E. coli	*Escherichia coli*
e.g.	Latin: Exempli gratia (for example)
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine-tetraacetic acid
EGFP	Enhanced green fluorescence protein
Et al.	Latin: Et alii (and others)
EtBr	Ethidium bromide
f	Forward

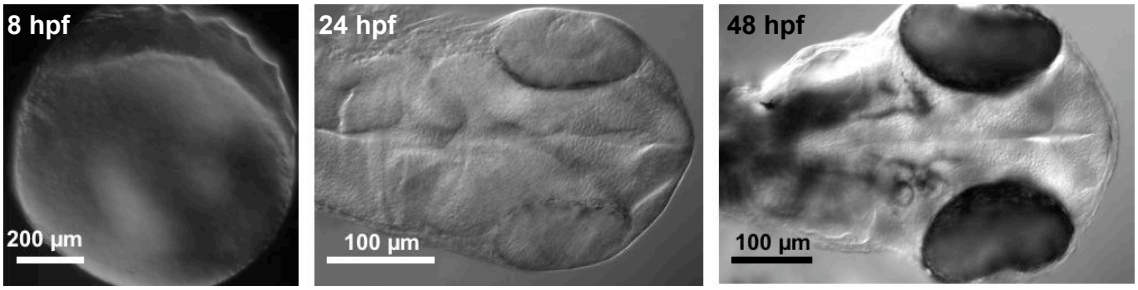
FCS	Fetal calf serum
G	Guanine nucleobase
g	Gravitational acceleration
G/C	Guanine/Cytosine nucleobases
Gb	Giga base pair, unit of length
GFP	Green fluorescence protein
GPI	Glycosylphosphatidylinositol
GSS	Gerstmann-Sträussler-Scheinker disease
h	Hindbrain
H+L	Heavy- and light-chain (immunoglobulin)
H_2O	Water
Ha	Hamster
HCl	Hydrochloric acid
HD	Hydrophobic domain
hPa	Hector pascal, unit of pressure
hpf	Hours post fertilization
hphs	Hours post heat shock
hr	Hour
HRP	Horseradish peroxidase
hs	Heat shock
Hsp	Heat-shock protein
ICP	Islet-1 core promoter
IF	Immunofluorescence
IgG	Immunoglobulin G
In situ	Latin: in the place (where it occurs)
In vivo	Latin: in the living
Isl1	Islet-1
kb	Kilo bases
kbp	Kilo base pairs
KCl	Potassium chloride
kDa	Kilo Dalton
l	Liter, unit of volume
L	Leader
LB	Luria-Bertani-Broth; bacterial media
LIM-HD TF	Lin-11, Isl1, mec-3-homeodomain transcription factor
llg	Lateral line ganglion
LS	Lehrstuhl (chair)
M	Molar mass, unit of one mole of a chemical element
m	Midbrain
ME	2-mercaptoethanol
MEM	Minimal essential medium
mg	Milligram, unit of mass
$MgCl_2$	Magnesium chloride
$MgSo_4$	Magnesium sulfate
Milli-Q water	Sterile deionized water
min	Minute, unit of time
ml	Milliliter, unit of volume
mn	Motor neuron
mo	Mouse
mol	Mole, unit of the amount of substance

mRNA	Messenger Ribonucleic Acid
N2a cell line	Neuro-2a (mouse neuroblastoma) cell line
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate
NaN_3	Sodium azide
nc	Notochord
NCAM	Neuronal cell adhesion molecule
NEB	New England Biolabs
ng	Nanogram, unit of mass
nl	Nanoliter, unit of volume
nm	Neuromeres
NMR	Nuclear magnetic resonance
N-terminus	Amino-terminus ($-NH_2$)
nvCJD	New variant Creutzfeldt-Jakob disease
OD	Optical density
ORF	Open reading frame
P/S	Penicillin/Streptomycin
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline triton X-100
PBS-TD	Phosphate-buffered saline triton X-100 dimethyl sulfoxide
pc	Posterior commissure
PCR	Polymerase chain reaction
pEGFP	Vector with EGFP tag
PFA	Paraformaldehyde
pg	Picogram, unit of mass
pH	= -log [H]
pmol	Picomole, unit of the amount of substance
poc	Postoptic commissure
Prn2	Shadoo2/PrP-rel-1 gene
Prnd	Doppel protein gene
Prnp/Prnprs	Prion protein gene
PrP	Prion protein
$PrP^{0/0}$ mice	PrP knock out mice
PrP^{C}	Cellular prion protein
PrP-rel	Prion protein related protein
PrP^{Sc}	Scrapie prion protein
PVA	Polyvinyl alcohol
r	Reverse
Ra	Rat
Rassf	Ras association domain family
rb	Rohon-Beard neuron
rER	Rough endoplasmatic reticulum
RNA	Ribonucleic Acid
rpm	Rotations per minute
s	Second, unit of time
sc	Spinal cord
Scl	Solute carrier family

SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ShaPrP	Syrian hamster prion
Sho	Shadoo protein
Slk	STE20-like kinase
SOB	Super optimal broth
SOC	Super optimal catabolite repression (from LB/SOB)
SOD	Superoxide dismutase
SP	Signal peptide
Sp1	Transcription factor Sp1
T	Thymine nucleobase
t	Telencephalon
TAE	Tris-Acetate-EDTA
Taq polymerase	*Thermus aquaticus* polymerase
TATA box	Cis-regulatory element with the core DNA sequence 5'-TATAA-3'
T-cell	Thymus-cell
TFA	Tierforschungsanlage (animal facility)
tg	Trigeminal ganglion
TRIS	Tris(hydroxymethyl)-aminomethan
TSE	Transmissible spongiform encephalopathy
TUNEL	Terminal deoxyribotransferase (TdT)- uridintriphosphat (UTP) nick-end labeling
u	Units of catalytic activity (enzyme)
UTR	Untranslated region
UV	Ultraviolet
V	Volt, unit of electric potential difference
v/v	volume/volume
VIS	Visible light
w/v	weight/volume
WB	Western blot
WPI	World Precision Instruments
wt	Wild type
xe	*Xenopus laevis*
zCR	zCREST (zebrafish conserved regulatory element for Isl1)
zCREST	Zebrafish conserved regulatory element for Isl1
ZF	Zebrafish

Annotations

- The zebrafish microinjection experiments were repeated at least twice and comparable results were obtained.
- Lethality of microinjected embryos during experiments at 24 hpf was in an averaged 52% (n > 1000) and from 24 hpf to 48 hpf, the lethality was in an averaged 23% (n > 200) (not shown).
- Stages of zebrafish development are described after Kimmel *et al.* (KIMMEL *et al.* 1995).
- If not otherwise noted, in dorsal and lateral zebrafish images, anterior is to the right and in lateral pictures, dorsal is up.
- Scale for zebrafish embryo images:



- Registered trademarks and protected brands are not particularly marked. The lack of such references may not be concluded as a free brand.

1 Introduction

1.1 The prion protein

1.1.1 A brief overview

Prion disorders belong to the most enigmatic diseases because of their unique mechanism of infection. The infectious agents, prions, cause fatal neurodegenerative disorders, also termed transmissible spongiform encephalopathies (TSEs) in a range of mammalian species (COLLINGE 1997). In humans, prion diseases are grouped into sporadic, genetic or infectious forms including the dementia causing Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), and Kuru. In other mammals, the ataxic illness Scrapie is found in sheep and goats, and bovine spongiform encephalopathy (BSE) in cattle. Although the clinical symptoms differ between individual diseases, they all cause the progressive loss of brain function due to the accumulation of misfolded protease resistant prion protein and the concomitant brain vacuolation, astrogliosis and neuronal apoptosis, which inevitably lead to death. Currently, prions are defined as small transmissible proteinaceous particles that are devoid of nucleic acids and consist exclusively of the misfolded isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}) (PRUSINER 1989). Both PrP isoforms share the same primary structure (amino acid sequence) but differ greatly in their tertiary structure. Thus, while the normal isoform, a ubiquitously expressed cell-surface glycoprotein, PrP^{C} (RUDD *et al.* 2001) is mainly α-helical, the pathogenic isoform exhibits primarily a β-sheet structure (PAN *et al.* 1993).

1.1.2 Historical background of prion research

The history of prion observations is long and began as early as 1730 in the United Kingdom with the first observation of an endemic disease in sheep, called Scrapie. The name Scrapie was chosen because diseased animals scraped their bodies against rocks (DESLYS and PICOT 2002). That Scrapie is transmissible to other sheep was first demonstrated by two French veterinarians (CUILLÉ and CHELLE 1939), who infected healthy sheep by inoculation of brain emulsions from sick animals. In humans, a neurodegenerative disorder that causes progressive dementia was first described in 1920 and 1921 by two German physicians, Hans Creutzfeldt and Alfons Jakob, whose names were later used to coin the term “Creutzfeld-Jakob disease” (CREUTZFELDT 1920; JAKOB 1921). Somewhat later, in 1957, Daniel Carleton Gajdusek and Vincent Zigas described Kuru, another human neurodegenerative disease that leads to dementia, loss of balance and

death. But while CJD affected people worldwide, Kuru was observed only among the Fore people living in the highlands of Papua New Guinea (ZIGAS and GAJDUSEK 1957). It was not clear if Kuru is a transmitted or genetically inherited disease since only woman and children fell ill and trials to inoculate diverse animal species failed (DESLYS and PICOT 2002). The similarity of the histological brain lesions between Scrapie and Kuru was noticed in 1959 by William Hadlow and led to intracerebral inoculation studies in primates. Seven years later, these studies showed that Kuru is still transmissible. In fact, it was transmitted within the Fore people by a ritualistic cannibalism (GAJDUSEK *et al.* 1966). Twenty-eight years later, it was shown that CJD is transmissible to chimpanzees (GIBBS *et al.* 1994). Until 1982, the molecular structure of the infectious agent had remained elusive and hypotheses described it as a slow virus or virinoid (DIENER *et al.* 1982; PRUSINER 1982), but observations that the Scrapie and CJD agents are extremely resistant to UV (ultraviolet) and ionizing radiation, which normally destroy nucleic acids (ALPER *et al.* 1967), led Stanley Prusiner to suggest that the agent could be exclusively made out of protein. He postulated the "protein-only" hypothesis and named the agent "prion", derived from "*pro*teinaceous" and "*in*fectious" (PRUSINER 1982). His hypothesis later was completed by the proposal that the prion is a conformational isoform of the host protein PrP^{C} and promotes the conversion of itself into further PrP^{Sc}, with PrP^{Sc} acting as a template (PRUSINER 1991). From this point on, prion research made rapid advances and Prusiner's hypothesis gained strong support. The initial isolation and sequencing of the prion protein from infectious tissue (BOLTON *et al.* 1982) allowed the identification of *Prnp*, its encoding gene. Once *Prnp* was cloned, it became evident that PrP^{C} was a normal cellular protein encoded by the host (CHESEBRO *et al.* 1985; OESCH *et al.* 1985). One year later, Konrad Basler showed that the same host gene, a single copy gene, encodes PrP^{C} as well as PrP^{Sc} (BASLER *et al.* 1986) and another group demonstrated that the two isoforms consist of the same amino acid sequence and post-translational modifications (STAHL *et al.* 1993). The next milestone in prion research was the generation of PrP knockout mice. Homozygote knockout mice were expected to be born unhealthy or even dead, since the single copy gene for PrP is highly conserved among vertebrates and expressed in the CNS during development (WEISSMANN and BUELER 2004). Nevertheless, Charles Weissmann generated the first of several lines of mice devoid of PrP^{C}. All mice were surprisingly viable and showed no obvious phenotype (BUELER *et al.* 1992). However, these mice provided the "final proof" of the "protein-only" hypothesis, because they failed to develop any disease after inoculation with brain homogenate from Scrapie-sick mice, showing that PrP^{C} is required for the susceptibility to scrapie (BUELER *et al.* 1993). In 1994, the establishment of a cell-free *in vitro* conversion system gave another hint of the direct conversion of PrP^{C} into PrP^{Sc}. Coincubation of purified radiolabeled PrP^{C} and cold PrP^{Sc} led to the formation of radiolabeled PrP^{Sc} (KOCISKO *et al.* 1994). Further studies on the structural features of PrP^{C} and PrP^{Sc} brought clarity into the conversion and propagation mecha-

nisms. For instance, using solution phase nuclear magnetic resonance (NMR), the structures of the cellular prion protein in mice, Syrian hamsters and humans (ZAHN *et al.* 2000) as well as those of chickens, turtles, frogs (CALZOLAI *et al.* 2005), cats, dogs, pigs, sheeps (LYSEK *et al.* 2005) and others have been resolved. For PrP^{Sc}, however, it has not yet been possible to elucidate a 3-D structure with high-resolution methods (WILLE *et al.* 2002). Although the mammalian prion protein is highly conserved among species and exhibits an overall similar tertiary protein structure, the amino acid sequence differs between species. This minimal diversity often leads to differences in susceptibility or prolongation of the incubation time for prion infection between different donor and host species, a phenomenon referred to as the "species barrier" (PRUSINER 1998). Inoculation studies across species have shown that wild type mice cannot be infected with Syrian hamster prions (ShaPrP), whereas transgenic mice expressing ShaPrP are susceptible to infection (PRUSINER 1997). During the BSE crisis in the 1990s, which was due to the feeding of contaminated meat and bone meal to cattle in Europe, the "species barrier" became an interesting subject and a new variant of CJD (nvCJD) was thought to be transmitted from infected beef to humans (DESLYS and PICOT 2002). 52 cases of nvCJD had been reported by the end of 1999 (ZEIDLER and IRONSIDE 2000). There is now only evidence of a possible therapy during early stages of human prion diseases. In prion infected mice, neurodegeneration can be reversed upon PrP^{C} depletion in neurons (MALLUCCI *et al.* 2007).

Despite these and other recent advances, there are still many unresolved questions in prion research. For example, it is not clear how PrP^{Sc} reaches the brain after oral entry in an organism and how it converts PrP^{C} to become PrP^{Sc}. Also, the pathogenic mechanisms and physiological function of PrP^{C} remain largely elusive. (AGUZZI and POLYMENIDOU 2004).

1.1.3 Structural features of prion proteins

The *Prn* gene family consists of two genes in mammals: *Prnp*, which encodes PrP[1] and downstream of it *Prnd*, which encodes Dpl (doppel). Both proteins display quite similar tertiary structures, although they share no more than about 25% amino acid similarity and likely play different physiological roles (MO *et al.* 2001). In mammals, PrP is ubiquitously expressed in many cell types, but predominantly found on the cell surface of neurons and immune cells (COLLINGE 2001), where it is prevalently localized to microdomains which act as signaling platforms for glycosylphosphatidylinositol (GPI)- anchored proteins like PrP or Thy-1 (STUERMER *et al.* 2004). During embryogenesis, PrP mRNA is first detectable in the developing CNS of mice and chicken with increasing levels until adulthood (HARRIS *et al.* 1993; MANSON *et al.* 1992). Structurally the ap-

[1] From here on PrP signify PrP^{C}

proximately two hundred amino acid containing mature prion protein is composed of three distinct domains that are highly conserved among vertebrates (Figure 1.1 A): the flexible amino (N)- terminal tail, consisting of the repetitive region; a central conserved hydrophobic stretch; and a stable carboxy (C)- terminal globular domain with three α-helices, two short anti-parallel β-sheets and a GPI-anchor (DONNE *et al.* 1997). During disease, the α-helix rich globular domain of PrP^C assumes an abnormal β-sheet rich conformation (PAN *et al.* 1993). The biosynthesis of PrP follows the typical pathways known for cell membrane proteins. PrP is synthesized in the rough endoplasmatic reticulum (rER) and posttranslationally modified during its transition through the rER and the Golgi apparatus. These modifications include cleavage of the N-terminal signal peptide, addition of two *N*-linked oligosaccharide chains, formation of a disulfide bond between two α-helices in the globular domain, and attachment of the GPI-anchor (HARRIS 1999).

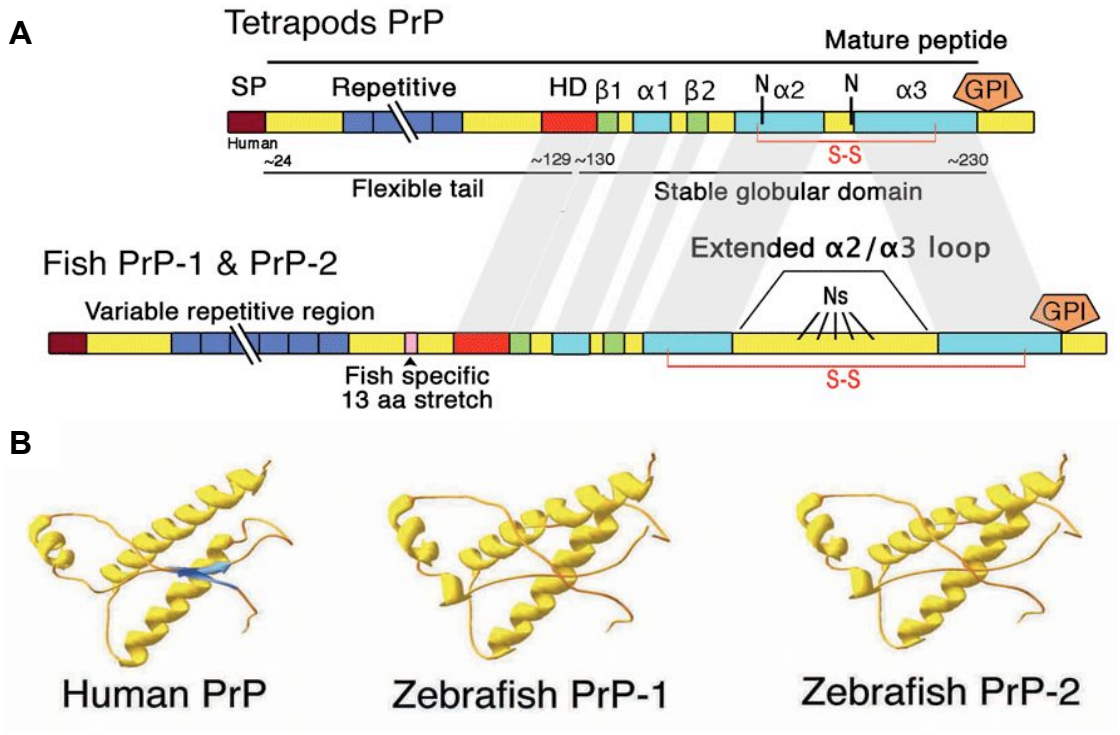


Figure 1.1: Structures of tetrapod and teleost fish PrPs. **(A)** shows the major structural landmarks of PrP, beginning with a signal peptide (SP) for membrane targeting, followed by the variable repetitive region (blue), the hydrophobic domain (HD, red), the globular domain with two β-sheets (green) and three α-helices (cyan), and finally the GPI anchor. Helix two and three are linked via a disulfide bridge (S-S). N symbolizes the glycosylation sites; aa, amino acid. **(B)** Modelled ribbon structure of the human PrP and predicted structures for fish PrP-1 and -2 (RIVERA-MILLA *et al.* 2006).

Interestingly, all known PrP structures contain highly similar domains and most notably, the stable globular fold. The PrP amino acid sequence is indeed strongly conserved within vertebrate classes but highly divergent between different classes, with similarity scores of only about 30% (RIVERA-MILLA *et al.* 2006). Despite these differences in primary sequence, the PrP structures of mammals, birds, reptiles and amphibians show minimal variation (BILLETER *et al.* 1997; CALZOLAI *et al.* 2005; LYSEK *et al.* 2005). In fish, two different orthologous PrP loci have been reported, PrP-1 and -2 (RIVERA-MILLA *et al.* 2006). Having originated from an ancient genome duplication event in fish, PrP-1 and -2 differ in their length and amino acid composition but structural models predict tertiary structures similar to all known PrPs (Figure 1.1 B) (RIVERA-MILLA *et al.* 2006).

1.1.4 The unknown natural role of PrP^C

Although much is known about PrP's pathogenic properties (1.1.2), the normal function of the prion protein remains largely elusive. This is partly due to the fact, that *Prnp* knock out mice, now available fore fifteen years, show no obvious phenotype (BUELER *et al.* 1992). Some mice were reported to show minor abnormalities (COLLINGE *et al.* 1994; FLECHSIG *et al.* 2003); however this might be due to genetic background of the mice utilized rather than because of an indispensable function of PrP. So far, the resistance of PrP deficient mice to prion infection is the only certain PrP "phenotype" observed in these animals (BUELER *et al.* 1993). However, the presence of highly conserved PrP structures in a variety of species from fish to humans (1.1.3) strongly suggests an essential physiological role for PrP rather than the evolution of a host protein that can only trigger prion disease. To date, numerous cellular functions have been proposed for PrP. Because of its location on neuronal cell membranes, it might perform signaling or cell adhesion roles, similar to cell adhesion molecules (CAMs) (SCHMITT-ULMS *et al.* 2001). For example, PrP has been identified as a signaling molecule activating tyrosine kinases (MOUILLET-RICHARD *et al.* 2000). Additionally, several interaction partners have been identified using a yeast two-hybrid assay but functional relevance is not yet understood (SPIELHAUPTER and SCHATZL 2001). PrP has also been implicated in promoting neurite outgrowth through direct interaction with NCAMs (SANTUCCIONE *et al.* 2005). Furthermore, a neuroprotective signal is though to be transduced by PrP and probably lost during its conversion to PrP^{Sc} (CHIARINI *et al.* 2002) but on the contrary, *in vivo* antibody mediated cross-linking of PrP, has been found to induce apoptosis in neurons (SOLFOROSI *et al.* 2004). Recently it has been shown that expression of PrP in developmental and adult neuronal progenitor cells increases cellular proliferation and differentiation and thus could play a role in neurogenesis (STEELE *et al.* 2006). Since the PrP repetitive region in the flexible N-terminus has been reported to bind copper ions (BROWN *et al.* 1997) it may play a role in copper metabolism by

stimulating endocytosis of copper-bound PrP from the cell surface (PAULY and HARRIS 1998). In association with copper, PrP has also been implicated to have an enzymatic function similar to the superoxide dismutase (SOD), which catalyzes the disproportionation of superoxide to oxygen and hydrogen peroxide (BROWN *et al.* 1999). Furthermore PrP has been found on the surface of lymphocytes, stimulating their activation (CASHMAN *et al.* 1990), and has been reported to trigger signal transduction after antibody cross-linking on T-(thymus) cells (STUERMER *et al.* 2004). However, all these proposed functions do not add up to one common molecular mechanism. The fragmentized evidence might suggest that PrP could mediate signal transduction which leads either to a neuroprotective signal of PrP, which is lost after prion infection, or to a toxic signal of PrP^{Sc} (HARRIS and TRUE 2006). In order to understand the molecular mechanism of the disease, it is important to focus also on the cellular function since disease and function are "two sides of the same coin". On this note, it is interesting to mention that transgenic mice which express a secreted form of PrP are able to accumulate PrP^{Sc} after Scrapie infection but fail to develop neurodegeneration (CHESEBRO *et al.* 2005). Overall, the evolutionarily highly conserved structure of PrP might imply a conservation of an important function in all vertebrates. Therefore, it would be a logical extension of past research to investigate this conserved function in a simple vertebrate like the zebrafish.

1.2 Zebrafish

1.2.1 The zebrafish as a model organism

The teleost zebrafish (*Danio rerio*), a tropical fish belonging to the family of Cyprinidae, is a useful tool for developmental and genetic studies. Compared to other higher vertebrate model organisms, the zebrafish possess many advantages. Adult zebrafish reach a length of no more than 3.8 cm and thus can be kept in fairly large numbers in adequate space. Zebrafish live for around five years and produce one hundred to five hundred synchronously developing eggs per spawning. The externally fertilized eggs can easily be observed due to optical clarity of the chorion and external development and thus, the embryos can be physically manipulated (NÜSSLEIN-VOLHARD and DAHM 2002). Because of the increasing popularity of zebrafish research, numerous protocols, mutants, genetic markers and publications are available (SPRAGUE *et al.* 2006). Zebrafish embryos develop rapidly; the four-cell stage is reached within one hour. The blastula is formed between two to four hours post fertilization (hpf) and after about 24 hpf the embryo already exhibits thirty somites, a compartmentalized brain and all the important organs (KIMMEL *et al.* 1995). The zebrafish genome is divided into twenty-five chromosomes (1*n*) and is 1.8 giga base pairs (Gb) in size. This is roughly half the size of tetrapod genomes like mice (3.4 Gb) and humans (3.3 Gb). To date, almost 16,000 genes have been identified in the zebrafish genome (ENSEMBL DATABASE 2007). How-

ever, compared to tetrapods, teleost fish often possess two orthologous duplicated genes instead of one. A genome duplication is thought to have occurred in an early fish ancestor before the teleost radiation, and led either to a secondary loss of one gene or, in many cases, to a segregation of functions of the new genes with distinct spatial and temporal expression patterns. In theory, the new genes partitioned their ancestral functions in two more specifically restricted functions (FORCE *et al.* 1999). In many cases, the function of each gene is less complex than the ancestral function and thus easier to investigate (NÜSSLEIN-VOLHARD and DAHM 2002).

1.2.2 Overview of zebrafish development

The different stages of zebrafish development offer the possibility to address various requests regarding specific anatomical structures. The development of a zebrafish begins rapidly after fertilization of the egg. Following sweeping cytoplasmatic movements, the first step is the formation of a single cell. About forty minutes after fertilization, the first cleavage occurs. Henceforth, cells divide every fifteen minutes. The blastula begins to form after about 2 hpf and the gastrulation at roughly 5 hpf. At the end of the gastrulation period, cells that later form the epidermis, neural crest and central nervous system can already be identified (Figure 1.2 A-E).

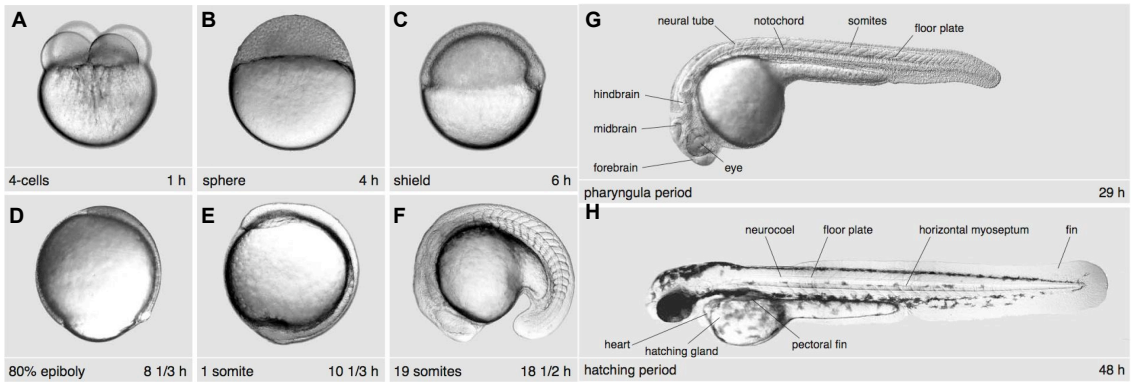


Figure 1.2: Important stages of the embryonic development of zebrafish during the first 48 hpf (HAFFTER *et al.* 1996).

Between 10 and 24 hpf, somites, neuromeres and primary organs develop and the first neurons (primary neurons) begin to stretch out their axons. From around 14 hpf onwards, the subdivision of the neural keel starts and at 24 hpf, the brain of the zebrafish embryo can be divided into forebrain, midbrain and hindbrain (Figure 1.2 F, G). During the pharyngula period, from 24 to 48 hpf, the body axis elongates, the heartbeat becomes visible, fins begin to form, and pigmentation occurs (Figure 1.2 H). After 72 hpf the morphogenesis of organs is completed, the embryos hatch and immediately begin to swim (KIMMEL *et al.* 1995).

1.3 Genomic analyses and expression of prion genes

1.3.1 *Prnp* genomic neighbors in zebrafish

As mentioned before, the zebrafish have two duplicated PrP loci, *Prnp-1* and *-2* located on chromosome 10 and chromosome 25, respectively. Comparisons of gene order (syntenic) between mammalian and fish homologous chromosomes reveal that the zebrafish PrP duplicated regions are organized as a mosaic of different chromosomes. Similar to mammals, zebrafish have a prion protein related gene (*PrP-rel-1* and *-2*) directly downstream to *Prnp* with some of the typical PrP identifying motives (RIVERA-MILLA *et al.* 2006), although the relationship with Dpl remains to be clarified. In humans and mice, the genes downstream of the *Prnp* locus are *Rassf2* (Ras association domain family 2) and *Slc23a1* (solute carrier family 23 member 1). In zebrafish, the *Prnp-1* locus is flanked upstream by *slk* (STE20-like kinase) and downstream by *Rassf2* (Figure 1.3 A) (PREMZL *et al.* 2004) whereas unknown genes flank the *Prnp-2* locus (Figure 1.3 B). Nevertheless, according to the Ensembl Web site, the PrP-1 downstream neighbor of *Prnp-1* is *Sprn2* (ENSEMBL DATABASE 2007), coding for Shadoo2 (Sho2, PrP-rel-1)), a PrP related protein (MIESBAUER *et al.* 2006). Gene homologies upstream of PrP-1 are not evident at this point from the Ensembl database.

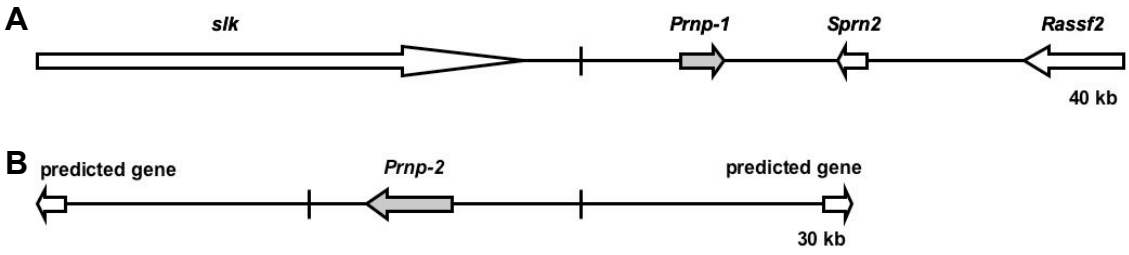


Figure 1.3: Genomic loci of zebrafish **(A)** PrP-1 on chromosome 10 (*Prnp-1*) and **(B)** PrP-2 on chromosome 25 (*Prnp-2*). Modified from Premzl et al. (PREMZL *et al.* 2004).

Given the differences between the PrP genomic neighborhoods of mammals and fish, it is likely that the regions have undergone multiple chromosomal rearrangements (RIVERA-MILLA *et al.* 2006). Such rearrangements need to be considered when searching intergenic and intronic regions (the region 5' and 3' of the gene and the intron) for promoter or regulatory elements (i.e. enhancing- or silencing elements).

1.3.2 *Prnp* gene organization

In many mammalian species, the promoter region as well as conserved motifs and transcription factor binding sites (*cis*-elements) of the PrP gene have already been characterized. PrP is among the housekeeping genes (constitutively constant expressed genes)

that usually exhibit many Sp1 transcription factor binding sites (BAILLY *et al.* 2004). Sp1 sites have been shown to act as strong activators for genes (ANDERSON and FREYTAG 1991). Other conserved features among mammalian PrP promoter regions are their guanine/cytosine- (G/C) rich stretches, AP-1 and -2 binding sites, four strongly conserved sequence stretches (motifs 1-4), and the lack of a TATA box, which in many genes is important for the initiation of transcription (Figure 1.4) (BASLER *et al.* 1986; PUCKETT *et al.* 1991; WESTAWAY *et al.* 1994a).

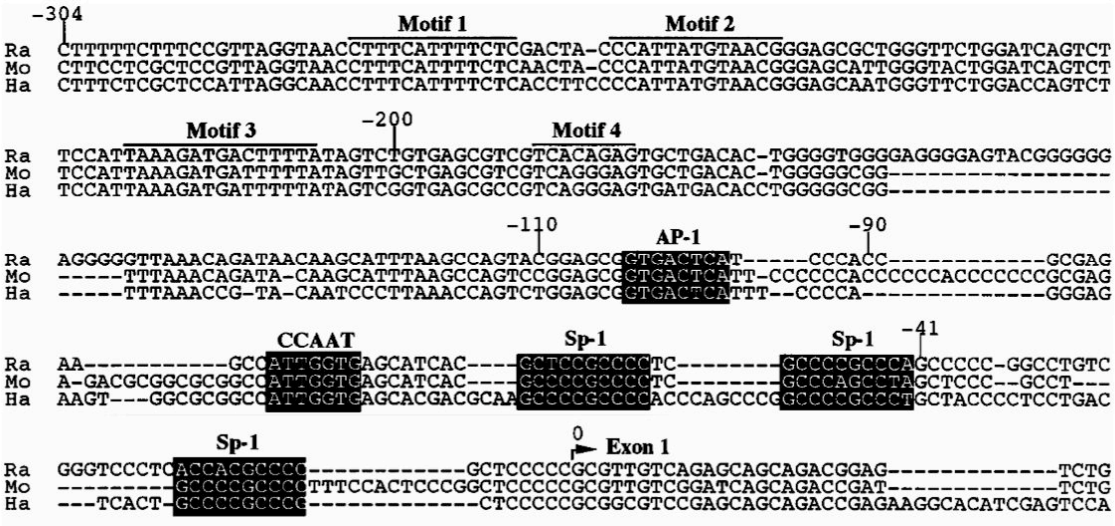


Figure 1.4: Characteristic mammalian *Prnp* promoter region that exhibit four conserved sequence stretches (motif 1-4) an AP-1 and three Sp-1 transcription factor binding sites and a CCAAT box. Ra: rat, Mo: mouse, Ha: hamster (SAEKI *et al.* 1996).

Some differences can be observed in the genetic structure of mammalian prion genes. In rodent species like rats and mice, *Prnp* is made of three exons with the last exon coding for the entire open reading frame (ORF) and the 3' untranslated region (UTR) (SAEKI *et al.* 1996; WESTAWAY *et al.* 1994a). A three exon structure with a short intron one and a large intron two is also found in sheep (LEE *et al.* 1998) and cattle (INOUE *et al.* 1997), whereas the mRNA of Syrian hamsters is transcribed alternatively in two or three exons (LI and BOLTON 1997) and human *Prnp* exhibits only two exons, with exon two missing (LEE *et al.* 1998). For zebrafish PrP, a two-exon human like structure has been reported (COTTO *ET AL.* 2005). Using expression studies in transgenic mice, Charles Weissmann's group showed that this conserved intron-exon structure is important for transcription. Three different PrP constructs were designed, the first one contained no introns and was not expressed, the second one included the first intron and was expressed unspecifically, and only the whole genomic construct showed tissue-specific expression (FISCHER *et al.* 1996). Similar results were reported for the bovine prion promoter where intron one is also required for the expression of a reporter enzyme in a bovine cell culture (INOUE *et al.* 1997). Besides eutherian mammals, the *Prnp* gene has also been analyzed in Marsu-

pialia (Metatheria), a more primitive infraclass of theria including opossums and wallabies. This study showed, that wallabies also exhibit only two exons with some resemblances in the G/C content and conserved motifs compared to eutherians (PREMZL *et al.* 2005). However, for non-mammalian vertebrate species, analyses of the prion protein promoter are so far unavailable and promoter comparisons between mammals and zebrafish are rare. Nevertheless, regulatory sequences in the zebrafish prion promoter region should be similar enough to those in mammals in order to detect conserved motifs (ROTHENBERG 2001).

1.3.3 Developmental expression pattern of endogenous zebrafish PrP

During zebrafish development, PrP-1 and -2 show distinct spatiotemporal expression patterns, indicative of highly restricted, differential gene regulation. Analysis by *in situ* hybridization demonstrated that PrP-1 RNA is maternally ubiquitously expressed around 2.5 hpf (Figure 1.5; A) with decreasing levels after gastrulation whereas PrP-2 is not detectable at this stage (Figure 1.5; C). At 30 hpf, PrP-1 is only detectable at low levels (Figure 1.5; B) but PrP-2 exhibits strong expression in the central nervous system (CNS), notably in the brain, trigeminal ganglion neurons, neuromeres and in some parts of the peripheral nervous system like Rohon-Beard neurons and lateral line ganglia (Figure 1.5; D) (MÁLAGA-TRILLO *et al*. in review by Cell-press).

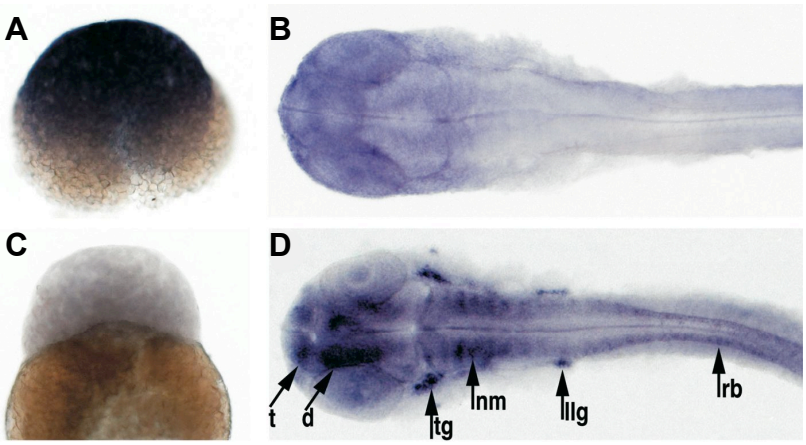


Figure 1.5: Expression patterns of zebrafish PrP-1 **(A; B)** and -2 **(C; D)** at 2.5 hpf **(A; C)** and 30 hpf **(B; D)**. RNA *in situ* hybridization detected PrP-1 ubiquitously distributed early in development whereas PrP-2 is late and neuronal expressed (t, telencephalon; d, diencephalons; tg, trigeminal ganglion; nm, neuromeres; llg, lateral line ganglion; rb, Rohon-Beard sensory neurons (MÁLAGA-TRILLO *et al*. in review by Cell-press).

Analogous experiments in chicken and mouse embryos show PrP expression throughout the developing CNS (HARRIS *et al.* 1993; MANSON *et al.* 1992) comparable to PrP-2. These results suggest sub-functionalization of PrP-1 and -2, as mentioned in 1.2.1, with PrP-2 being the ortholog of the PrP of higher vertebrates.

1.4 Targeted PrP expression in zebrafish neurons

1.4.1 PrP loss- and gain-of-function in zebrafish

A powerful method for studying the function of a protein *in vivo* is to analyze its loss- and gain-of-function phenotypes by knockdown or overexpression of the gene. Unlike $PrP^{0/0}$ mice, which show no obvious phenotype (BUELER *et al.* 1992), the knockdown of zebrafish PrP-1 and -2 with modified antisense RNA oligonucleotides (morpholinos) has revealed important roles in early and late development. The knockdown of PrP-1 causes an early developmental arrest that leads to embryonic death. In contrast, embryos without PrP-2 reach the larval stages, but possess malformed heads and eyes. Ectopic overexpression of *in vitro* synthesized PrP mRNA in zebrafish embryos likewise affected their development, but for both PrP in a similar manner. After asymmetric epiboly, embryos develop deformed brains and reduced or fused eyes at later stages (Figure 1.6) (MÁLAGA-TRILLO *et al.* in review by Cell-press). These results could imply that PrP-1 and -2 indeed share the same function but under different regulation. But as RNA/DNA injections into zebrafish embryos need to be performed at stages no later than the four-cell stage, there is a technical limitation for late and specific overexpression of PrP-2, as overexpression in early stages is likely to mask later effects. Moreover, RNA is actively degraded during early development. An alternative for late and specific expression of PrP could be to place it under the temporal and spatial control of known regulatory elements, such as the *Islet-1* gene enhancers, which target the protein to late developing neurons.

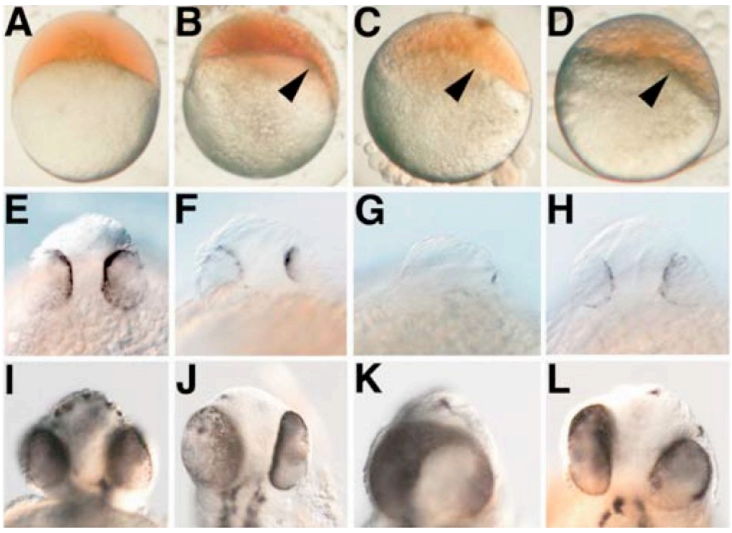


Figure 1.6: Ectopic PrP mRNA overexpression affects normal zebrafish development. Control embryos **(A, E, I)** develop normal whereas ectopic overexpression of PrP-1 **(B, F, J)**, PrP-2 **(C, G, K)** and PrP-mo **(D, H, L)** results in asymmetric epiboly **(B-D)**, smaller brains **(F-H)** and malformed or fused eyes **(J-L)** (MÁLAGA-TRILLO *et al.* in review by Cell-press).

1.4.2 Islet-1 and its enhancers

The Islet-1 (Isl1) protein belongs to the LIM- (lin-11, Isl1, mec-3) homeodomain transcription factors (LIM-HD TF), a family of genes involved in the regulation of other

developmentally important genes (homeobox gene family) (SANCHEZ-GARCIA *et al.* 1993). It was originally identified as a homeobox gene that enhanced the expression of the insulin gene in the rat pancreas (KARLSSON *et al.* 1990) but it is also expressed in a subset of neurons including primary sensory and motor neurons (THOR *et al.* 1991). For specific expression of Isl1, three regulatory elements, zCREST1-3 (zebrafish conserved regulatory element for Isl1 1-3) have recently been identified (Figure 1.7) (HIGASHIJIMA *et al.* 2000; UEMURA *et al.* 2005).

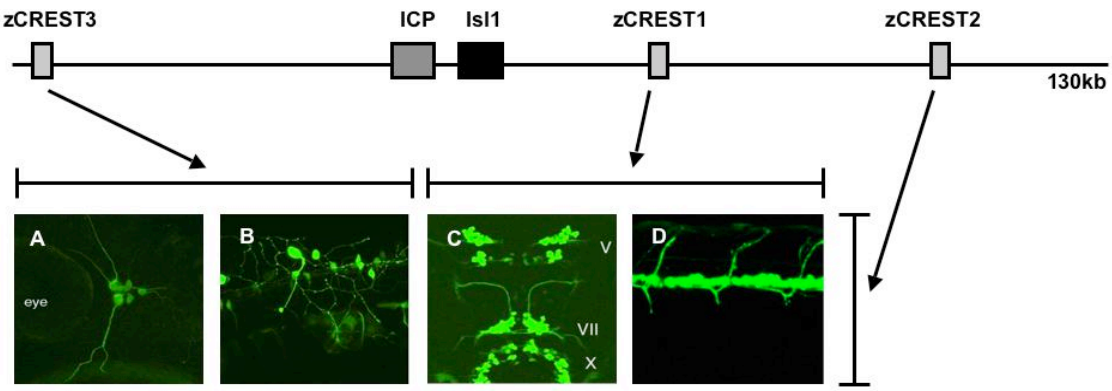


Figure 1.7: *Isl1* gene locus and expression examples of zCREST-GFP microinjected zebrafish embryos. zCREST 3 shows enhancer activity in primary sensory neurons such as trigeminal ganglion cells **(A)** and Rohon-Beard neurons **(B).** zCREST1 drives the expression in primary motor neurons like different cranial motor neurons **(C)** and spinal motor neurons **(D).** zCREST2 exhibits a more broad expression pattern in sensory and motor neurons **(A-D)**. Black box: *Isl1* gene; dark grey box: ICP (*Isl1* core promoter, 4.1kb) for the basal expression; light grey boxes: zCREST 1-3 enhancer elements. Modified from Uemura *et al*. (UEMURA *et al.* 2005).

Injections of green fluorescent protein (GFP) fused zCREST constructs (zCREST-GFP) into zebrafish embryos have revealed the specificity of these *Isl1* enhancers. The roughly 800 bp fragment zCREST1 downstream of the Isl1 gene can drive GFP expression to primary cranial and spinal motor neurons. zCREST3 (~420 bp), which lays upstream of *Isl1* and enhances GFP expression in primary sensory neurons, such as trigeminal ganglion neurons and Rohon-Beard neurons (UEMURA *et al.* 2005). Notably, this expression pattern largely overlaps with the expression pattern observed for PrP-2 in zebrafish embryos (1.3.3). The zCREST2 (~600 bp) enhancer element drives GPF expression in both sensory and motor neurons (UEMURA *et al.* 2005). These enhancer elements, together with temporally regulated promoters such as a heat inducible heat-shock protein (Hsp) promoter, offer a nice tool for targeted expression of a gene of interest.

1.5 Objectives

The general aims of this diploma thesis were to identify and characterize the regulatory sequences driving the expression of PrP-1 and -2 in zebrafish and to gain preliminary insight into the effects of targeted overexpression of PrP in zebrafish neurons. The zebrafish provides an ideal model to investigate the elusive molecular functions of PrP in a simple vertebrate. Transgenic zebrafish expressing reporter genes such as GFP under tissue-specific promoters provide an invaluable means to visualize the behavior of distinct cell populations or organs under genetic manipulations.

In order to characterize the regulatory sequences of PrP in zebrafish, the following specific aims were undertaken:

(1) Bioinformatics characterization of putative regulatory sequences for PrP using a genome database (ENSEMBL DATABASE 2007).
(2) Amplification of the corresponding genomic sequences by polymerase chain reaction (PCR) using whole genomic zebrafish DNA as template.
(3) Cloning of the amplified sequences upstream of the enhanced green fluorescence protein (EGFP) in a promoterless vector.
(4) Activity tests in order to detect EGFP expression by injection of the cloned constructs into zebrafish embryos.

These experiments were designed to help us understand the regulatory basis for the restricted expression patterns of PrP-1 and -2 during development and as preparative work towards the generation of stable transgenic lines which express for instance mouse PrP or zebrafish PrP-1 under the control of the promoter/enhancer of PrP-2. Such transgenic zebrafish will be useful tools to study PrP functional aspects *in vivo*.

In order to overexpress PrP specifically in neurons, the following aims were pursued:

(1) Identification of promoter/enhancer elements able to drive protein expression specifically in neurons by bioinformatics search.
(2) Amplification of these promoter/enhancer elements by PCR using whole genomic zebrafish DNA as template.
(3) Cloning of the promoter/enhancer elements into an EGFP vector.
(4) Activity tests by injection of the cloned constructs into zebrafish embryos in order to examine the specificity of the expression patterns.
(5) Subcloning of EGFP-tagged PrPs from different vertebrate species downstream of the neuronal expressing promoter/enhancer elements.
(6) Analysis of the effects of neuronal overexpression of PrP in transient transgenic zebrafish.

These experiments will help us to characterize the neuronal function of PrP and to overcome the technical limitation of ectopical overexpression of PrP-2, which masks later

effects due to early injection (1.4.1). As PrP-2 is expressed late and neuronal in zebrafish, overexpression needs to be targeted to developing neurons to investigate their effects.

2 Materials[2]

2.1 Molecular biology

2.1.1 Oligonucleotides

Oligonucleotides were purchased from Operon (Köln, Germany) and used for PCR, colony PCR and sequencing. Specific restriction sequences for enzyme recognition were added to allow ligation into the corresponding vector sites.

Table 2.1: Primers used to amplify the genomic sequences up- and downstream of the zebrafish *Prnp-1* gene, for sequencing and colony PCR. Restriction sites are highlighted in bold. Small types (gg) are bases added to prevent the loss of restriction sites during oligosynthesis. f: forward, r: reverse.

Name	Restriction site added	Sequence
PrP1.A1-NdeI-f	*Nde*I	5'- gg**CATATG**CGGATGTTGTATGTGTGTCTTCAGG
PrP1.A1-NheI-f	*Nhe*I	5'- gg**GCTAGC**CGGATGTTGTATGTGTGTCTTCAGG
PrP1.A2-NdeI-f	*Nde*I	5'- gg**CATATG**AGTCAGCGTGATCTCTGTTGAGCAG
PrP1.A2-NheI-f	*Nhe*I	5'- gg**GCTAGC**AGTCAGCGTGATCTCTGTTGAGCAG
PrP1.A3-NheI-r	*Nhe*I	3'- gg**GCTAGC**AGGTTGAAGAAGCGTCTCTCCCGCA
PrP1.A3-AgeI-r	*Age*I	3'- gg**ACCGGT**AGGTTGAAGAAGCGTCTCTCCCGCA
PrP1.in-NheI-r	*Nhe*I	3'- gg**GCTAGC**GGGAACAGTCTTGCTTACAGTGCCT
PrP1.in-AgeI-r	*Age*I	3'- gg**ACCGGT**GGGAACAGTCTTGCTTACAGTGCCT
PrP1.in-NdeI-r	*Nde*I	3'- gg**CATATG**GGGAACAGTCTTGCTTACAGTGCCT
PrP1.out-NdeI-f	*Nde*I	5'- gg**CATATG**TTTTACACAACAGATGCCCTTTCAGCC
PrP1.B1-NheI-r	*Nhe*I	3'- gg**GCTAGC**CTTCTCATCACACTGGGCACGGTCG
PrP1.B2-NdeI-f	*Nde*I	5'- gg**CATATG**GCAGCCCTACTACAGATACAGCACC
PrP1.B2-NheI-r	*Nhe*I	3'- gg**GCTAGC**GGTGCTGTATCTGTAGTAGGGCTGC
PrP1.A1/1-NheI-r	*Nhe*I	3'- gg**GCTAGC**GACTGCATCCATACATCTCTGC
PrP1.2/A3-NdeI-f	*Nde*I	5'- gg**CATATG**GCAGAGATGTATGGATGCAGTC
PrP1.A1/A3mid-NdeI-f	*Nde*I	5'- gg**CATATG**GTTCAGTTACATTCAGTTTGG
PrP1.A1/A3mid-NheI-r	*Nhe*I	3'- gg**GCTAGC**GATGAGTTGTCATAAGCTGTC
PrP1.4/A3-NdeI-f	*Nde*I	5'- gg**CATATG**GACAGCTTATGACAACTCATC
PrP1.A1/A3-seq-f	-	5'- CTTCTCACACTTCAGCCTCTACATC
PrP1.A2/in-seq-f	-	5'- TAGCCTTATCCTTCGTGGCATAC
PrP1.B2/B1-seq-r	-	3'- GGCTCCAGGACCCAGATTGGTGC

[2] Unless otherwise stated Milli-Q water was used for all media, buffers and solutions.

Table 2.2: Primers used to amplify the genomic sequences up- and downstream of the zebrafish *Prnp-2* gene, for sequencing and colony PCR. Restriction sites are highlighted in bold. Small types (gg) are bases added to prevent the loss of restriction sites during oligosynthesis. f: forward, r: reverse.

Name	Restriction site added	Sequence
PrP2.A1-f	-	5'- GCTATTCCACCATCTGCGTCTGTTC
PrP2.A2-f	-	5'- CCACTTCATGTGATGGAGCACAATGC
PrP2.A2-r	-	3'- GCATTGTGCTCCATCACATGAAGTGG
PrP2.A3-f	-	5'- GTACAGGTATGCAGTTGCAGCAAGC
PrP2.A3-NdeI-f	*Nde*I	5'- gg**CATATG**GTACAGGTATGCAGTTGCAGCAAGC
PrP2.A3-r	-	3'- GCTTGCTGCAACTGCATACCTGTAC
PrP2.A3-NheI-r	*Nhe*I	3'- gg**GCTAGC**GCTTGCTGCAACTGCATACCTGTAC
PrP2.A4-f	-	5'- CACAACTGACAAATAGTCGCAGGCA
PrP2.A4.2-Nde-f	*Nde*I	5'- gg**CATATG**GGTGTTTACGACATCACTTAGAAGGCAGC
PrP2.A4-r	-	3'- TGCCTGCGACTATTTGTCAGTTGTG
PrP2.A4-NheI-r	*Nhe*I	3'- gg**GCTAGC**TGCCTGCGACTATTTGTCAGTTGTG
PrP2.A5-f	-	5'- GTCGCGCATCCAAGGGACAACAATC
PrP2.A5-NdeI-f	*Nde*I	5'- gg**CATATG**GTCGCGCATCCAAGGGACAACAATC
PrP2.A6-r	-	3'- GCTGTTTTAAGCGAGTCCTCTCTTG
PrP2.A6-NheI-r	*Nhe*I	3'- gg**GCTAGC**GCTGTTTTAAGCGAGTCCTCTCTTG
PrP2.A7-f	-	5'- GATGCTGGAGATTTGTTCAGCCACAG
PrP2.A7-NdeI-f	*Nde*I	5'- gg**CATATG**GATGCTGGAGATTTGTTCAGCCACAG
PrP2.A7-r	-	5'- CTGTGGCTGAACAAATCTCCAGCATC
PrP2.A7-NheI-r	*Nhe*I	5'- gg**GCTAGC**CTGTGGCTGAACAAATCTCCAGCATC
PrP2.in-r	-	3'- CCACAAGAGCCAGACAGAGCAATAG
PrP2.in.2-NheI-r	*Nhe*I	3'- gg**GCTAGC**CCTCATCATTTAATCTTCCAGATGTGG
PrP2.out-f	-	5'- CGTTCGTTAAATGTCCTCCACTGTTGG
PrP2.B3-f	-	5'- CCATGACCTTACGCTGACAGAGAAC
PrP2.B3-r	-	3'- GTTCTCTGTCAGCGTAAGGTCATGG
PrP2.B2-f	-	5'- CCGATTTGTGGCGAATCGTTAAAGTTTGCG
PrP2.B2-r	-	3'- CGCAAACTTTAACGATTCGCCACAAATCGG
PrP2.B1-r	-	3'- GTACCTTCTAAACCGCTCTCAGTGAC
PrP2.A4/A6-seq-r	-	3'- GTATGTTGTATACTGTAAACG

Table 2.3: Primers used to amplify the genomic sequences of the zebrafish *Islet-1* enhancers and the Hsp70 promoter region, for sequencing reactions and colony PCR. Restriction sites are highlighted in bold. Small types (gg) are bases added to prevent the loss of restriction sites during oligo synthesis. f: forward, r: reverse.

Name	Restriction site added	Sequence
zCREST1-AseI-f	*Ase*I	5'- g**ATTAAT**CTGAGTGGACCTGGCCACAGTCAAT
zCREST1-AseI-r	*Ase*I	3'- g**ATTAAT**GTCTTGGAATGAAACAGACCATTTT
zCREST2-AseI-f	*Ase*I	5'- g**ATTAAT**GTGCAGCTTTAGACATTTAAAATTG
zCREST2-AseI-r	*Ase*I	3'- g**ATTAAT**CAGCACCATAATTCACCACGGTGTG
zCREST3-NdeI-f	*Nde*I	5'- g**CATATG**GTAACAGGATGTGACACGTCGTCTG
zCREST3-NdeI-r	*Nde*I	3'- g**CATATG**GCCTGCTGCTGGTGTCATTTACTGG
zCREST2-NdeI-f	*Nde*I	5'- gg**CATATG**GTGCAGCTTTAGACATTTAAAATTG
zCREST2/3-f	-	5'- CGTGGTGAATTATGGTGCTG**G**TAACAGGATGTGACACGTC
zCREST2/3-r	-	3'- GACGTGTCACATCCTGTTA**C**CAGCACCATAATTCACCACG
ZF-Hsp70-Chr3-f	-	5'- CAATCCGTCAGATTCTGGAGTGCATTACAGC
ZF-Hsp70-Chr3-r	-	3'- GTAGGTGGTGCCCAGGTCAATGCCAATAGCG
ZF-Hsp70-Chr8-f	-	5'- GATCCTTCAGGGGTGTCGCTTGGTGATTTCC
ZF-Hsp70-Chr8-r	-	3'- GTAGGTGGTGCCCAGGTCAATGCCAATAGCG
ZF-Hsp70Chr8-AseI-f	*AseI*	5'- gg**ATTAAT**GATCCTTCAGGGGTGTCGCTTG
ZF-Hsp70Chr8-NheI-r	*NheI*	3'- gg**GCTAGC**GTAGGTGGTGCCCAGGTCAATG

Table 2.4: General primers, provided from the manufacture where the corresponding vectors were obtained or purchased from Operon (Köln, Germany). f: forward, r: reverse.

Name	Vector	Sequence
M13-f	pCRII-TOPO	5'- GTAAAACGACGGCCAG
M13-r	pCRII-TOPO	3'- GTCATAGCTGTTTCCTG
pEGFP-N1-r	pEGFP-C1	3'- GCCCTTGCTCACCATGGTGG
pEGFP CMV-r	pEGFP-C1	3'- GGCTATGAACTAATGACCCCG
pEGFP Ori-f	pEGFP-C1	5'- GTGGATAACCGTATTACCGC

2.1.2 Vectors

Name	Source
pCRII-TOPO	Invitrogen (Karlsruhe, Germany)
pEGFP-C1	BD Biosciences Clontech (Mountain View, CA, USA)
pDsRed-Monomer-N1	BD Biosciences Clontech (Mountain View, CA, USA)

2.1.3 Kits

Name	Source
Quick Ligation Kit	NEB (Frankfurt am Main, Germany)
Topo TA Cloning Kit	Invitrogen (Karlsruhe, Germany)
QIAprep Spin Miniprep Kit	Qiagen (Hilden, Germany)
GeneJET Plasmid Miniprep Kit	MBI-Fermentas (St. Leon-Roth, Germany)
peqGOLD Plasmid Miniprep Kit	PEQLAB (Erlangen, Germany)
QIAquick PCR Purification Kit	Qiagen (Hilden, Germany)
QIAquick Gel Extraction Kit	Qiagen (Hilden, Germany)

2.1.4 Enzymes

Name	Source
REDTaq DNA Polymerase (1u/µl)	Sigma-Aldrich (Deisenhofen, Germany)
*Bsh*TI (*Age*I) (10u/µl)	MBI-Fermentas (St. Leon-Roth, Germany)
*Eco*RI (10u/µl)	MBI-Fermentas (St. Leon-Roth, Germany)
*Nde*I (10u/µl)	MBI-Fermentas (St. Leon-Roth, Germany)
*Nhe*I (10u/µl)	NEB (Frankfurt am Main, Germany)
*Vsp*I (*Ase*I) (10u/µl)	MBI-Fermentas (St. Leon-Roth, Germany)
Calf Intestine Alkaline Phosphatase (1u/µl)	MBI-Fermentas (St. Leon-Roth, Germany)
T4 DNA Ligase (5u/µl)	MBI-Fermentas (St. Leon-Roth, Germany)
T4 DNA Ligase (1-3u/µl)	Promega (Mannheim, Germany)

2.1.5 Buffers for molecular biology

6 x DNA loading buffer for agarose gels	0,25% (w/v) bromophenol blue 0,25% (w/v) xylene cyanol FF 15% (w/v) Ficol (type 400) in water
50 x TAE buffer	2 M Tris acetate 1 M glacial acetic acid 0,5 M EDTA pH 8
1 x TAE buffer	0,04 M Tris acetate 0.02 M glacial acetic acid 0,0001 M EDTA
Ethidium bromide solution	1 x TAE 0.005 % (v/v) ethidium bromide

2.1.6 Other molecular biology reagents

Name	Source
6 x DNA loading buffer	PEQLAB (Erlangen, Germany)
dNTPs	PEQLAB
Lambda HindIII Digest	PEQLAB
peqGOLD 100bp DNA-Ladder	PEQLAB
peqGOLD 1kb DNA-Ladder	PEQLAB
peqGOLD Ladder-Mix	PEQLAB

2.1.7 Internet tools

Tool	Source
ClustalW	http://www.ch.embnet.org/software/ClustalW.html
Ensembl	http://www.ensembl.org
National center for biotechnology information	http://www.ncbi.nlm.nih.gov/
NEBcutter V2.0	http://tools.neb.com/NEBcutter2/index.php
NetPrimer	http://www.premierbiosoft.com/netprimer/index.html
Reverse complement	http://bioinformatics.org/sms/rev_comp.html
The Zebrafish Model Organism Database	http://zfin.org

2.2 Organisms, buffers, media, equipment

2.2.1 Organisms

Name	Source
E. coli strain; chemically competent cells TOP10F' / TOP10	Invitrogen (Karlsruhe, Germany)
Neuro-2a (N2a) cell line (ATCC CCL-131); cell line derived from mouse neuroblastoma	Kindly provided by LS Bürkle (University of Konstanz, Germany)
Zebrafish wt (wild type)	Maintained at the TFA (University of Konstanz, Germany)

2.2.2 Media and antibiotics

Bacteria cell culture media	
LB (Luria-Bertani-Broth)	1% (w/v) bacto tryptone 0,5% (w/v) bacto yeast extract 1% (w/v) NaCl pH 7.0, autoclaved
LB agar	1% (w/v) bacto tryptone 0,5% (w/v) bacto yeast extract 1% (w/v) NaCl 1.5% (w/v) bacto agar pH 7.0, autoclaved
SOC (Invitrogen)	2% (w/v) bacto tryptone 0.5% (w/v) bacto yeast extract 10 mM NaCl 2.5 mM Kcl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose pH 7.4, autoclaved

N2a cell culture media	
1 x MEM with Earle's salts, L-glutamine, P/S (penicillin/streptomycin)	10% (v/v) FCS 0.06 mg/ml penicillin 0.1 mg/ml streptomycin 2 mM L-Glutamine

Zebrafish media	
Embryo medium E3	5 mM NaCl 0.17 mM KCl 0.33 mM $CaCl_2$ 0.33 mM $MgSO_4$ 0.5% methylene blue autoclaved

Antibiotics	**Stock solution**	**Working solution**
Ampicillin	50 mg/ml	0.1 mg/ml
Kanamycin	50 mg/ml	0.05 mg/ml
Penicillin	6 mg/ml	0.06 mg/ml
Streptomycin	10 mg/ml	0.1 mg/ml

2.2.3 Solutions and buffers

Zebrafish handling	
Injection solution	0.15 M KCl 33% (v/v) 0.5% Phenol red in DPBS
$NaPO_4$ buffer	0.4M NaH_2PO_4 0.4M Na_2HPO_4
10 x PBS	95.5 g/l PBS Dulbecco
4% PFA in PO_4	4% (w/v) PFA 0.1M $NaPO_4$ buffer pH 7.4
4% PFA in PBS	4% (w/v) PFA 1x PBS pH 7.4
PBS-T (permeabilization solution)	1 x PBS 0.1% (v/v) Triton X-100
PBS-DT	1 x PBS 0.1% (v/v) Triton X-100 1% (v/v) DMSO
Blocking solution	PBS-DT 10% goat serum
Mowiol mounting medium	35% (w/v) Mowiol 50% (v/v) 2M Tris-Hcl (pH 8) 25% (v/v) Glycerin 0.8% (w/v) Thymerosal
30 x Pronase	30% (w/v) Pronase E/Actinase E pH 7

1 x Pronase	1 x Pronase Embryo medium E3

SDS-PAGE	
Separating gel buffer	1.5 M Tris-HCl pH 8.8 0.4% (w/v) SDS
Stacking gel buffer	1 M Tris-HCl pH 6.8 0.8% (w/v) SDS
10 x running buffer (Lämmli buffer)	0.25 M Tris-HCl 2 M Glycine 1% (w/v) SDS (sodium dodecyl sulfate) pH 8.3
1 x running buffer (Lämmli buffer)	10% (v/v) 10x running buffer
6 x protein loading buffer	10% (v/v) 2-mercaptoethanol 6.67% (w/v) SDS 3% (v/v) 1M Tris-HCl 0.1% (v/v) Bromophenol blue

Western blot	
1 x Blotting buffer	0.2 mM Tris-HCl 0.15 M Glycine 25% (v/v) Methanol
10 x PBS	95.5 g/l PBS Dulbecco
Blocking solution	1 x PBS 5% (w/v) Non-fat milk powder
Stripping solution	1% (v/v) SDS solution 1 x PBS

2.2.4 Antibodies

Primary antibodies	Dilution	Source
Monoclonal mouse anti-acetylated tubulin IgG (Catalog number: T7451)	1:500 (IF)	Sigma-Aldrich (Deisenhofen, Germany)
Monoclonal mouse anti-GFP IgG (Catalog number: 11814460001)	1:2000 (WB)	Roche (Mannheim, Germany)
Polyclonal rabbit anti-DsRed (living colors) (Catalog number: 632496)	1:2000 (WB)	Clontech (Saint-Germain-en-Laye, France)

Secondary antibodies	Dilution	Source
Polyclonal donkey anti-mouse IgG (H+L) Cy3	1:1000 (IF)	Jackson ImmunoReasearch (Newmarket, England)
Polyclonal goat anti-mouse IgG (H+L) HRP	1:10000 (WB)	Jackson ImmunoReasearch
Polyclonal goat anti-rabbit IgG (H+L) HRP	1:10000 (WB)	Jackson ImmunoReasearch

2.2.5 Protein size marker

Name	Source
Precision Plus Protein all blue standards	Bio-Rad (München, Germany)

2.2.6 Chemicals

Name	Source
0.05% Trypsin with 0.02 % EDTA	Invitrogen (Karlsruhe, Germany)
1 x MEM (minimal essential medium) with Earle's salts, without L-glutamine	Invitrogen (Karlsruhe, Germany)
Acrylamid/Bisacrylamid	Roth (Karlsruhe, Germany)
Ammoniumpersulfat (APS)	Sigma-Aldrich (Deisenhofen, Germany)
Calcium chloride ($CaCl_2$)	Riedel-de-Häen (Seelze, Germany)
Cur guard tonic	Dupla (Grafschaft-Gelsdorf, Germany)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (Deisenhofen, Germany)
Dulbecco's phosphate-buffered saline (D-PBS)	Invitrogen (Karlsruhe, Germany)
ECL-Solutions (SuperSignal west pico)	Perbio science (Bonn, Germany)
EDTA (ethylenediaminetetraacetic acid)	Roth (Karlsruhe, Germany)
Ethidium bromide	Sigma-Aldrich (Deisenhofen, Germany)
FCS (fetal calf serum)	Invitrogen (Karlsruhe, Germany)
Glycine	Roth (Karlsruhe, Germany)
Goat serum	Sigma-Aldrich (Deisenhofen, Germany)
Isopropanol	Fisher Scienific (Schwerte, Germany)
L-Glutamine	Invitrogen (Karlsruhe, Germany)
Lipofectamine transfection reagent	Invitrogen (Karlsruhe, Germany)
Magnesium sulfate ($MgSO_4$)	Merck (Darmstadt, Germany)
Methylene blue	Merck (Darmstadt, Germany)

Milk powder (Rapilait)	Migros (Zürich, Switzerland)
Mowiol 4-88	Hoechst (Frankfurt, Germany)
N,N,N',N'-Tetramethyl-ethylenediamine (TEMED)	Serva (Heidelberg, Germany)
NEEO Agarose	Roth (Karlsruhe, Germany)
Paraformaldehyde (PFA)	Riedel-de-Häen (Seelze, Germany)
Phenol red solution 0.5% in DBPS	Sigma-Aldrich (Deisenhofen, Germany)
Phosphate-buffered saline (PBS) dulbecco	Biochrom AG (Berlin, Germany)
Phosphate-buffered saline (PBS) sterile	Invitrogen (Karlsruhe, Germany)
Ponceau S	Sigma-Aldrich (Deisenhofen, Germany)
Potassium chloride (KCl)	Fluka (Buchs, Switzerland)
Pronase E	Sigma-Aldrich (Deisenhofen, Germany)
SDS (sodium dodecyl sulfate)	Serva (Heidelberg, Germany)
Sodium azide (NaN_3)	Merck (Darmstadt, Germany)
Sodium chloride (NaCl)	Merck (Darmstadt, Germany)
Sodium phosphate dibasic (Na_2HPO_4)	Merck (Darmstadt, Germany)
Sodium phosphate monobasic (NaH_2PO_4)	Merck (Darmstadt, Germany)
TRIS (Tris(hydroxymethyl)-aminomethane	Sigma-Aldrich (Deisenhofen, Germany)
Triton X-100	Sigma-Aldrich (Deisenhofen, Germany)
Vaseline	Mainland Pharmazeutische Fabrik (Frankfurt, Germany)
Water conditioner	Original-Bio-Natural (Essen, Germany)
Bromophenolblue	Merck (Darmstadt, Germany)
β-Mercaptoethanol	Sigma-Aldrich (Deisenhofen, Germany)

2.2.7 Laboratory equipment

2.2.7.1 Molecular biology

Name	Specifications	Manufacturer
PCR thermal cycler	DNA Engine PTC-200 DNA Engine DYAD PTC-0220	Genetic Technologies (Miami, FL, USA)

2.2.7.2 Electrophoresis and gel documentation

Name	Specifications	Manufacturer
Electrophoresis power supplies	Consort E831 (for DNA) PS500X (for SDS-PAGE) EPS 500/400 (for WB)	Consort (Turnhout, Bellgium) Hoefer Pharmacia Biotech (San Fransisco, CA, USA)
Electrophoresis units	PerfectBlue Gel System Mini (for DNA) SE 250 (for SDS-PAGE) TE22 (for WB)	PEQLAB (Erlangen, Germany) GE healthcare (München, Germany) Hoefer Pharmacia Biotech (San Fransisco, CA, USA)
Gel imager	Gel Doc 1000	Bio-Rad (München, Germany)
Integration Control Unit	Integration Control Unit	Bio-Rad (München, Germany)
Printer	UP-860 CE	Sony (Köln, Germany)
UV screen	366nm	Bachofer (Reutlingen, Germany)
Video monitor	WV-BM 900	Panasonic (Hamburg, Germany)

2.2.7.3 Microscopy

Name	Specifications	Manufacturer
Cold light sources	KL 1500 LCD KL 1500 electronic	Zeiss (Göttingen, Germany)
Fluorescence microscope	Axioplan 2 imaging	Zeiss (Göttingen, Germany)
Fluorescence stereomicroscope	MZ FLIII	Leica (Bensheim, Germany)
Inverted microscope	Axiovert 25	Zeiss (Göttingen, Germany)
Laser scanning microscope	Axiovert 200M	Zeiss (Göttingen, Germany)
Laser scanning module	LSM 510	Zeiss (Göttingen, Germany)
Stereomicroscopes	Stemi 2000-C Stemi SV 11	Zeiss (Göttingen, Germany)

2.2.7.4 Plastic ware

Name	Specifications	Manufacturer
Cell culture dishes, sterile	145mm 100mm	Greiner Bio-One (Frickenhausen, Germany)
Cell culture plates	12-well	Sigma-Aldrich (Deisenhofen, Germany)
Cell culture tubes, sterile	Polystyrene, round, 12ml	Greiner Bio-One (Frickenhausen, Germany)
Polypropylene tubes, sterile	conical, 50ml conical, 15ml	Greiner Bio-One (Frickenhausen, Germany)
Reaction tubes	2 ml, 1.5 ml, 0.5 ml	Eppendorf (Wesseling-Berzdorf, Germany)
Reaction tubes (PCR)	Thermo tubes 0.2 ml	PEQLAB (Erlangen, Germany)
Tissue culture flasks	75 cm^2	TPP (Trasadingen, Switzerland)

2.2.7.5 Glassware

Name	Specifications	Manufacturer
Cover glasses	Cover glasses	Roth (Karlsruhe, Germany)
Glass capillaries	TW100F-4	WPI (Berlin, Germany)
Glass pasteur pipettes	Glass pasteur pipettes	Brand (Wertheim, Germany)
Microscope slides	Microscope slides	Menzel (Braunschweig, Germany)

2.2.7.6 Electrical equipment

Name	Specifications	Manufacturer
Digital camera	AxioCam HRm Zeiss	Zeiss (Göttingen, Germany)
Film processor	SRX-201	Konica Minolta (München, Germany)
Freezer	-86°C freezer Comfort -20°C	Forma Scientific (Waltham, MA, USA) Liebherr (Biberach an der Riss, Germany)
Incubation shaker	HT-Z52	Infors-HT (Bottmingen, Swiitzerland)
Incubators	B 6060 BK 500	Heraeus (Fellbach, Germany)
Magnet stirrer	RCT	Ikamag (Staufen, Germany)
Micromanipulator	Micromanipulator	Bachofer (Reutlingen, Germany)
Microwave oven	Privileg 7032	Privileg (Fürth, Germany)
pH meter	PH-meter 766 calimatic	Buddeberg (Mannheim, Germany)
Scales	AE163 1265MP	Mettler Toledo (Giessen, Germany) Sartorius (Göttingen, Germany)
Thermomixer	Thermomixer 5436	Eppendorf (Wesseling-Berzdorf, Germany)
Transjector	5240	Eppendorf (Wesseling-Berzdorf, Germany)
UV/VIS Spectrophotometer	DU 530 life science	Beckman Coulter (Krefeld, Germany)
Vortex	VF2	Ikamag (Staufen, Germany)
Water preparation unit	Milli-Q, Q-Gard 3	Millipore (Schwalbach, Germany)

2.2.7.7 Centrifuges

Name	Specifications	Manufacturer
Centrifuges	Biofuge pico Multifuge 4 KR Centrifuge 5424 Centrifuge 5402	Heraeus (Fellbach, Germany) Eppendorf (Wesseling-Berzdorf, Germany)
Rotor for centrifuge 5402	F-45-18-11	Eppendorf (Wesseling-Berzdorf, Germany)
Rotor for centrifuge 5424	FA-45-24-11	Eppendorf (Wesseling-Berzdorf, Germany)
Rotor for centrifuge biofuge pico	75003328	Heraeus (Fellbach, Germany)
Rotor for centrifuge multifuge4 KR	75006475	Heraeus (Fellbach, Germany)

2.2.7.8 Others

Name	Specifications	Manufacturer
Film	Hyperfilm	GE healthcare (München, Germany)
Laboratory gloves	Nitril Examination Gloves Safeskin Powder-free latex gloves	VWR (Darmstadt, Germany) Kimberly-Clark (Koblenz, Germany)
pH indicator test stripes	Neutralit	Merck (Darmstadt, Germany)
Pipettes (I)	Pipetman P1000 Pipetman P200 Pipetman P20 Pipetman P10	Gilson (Villieres le Bel, France)
Pipettes (II)	Research variable 5000 Research variable 1000 Research variable 200 Research variable 20 Research variable 10 Research variable 2.5	Eppendorf (Wesseling-Berzdorf, Germany)

2.2.8 Software

Software	Supplier
Adobe Photoshop CS	Adobe systems GmbH (München, Germany)
Axio Vision	Zeiss (Göttingen, Germany)
EndNote 8.0	Thomson ISI ResearchSoft (Stamford, CT, USA)
Microsoft Excel 2004	Microsoft Deutschland GmbH (Unterschleißheim, Germany)
Microsoft PowerPoint 2004	Microsoft Deutschland GmbH (Unterschleißheim, Germany)
Microsoft Word 2004	Microsoft Deutschland GmbH (Unterschleißheim, Germany)

3 Methods

3.1 Molecular Methods

3.1.1 Primer design

In order to amplify DNA fragments using the polymerase chain reaction (PCR; 3.1.2) and for sequencing reactions, short, synthetic DNA oligonucleotides are required to prime the synthesis of a new DNA strand. The primers used in this study were manually designed on the basis of existing DNA sequences (exported as FASTA text files from Ensembl or the genbank NIH genetic sequence database; ENSEMBL DATABASE 2007, GENBANK NIH GENETIC SEQUENCE DATABASE 2007), followed by an improvement of the conditions through an automated analysis using the Internet tool NetPrimer (Figure 3.1) (PREMIER BIOSOFT NETPRIMER).

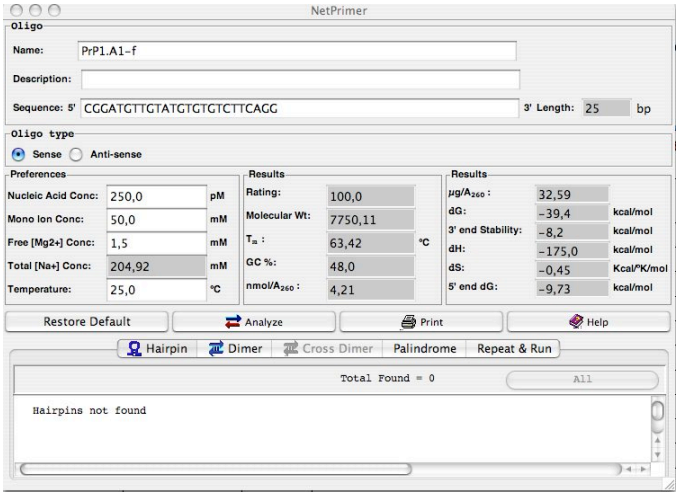


Figure 3.1: Interface of the free Internet tool NetPrimer (Premier Biosoft). The given information about the selected primer helped to estimate how well the primer will associate with the DNA template.

The strength of the binding between two complementary DNA strands depends strongly on the G/C content (guanine and cytosine are joined by three hydrogen bonds whereas adenine and thymine form only two) and on the length of the molecules involved. Accordingly, the primers used in this study were optimized to fulfill the following requirements:

- A length of not less than 18 nucleotides and no longer than 30 nucleotides.
- The G/C content should be around 50%.
- Nucleotides were not allowed to occur more than twice in a row.
- The primer should begin and end with either G or C to enhance adhesion.
- No repetitive sequences should occur in the primer.

As bases at the 5'-end of the primer are less critical for annealing, specific restriction sites for further cloning of the PCR products were added at this end. Selected primers were ordered from Operon (Köln, Germany). Before opening, tubes were shortly centrifuged at 16,060 x g for one minute to ensure that all lyophilized primers reside at the bottom of the tube. Subsequently, the lyophilizate was resuspended with sterile Milli-Q water and adjusted to a concentration of 100 µM. This stock solution was further diluted to the final working solution of 20 µM.

3.1.2 PCR (Polymerase chain reaction)

The polymerase chain reaction (PCR) allows for the *in vitro* production of a multitude of identical copies of a specific DNA fragment from minute quantities of source DNA (PEAKE 1989). The working principle is the cyclic repetition of targeted DNA synthesis (Table 3.1), which is carried out in an automated thermal cycler and begins with the denaturation of the double-stranded DNA template at 94°C.

Table 3.1: Thermal profile of the PCR reaction. Annealing temperatures and elongation times were adjusted for each experiment.

Cycle	Temperature [°C]	Duration [s]	Repetition
Initial denaturation	94°C	180 s	-
Denaturation Annealing Elongation	94°C 55°C -65°C 72°C	30 s 30 s 30 s to 180 s	30 cycles
Final elongation	72°C	300 s	-
End of program	4°C	∞	-

Depending on the melting temperature of primers used (3.1.1), the temperature then decreases to 50°C-65°C, allowing the hybridization of primers with the complementary sequence in order to start the amplification reaction. A thermostable polymerase then elongates the new complementary strand at 72°C (the optimal temperature for most thermostable polymerases used) by inserting single dNTPs at the primer's free 3'OH-ends. These steps are repeated 30 times and the amount of amplified DNA fragments is exponentially increased. The contents of the PCR mixture are given in table 3.2.

Substance	Volume [µl]
Template DNA (~3 ng/µl)	1 µl
10 x reaction buffer	2µl
dNTPs (10mM)	0.5 µl
forward primer (20µM)	0.3 µl
reverse primer (20µM)	0.3 µl
Thermostable polymerase (1u/µl)	1µl
Milli-Q water	14.9 µl

Table 3.2: Contents of the PCR reaction mixture. Preparation took place on ice in 0.2 ml reaction tubes to final volumes between 20µl and 50µl. Volumes here are given for a final size of 20 µl and were adjusted for other volumes.

As the PCR product is a fragment of a defined length, the analysis and purification was performed by agarose gel electrophoresis (3.1.4), thus PCR products were isolated for further cloning.

3.1.3 Restriction digest

In order to prepare DNA for cloning or to verify cloned inserts (analytical digest), PCR products and plasmids were digested with specific restriction endonucleases (2.1.4).

For analytical purposes, 1 µg of DNA was mixed with 1 µl of the corresponding 10 x reaction buffer, five units (u) of the desired restriction enzyme and adjusted to a final volume of 10 µl in a 1.5 ml reaction tube. Samples were then incubated at 37°C for one to two hours and analyzed by agarose gel electrophoresis (3.1.4).

For preparative digests, typically 5 to 10 µg of DNA were digested overnight with the corresponding 10 x reaction buffer and an appropriate amount of restriction enzyme according to the manufacturer's recommendations in a total volume of up to 40 µl at 37°C.

Synchronous double digests with two restriction endonucleases were performed only if the conditions fit together. Otherwise the digest was carried out consecutively.

3.1.4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to analyze PCR products, ligations and restriction digests by size, to estimate concentrations of DNA by comparison to known molecular weight markers and to purify DNA. Under physiological conditions, DNA carries one negative charge per nucleotide and thus migrates in an electric field from the cathode to the anode. Therefore, the shortest DNA fragment covers the longest distance.

A 50 ml, 0.5 to 1% (w/v) agarose gel was freshly made by boiling agarose in 1 x Tris-Acetate-EDTA (TAE) buffer. Upon cooling down to about 60°C, 0.0005 µl/ml ethidium bromide (EtBr) was added to later visualize the DNA, as EtBr intercalates in doublestranded DNA and fluoresces when excited at 302 nm. The mixture then was poured into a gel tray and a comb was plunged into the gel to create sample pockets. When the gel became solid, the gel without the comb was transferred to an electrophoresis unit filled with 0.0005 µl/ml EtBr in 1 x TAE buffer. EtBr was added to prevent a leakage of EtBr out of the gel because it migrates in the electric field in the opposite direction as DNA. The samples, completed with 6 x loading buffer and 6 µl of a molecular weight marker in order to determine the size of the DNA fragments, were pipetted into the pockets. A voltage between 80 and 120 V was applied and the gel was allowed to run for approximately 30 min until the smallest fragment of the molecular weight marker reached the very end of the gel. In order to detect and document the DNA, the gel was illuminated with a UV (ultraviolet) lamp.

3.1.5 Gel extraction

Gel extraction was performed to isolate and purify the DNA fragments from an electrophoresis gel (3.1.4). Gels were transferred onto an UV screen and the fragments of interest were cut out of the gel using a razor blade and transferred to reaction tubes. The DNA fragments were extracted from the gel slice with a Gel Extraction kit (Qiagen), following the manufacturers protocol.

3.1.6 Topo cloning

The Topo cloning kit (Invitrogen) provides a fast method to insert *Taq* (*Thermus aquaticus*) polymerase amplified PCR products into a special vector. This vector (pCRII-Topo) is modified with a single 3'-thymidine overhang and a covalently bound topoisomerase to insert the PCR products. For the cloning, 2 µl of fresh PCR products, 0.5 µl of salt solution (1.2 M NaCl, 0.06 M $MgCl_2$), to enhance the DNA uptake during transformation, and 0.5 µl Topo vector were mixed well and incubated at room temperature for thirty minutes. Afterwards the samples were chilled on ice and transformation was immediately performed (3.1.8).

3.1.7 Sequencing

Shotgun-sequencing reactions were run by the Sequence laboratories (Seqlab, Göttingen, Germany). Normally, 0.6 µg Topo vector including the cloned PCR products were sent to the company along with one forward (M13-f) or reverse primer (M13-r), in a final volume of 7 µl. Sequences were retrieved from an online sever and analyzed using ClustalW and other sequence analysis tools mentioned in 2.1.7 and 2.2.8.

3.1.8 Ligation

The ligation reaction was used to insert the DNA fragment of interest into a plasmid. Vector and inserts were first linearized with restriction endonucleases (3.1.3) to produce compatible ends, and then ligated with T4 DNA ligase (an enzyme, originally isolated from bacteriophage T4), which catalyzes the formation of phosphodiester bonds between juxtaposed 5'-phosphate and 3' hydroxyl groups.

Prior to ligation, vectors were first dephosphorylated with calf intestine alkaline phosphatase (CIAP), an enzyme that catalyzes the release of 5'- and 3'- phosphate termini to avoid self-ligation of the vector ends. 50 to 200 ng of this vector DNA and a 3-fold increased molar ratio of insert DNA were mixed together with 10 x ligation buffer and five units T4 DNA ligase and adjusted to a final volume of 10 µl with Milli-Q water. The molecular weight of 1 µg DNA was determined as $M_{pmol} = 1520.1 \times N_{bp}^{-0.9997}$. The reaction ran overnight in a thermal cycler (Table 3.3) for optimal ligation conditions.

Table 3.3: Thermal profile of the ligation reaction.

Cycle	Temperature [°C]	Duration [min]	Speciality
Ligation	24°C	15 min	-
	23°C	120 min	-
	22°C	120 min	-
	22°C	15 min	Decreasing temperature at 0.5°C every 15 min for 35 times.
	4°C	∞	-

Although T4 DNA ligase (Fermentas) is most active at 22°C, the optimal reaction temperature needs to be balanced with the melting temperature of the sticky ends of the plasmid and the DNA fragment being ligated. The shorter the overhang is, the lower the temperature has to be chosen. Following incubation, the enzyme was normally heat-inactivated for 10 min at 65°C and chilled on ice to cool down. 5 µl of the reaction were used to transform chemically competent *E. coli* cells (3.1.9).

3.1.9 Transformation of chemically competent bacteria

Transformation is the process during which competent cells, in this case chemically competent *E. coli* bacteria (Invitrogen), take up extra-chromosomal DNA. 25 µl of frozen chemically competent cells, thawed on ice, were added to 5 µl of the ligation reaction (3.1.8) or 3 µl of the topo cloning reaction (3.1.6) and incubated on ice for thirty minutes. During this time, divalent cations permeabilize the cell walls of bacteria and the DNA, which attach to the cell wall and enter the cell during a brief heat shock of 45 s at 42°C. After the heat shock, cells were immediately transferred on ice and 200 µl of SOC medium (super optimal broth catabolite repression) at room temperature was added. The tubes were horizontally shaken (200 rpm) at 37°C for one hour to allow recovery of the cells and phenotypic expression of antibiotic resistances. Cells were then centrifuged for one minute at 16,060 x g, the supernatant was discarded and the cells in the remaining SOC medium resuspended. Approximately 50 µl of bacteria resuspension was plated out on a prewarmed selective LB (luria bertani) agar plate with either the antibiotic ampicillin or kanamycin and incubated overnight at 37°C to allow the growth of colonies.

3.1.10 Colony PCR

In order to find correctly ligated bacterial clones (positive clones), an adequate amount of colonies were picked using an inoculation loop from the agarose plate and directly transferred to a PCR reaction mixture (Table 3.2). The PCR (3.1.2) was conducted with specific primers (3.1.1) to determine if a colony contained the plasmid of interest. During the PCR denaturation step, cells are broken up and the DNA released for amplifica-

tion. The PCR product was analyzed in an agarose gel (3.1.4) and any colony which resulted in an amplification product of the expected size was assumed to contain the correct plasmid. Positive colonies were then picked again for a preparatory overnight culture (3.1.11).

3.1.11 Overnight culture

For long-term storage and to obtain plasmid DNA (3.1.13), 5 ml LB media containing an appropriate selective antibiotic were inoculated with picked positive colonies or alternatively with 20 µl bacteria from a dimethyl sulfoxide (DMSO) bacteria stock (3.1.12) and incubated in a shaker (200 rpm) at 37°C overnight or at least eight hours.

3.1.12 Maintenance of bacterial stocks

To maintain bacteria for further plasmid preparations (3.1.13), 500 µl 10% DMSO stocks were stored in a -80°C freezer. DMSO protects the bacterial cells from freezing damage due to ice formation.

3.1.13 Plasmid preparation

In order to obtain DNA constructs for further cloning, restriction digest, sequencing or even for injection into zebrafish embryos (3.2.3), small scale plasmid DNA preparation from a 5 ml overnight culture (3.1.11) was conducted using various kits and following the instructions of the manufacture (Qiagen, MBI-Fermentas, Peqlab). The volume of buffer used to eluate the vector DNA from the miniprep columns was always 60 µl and the obtained concentrations for Topo-constructs were between 0.2 and 0.5 µg/µl and for the pEGFP constructs 0.2 to 0.3 µg/µl, respectively.

3.1.14 Determination of DNA concentration

DNA concentration was measured in a quartz cuvette using an UV/VIS spectrophotometer at 260 nm and a dilution of 1:100 DNA in Milli-Q water. Milli-Q water served also as blank. An absorption of 1.0 corresponds to a DNA concentration of 0.05 µg/µl, hence the concentration of the samples results from: c [µg/µl] = A260 x 0.05 x dilution.

3.2 Zebrafish

3.2.1 Zebrafish care

The adult zebrafish (*Danio rerio*, wild type) were kept in swarms (males and females) in several tanks at a temperature of 28°C. The care and feeding of adults, the cleaning of the tanks, water changes and breeding was completely in the hands of our collaborators at the animal facility (TFA) of the University of Konstanz.

3.2.2 Raising fish

In order to collect eggs for microinjection, a male and a female zebrafish were kept separated overnight in a two-liter acrylic mating container, which contained a small bundle of plastic grass as a barrier in order to reduce aggression (Figure 3.2). The zebrafish pair was maintained under the same water-, temperature- and light-cycle conditions under which they are normally kept. To keep the water clean, 500 µl Cur tonic and 500 µl water conditioner were added to each container. The boxes also contained two removable inner grids (Figure 3.2 A), which separated the male and the female from each other during the night. The upper grid was removed the next morning to allow the fish to mate (Figure 3.2 B). The first eggs were laid and fertilized five to sixty minutes after removal of the upper grid and the lower grid now prevented the eggs from being eaten by allowing them to sink to the bottom of the container.

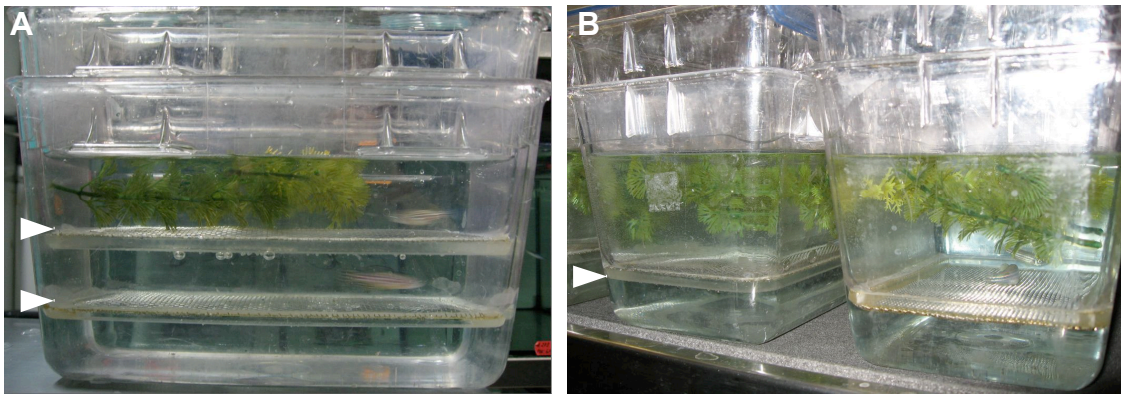


Figure 3.2: (A) Zebrafish mating container with two grids (arrows) that separate male and female and **(B)** only one grid (arrow) to separate the fertilized eggs from male and female.

After mating, eggs were collected in a 145 mm Petri dish with pre-warmed E3 medium after pouring the water from the mating container through a plastic tea strainer. The fish normally laid between one hundred and three hundred eggs which were immediately prepared for microinjection (3.2.3.2).

3.2.3 Microinjection into zebrafish embryos

3.2.3.1 Preparation of DNA

Plasmid DNA for injection was prepared using a Miniprep kit (3.1.13) and obtained at a concentration of roughly 0.3 µg/µl. Injection of high amounts of DNA often leads to abnormal or dead embryos; therefore the DNA was diluted with Milli-Q water, 0.225 M KCl solution and Phenol red to a concentration of 60 ng/µl or less. Phenol red (final concentration 0.05%) acted as a non-toxic injection tracer and helped to visualize the injected volume, which depended on the concentration and was normally a bolus of about 1/3 to 1/5 of the total cell diameter, which corresponds approximately to 2 - 5 nl. The salt solution (final concentration: 0.05 M) maintained physiological conditions. Under these circumstances, the total amount of microinjected DNA was around 0.1 to 0.3 ng per injection. From this microinjection solution, 4 µl were loaded into a glass capillary needle (Figure 3.3; A) with filament, using a pipette with a gel-loader tip. The capillary's tip was broken off before injection to give an opening of an approximated diameter of 1 to 10 µm.

3.2.3.2 Preparation of embryos

For each microinjection, twenty to one hundred embryos between the one- to four-cell stage from a mating couple were selected under a stereomicroscope and transferred to a microinjection plate with a pipette. The microinjection plate consists of six grooves, which holds the slippery and buoyant eggs stable during the injection procedure. The grooves in which the eggs get trapped contain a slope at one side to give access to the needle and a vertical wall on the opposite side to resist the pressure of the needle. Water remaining on the plate was removed before injection.

3.2.3.3 The microinjection procedure

The microinjection apparatus consists of a stereomicroscope to monitor the procedure, a micromanipulator that holds the glass capillary needle, and a pneumatic microinjector with an air compressor (Figure 3.3; B). The injection of DNA into embryos was carried out with a pressure from 100 hPa to 600 hPa over a period of 0.1 to 6.0 s. The pressure and time used for the injection depended on the diameter of the needle used: for thinner needles, more pressure was necessary to inject the accurate concentration of DNA. Injections were carried out at the earliest cleavage stages not exceeding the four-cell stage to reduce mosaicism. Where possible, injections were targeted directly at the forming cell, as this increases the chance for DNA to reach the nucleus and thus leads to a higher frequency of transgenic fish. Following injection, the embryos were collected again in a Petri dish by flooding the plate with pre-warmed E3 medium and were then allowed to develop at 28°C. Dead embryos were removed and E3 medium was changed regularly to keep the embryos healthy and to protect them from fungi and other parasites.

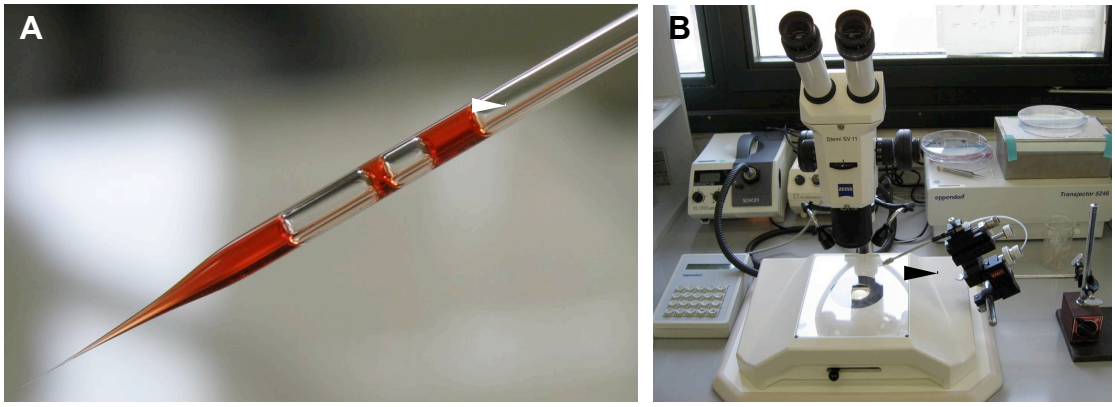


Figure 3.3: **(A)** Microinjection needle with filament (arrow) and phenol red solution. **(B)** Apparatus for microinjections into zebrafish embryos with stereomicroscope, micromanipulator (arrow) and microinjector.

3.2.4 Microscopy

3.2.4.1 Fixation

EGFP expression in embryos was controlled at various times after fertilization under a fluorescence stereomicroscope (MZ FLIII, Leica). Embryos with interesting patterns were collected in a reaction tube and chilled on ice for thirty minutes to anaesthetize. In order to preserve the embryos, the tissues were fixed overnight at 4°C in freshly defrosted 4% paraformaldehyde (PFA, in phosphate-buffered saline (PBS) or PO_4). It was important to use fresh PFA to prevent background staining. The PFA fixes tissues by cross-linking of proteins. The next day, embryos were washed three times in PBS for five minutes each to remove the PFA. Subsequently, embryos for glycerin mounts (3.2.4.4.1) were brought carefully in two steps (30% and 50% glycerin in PBS) into 70% glycerin in PBS. The water-glycerin mix acts as an optical reagent, the refraction index of which is between those of water and oil and thus comparable with the glass slide. Furthermore, glycerin exhibits almost no intrinsic fluorescence. Embryos for Mowiol mounts (3.2.4.4.2) were left in PBS.

3.2.4.2 Dechorionation

The acellular chorion surrounds, as a protective cover, the developing zebrafish until approximately 50 hpf. If embryos were needed prior to this age, they were gently dechorionated, either before or after fixation, by disrupting the chorion with tweezers (Figure 3.4) or with the enzyme pronase.

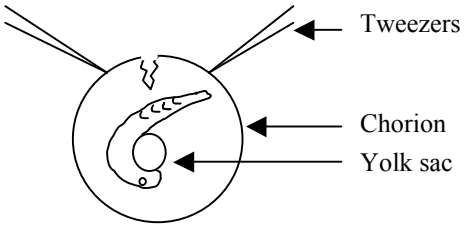


Figure 3.4: Dechorionation of zebrafish embryos with tweezers.

Pronase is a mixture of endo- and exo peptidases that cleaves almost any peptide bond. Hence, it can completely digest the chorion, which is composed of a protein fiber matrix. For dechorionization, embryos were pipetted in a 0.5 mg/ml pronase solution in E3-medium at 37°C and incubated for five to ten minutes. After that, three washing steps with prewarmed (28°C) E3 medium were carried out to remove the enzyme.

3.2.4.3 Zebrafish immunostaining

To mark specific neuronal tissue, embryos were stained using a monoclonal antibody against acetylated alpha tubulin. The first cytoskeletal elements that appear in neurons are microtubules, which are made up of alpha- and beta tubulin. But unlike other cells exhibiting normal tubulin, in neurons a part of the tubulin is modified with acetyl groups, which is targeted by the antibody.

After overnight fixation (3.2.4.1) in 4% PFA in PO_4, embryos were washed three times, five minutes each in 1 ml PBS containing Triton-X 100 (PBS-T) and washed once in PBS-T with DMSO (PBS-TD) to permeabilize cell membranes for antibody treatment. To minimize unspecific antibody binding, embryos were then incubated for five hours in 10% goat serum in PBS-DT while shaking at room temperature. The blocking solution was discarded and 250 µl primary anti-acetylated tubulin antibody, diluted 1:500 in blocking solution, was added and incubated overnight at 4°C. The next day, the primary antibody solution was removed and embryos were washed again thrice, five minutes each with 1 ml PBS-TD before the appropriate secondary antibody (donkey, anti-mouse) coupled to a red fluorescence dye (Cy3) and diluted 1:1000 in blocking solution

was added. Incubation period was once again overnight at 4°C. Subsequently, embryos were washed again three times in 1 ml PBS-T prior to mounting in Mowiol (3.2.4.4.2).

3.2.4.4 Mounting

Mounting, the last and a very essential part of preparing zebrafish embryos, was carried out in different ways. Depending on the manner of microscopy and the kind of cells that one wants to observe, whole or flat mounts, either in glycerin or mowiol, were conducted.

3.2.4.4.1 Whole and flat mounts

Whole mounting implies that the whole embryo, fixed, dechorionated and including the yolk, is placed in the optic media (70% glycerin in PBS) between a glass slide and a cover slip (Figure 3.5; A). In order to prevent the embryo from getting squeezed, small plastillin beads were used as spacers between the slides. The beads also allowed the rotation of the embryo to get a look from different perspectives.

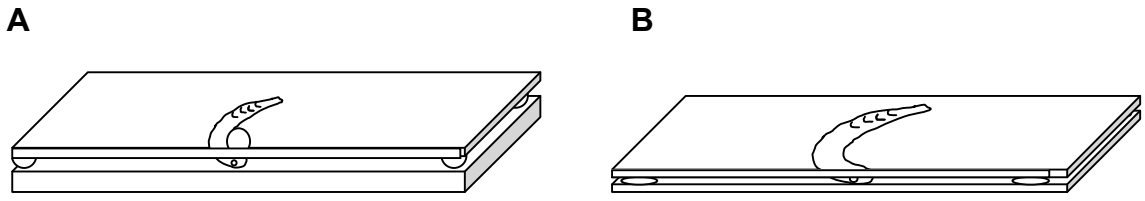


Figure 3.5: Zebrafish embryos embedded between glass slides in a whole- **(A)** and flat **(B)** mount for microscopy.

Flat mounting was carried out to better focus on specific cellular details (Figure 3.5; B). Therefore, the embryo was separated from its yolk sac and flattened on a cover slip after dechorionation. The second coverslip above the embryo was spaced with Vaseline dots instead of plastillin beads and pressed down until it touched the embryo. The remaining space was filled with 70% glycerin in PBS.

All embryos prepared in this manner were visualized and documented using a fluorescence microscope (Axioplan 2 imagin, Zeiss) and a monochrome digital camera (AxioCam HRm, Zeiss).

3.2.4.4.2 Mowiol mounts

For higher magnifications and for immuno stained embryos, the mounting was conducted in Mowiol. Mowiol is a polyvinyl alcohol (PVA) that hardens during drying and has good anti-fade characteristics. Flat mounts were prepared as described above but the second coverslip was spaced with small pieces of cut coverslip and the embedding me-

dia was Mowiol instead of glycerin. Embryos were embedded by orientating them in a dorsal or lateral position within a small drop of Mowiol. After approximately twenty minutes of drying, another drop of Mowiol was placed onto the embryo and covered with the second coverslip.

Embryos prepared in this way were visualized and documented like the glycerin mounts but also with a laser-scanning microscope (Axiovert 200, LSM 510 laser module, Zeiss) for documenting specific cellular patterns (not shown).

3.3 Cell culture

3.3.1 N2a cell culture

The adherent Neuro-2a (N2a) cell line is derived from a mouse neuroblastoma. The cells were maintained in minimal essential medium (MEM) completed with heat-inactivated fetal calf serum (FCS), L-glutamine and the antibiotics penicillin and streptomycin (P/S) in 75 cm^2 cell culture flasks at 37°C in 5% CO_2.

3.3.2 Passaging of N2a cells

As N2a cells double every 24 hr, cells were grown until confluence and then subcultured every two or three days in 1:4 or 1:6 dilutions into a new cell culture flask. All work with cells was performed in a laminar flow hood. For subculturing, old medium was aspirated and cells washed once with 10 ml sterile PBS to remove cell debris. After that, the cells were dissociated from the flask bottom with 0.05% trypsin and 0.02% EDTA (ethylenediaminetetraacetic acid) in Hank's salt solution. Trypsin is a serine protease that was used to cleave extracellular adhesion proteins from adherent N2a cells, and EDTA is a chelating agent that reversibly binds ions like Ca^{2+}, which are essential for cell adhesion. Subsequently, the cell homogenate was filled up to 10 ml with MEM[3], transferred to a 15 ml Falcon tube and centrifuged for five minutes at 154 x g. The supernatant was aspirated and the cell pellet resuspended with a sterile pipette in 6 ml MEM. Any potentially remaining trypsin was inactivated by the FCS contained in MEM. For the new culture, 1 or 1.5 ml (according to a 1:4 or 1:6 split) of the cell suspension was transferred into new 13.5 or 14 ml MEM in a cell culture flask. The density and general condition of the cells were controlled using an inverted microscope (Axiovert 25, Zeiss).

[3] MEM supplemented with FCS, L-glutamine and P/S if not otherwise stated

3.3.3 Transient transfection of N2a cells

To further test whether genomic fragments up- and downstream of the zebrafish prion genes exhibit promoter activity, the corresponding constructs were transiently transfected into N2a cells. One day prior to transfection, cells were subcultured (3.3.2) and 150 µl of the 6 ml cell suspension (corresponding to approximately 2-6 x 10^4 cells) in MEM were transferred into each well of a 12-well plate and supplemented with 850 µl MEM each. Cells were allowed to grow 24 hr at 37°C in 5% CO_2 until they reached approximately 80% of confluence. The next day, 1 µg of the construct and 1 µg of the co-transfection construct (as a control for efficiency) were mixed per experiment. The mixture was pipetted into 100 µl MEM (without any supplements) each and completed with 100 µl MEM (without any supplements) plus 4% transfection agent (Lipofectamine). After an incubation period of twenty minutes, during which the cationic liposomes of the Lipofectamine reagent were bound to the negatively charged vector DNA, the mixture was spread over the cells. Through the interaction of the liposome-vector complexes with the cell membrane of N2a cells, the vector DNA entered the cells. After an incubation of two hours at 37°C in 5% CO_2, the transfection medium was aspirated and instead 1 ml MEM was added. Now the cells were allowed to grow for 24 hr (37°C, 5% CO_2). During this time, the DNA was transported to the nucleus, transcribed and the protein expressed.

3.3.4 Harvesting of N2a cells for Western blot

To harvest the cells after transfection (3.3.3), the medium was aspirated and 1 ml MEM (without any supplements) plus 5 mM EDTA were added for five to ten minutes. The floating cells were resuspended through pipetting and transferred into reaction tubes. To transfer all cells, wells were rinsed once with 500 µl cold PBS, which was then also transferred to the corresponding reaction tube. Tubes were centrifuged for one minute at 2096 x g at 4°C and the supernatant discarded. To remove chemicals and serum, cells were washed thrice with cold PBS, always followed by one minute centrifugation at 2096 x g at 4°C. Finally, the cell pellet was resuspended in 50 µl PBS.

3.4 Protein analysis

3.4.1 SDS-PAGE

In order to separate proteins according to their molecular mass, SDS (sodium dodecyl sulfate) PAGE (polyacrylamid gel electrophoresis) was performed. SDS is an anionic detergent that denaturizes and negatively coats proteins, thus masking positive protein charges and linearizing the proteins so that they can be separated in an electric field due to their molecular mass. The discontinuous SDS-PAGE consists of two different gel

systems: The upper one concentrated the proteins together in one sharp band whereas the lower one subsequently separated by mass.

For the gel, two 10 x 8 cm glass slides were cleaned with 70% ethanol, rinsed once with Milli-Q water and clamped together with two 0.1 cm spacers in between. The slides were fit into a vertical holder with rubber seal to avoid leakage of the gel. The gel solutions were mixed (Table 3.4) and finally, ammonium persulfate (APS) as polymerization initiator (source of free radicals) and tetramethylethylenediamine (TEMED) as catalyst were pipetted into the solution. Roughly 5 ml of separating gel was poured directly in between the glass slides and covered with isopropanol to flatten the gel surface.

Table 3.4: SDS-PAGE separating and stacking gel mixtures for two gels at different percentages (separating gel). Numbers display volumes in ml.

Separating gel (10 ml)	**10%**	**12%**	**Stacking gel (6 ml)**
Milli-Q water	4.0	3.3	4.1
Separating gel buffer pH 8.8	2.6	2.6	-
Stacking gel buffer pH 6.8	-	-	0.81
Rotiphorese 30 % acrylamide/bisacrylamide	3.3	4.0	1.0
10 % (w/v) APS	0.1	0.1	0.06
TEMED	0.01	0.01	0.01

After polymerization of the gel, where the containing acrylamid/bisacrylamid formed a stable network, the isopropanol was removed and the upper part of the glass slides rinsed multiple times with Milli-Q water. APS and TEMED were added to the stacking gel solution and about 3 ml was poured onto the separating gel. A comb was plunged into the gel to create sample pockets. After polymerization of the stacking gel, the comb was removed and gels were either stored in a wet chamber at 4°C or directly clamped into a vertical gel electrophoresis unit.

In order to prepare the samples for SDS-PAGE, 10 µl of 6 x protein loading buffer with SDS, 2-mercaptoethanol (ME) and bromophenol blue were added to the 50 µl N2a cell suspension (3.3.4). The samples were heated at 95°C for five minutes to denaturize the proteins. ME was used to reduce disulfide bonds in proteins and bromophenol blue as negatively charged dye that migrates ahead of the proteins. The electrophoresis chamber was filled with 1 x running buffer and sample pockets were rinsed with the same buffer. 20 µl of the samples were applied to the pockets. In one pocket, 8 µl of a prestained molecular weight marker were added to monitor the separating process and later as efficiency control for Western blot (3.4.2). Empty pockets were filled with 8 µl 6 x protein loading buffer. Current was applied and the samples were allowed to enter the stacking gel at 110 V. For the separating gel, 130 V were applied. When the front of the loading dye reached the bottom of the gel, electrophoresis was stopped and the gel removed

from the glass slides. The stacking gel was discarded and the separating gel transferred to a nitrocellulose membrane for Western blot analyses (3.4.2).

3.4.2 Western Blot

In a Western blot (immunoblot), proteins are electrically transferred from a gel onto a support membrane and one can detect and identify distinct proteins from a protein mixture with specific antibodies.

3.4.2.1 Wet-blotting

Prior to wet-blotting, six sheets of filter paper the size of the gel, one nitrocellulose membrane the same size and two sponges were moistured in 1 x blotting buffer. A sandwich was arranged with the membrane and the SDS-gel in the middle, surrounded by three layers of filter paper and one sponge on each side. Air bubbles were carefully removed by smoothing over the sponges. The sandwich was clamped into a sieve and inserted into a wet blot chamber with the membrane on the side of the anode. In order to vertically transfer the proteins from the gel onto the membrane, the chamber was filled with cold 1 x blotting buffer and a current of 90 V was applied for three hours at 4°C. The molecular weight marker on the membrane served as control for efficiency. To visualize whether the proteins were transferred to the membrane, the membrane was reversibly stained with 0.1 % Ponceau S solution. Thereafter, the membrane was washed thrice in 1x PBS to remove the staining.

3.4.2.2 Immuno detection

Prior to antibody incubation, the membrane was blocked in 20 ml 5% milk powder in 1 x PBS (blocking solution) for one hour at room temperature[4]. The milk-proteins attached to any remaining sticky spot on the membrane in order to avoid unspecific binding of the antibodies. After blocking, the membrane was briefly washed with 1 x PBS. Subsequently, the membrane was incubated in a dilution of the primary antibody (2.2.4) in 20 ml 1% milk powder in 1 x PBS for one hour at room temperature or overnight at 4°C. To detect the primary, unmarked antibody that was bound to the target protein, the membrane then was incubated in a 1:10000 dilution of the secondary, horseradish peroxidase (HRP) conjugated antibody (2.2.4) in 1% milk powder in 20 ml 1 x PBS for one hour at room temperature. Used antibody solutions were stored with sodium azide (NaN_3) at 4°C in order to inhibit fungal or bacterial growth. But because NaN_3 also inhibits the reporter enzyme HRP, intensive washing steps with 1 x PBS in between these two antibody incubations were important. After antibody treatment, the membrane was washed again in 1 x PBS prior to the start of the reporter enzyme reaction with “En-

[4] All washing and incubation steps for Western blot were conducted on a shaker.

hanced Chemiluminescent (ECL) solution". Therefore, 750 µl of a luminol solution was activated with 750 µl of a hydrogen peroxide solution (oxidizing agent) and spread over the membrane. Under the presence of the catalyst HRP, luminol reacted with hydrogen peroxide under extinction of a blue light. Membranes were incubated for one minute before chemiluminescence was detected using a photographic film (Hyperfilm). After one to ten minutes exposure in a darkroom, the film was chemically processed with an automatically film processor machine (Konica).

3.4.2.3 Western blot stripping and re-probing

Following chemiluminescent detection of the first protein, it is possible to remove the antibodies and detect another protein with a second set of specific antibodies. Therefore, the membrane was washed in 15 ml 1% SDS in 1 x PBS for two hours at room temperature. As the proteins were denaturized by SDS, the antibodies lost the specific binding and dissolved from the proteins that were bound to the membrane. Three washing steps with 1 x PBS followed before the membrane was again blocked and incubated with the second set of antibodies as described (3.4.2.2).

4 Results

4.1 Analyses of the zebrafish prion protein promoter region

4.1.1 Bioinformatics analyses and amplification of PrP intergenic regions

In order to identify the regulatory sequences of the zebrafish prion protein gene, the *Prnp-1* and *-2* gene and the sequences adjacent to these genes were searched for in the Ensembl zebrafish genome database (ENSEMBL DATABASE 2007). Ensembl is an open source Web site that displays the genomes and related biological information of various species (BIRNEY *et al.* 2004). A search with the basic local alignment search tool (BLAST) for the known zebrafish *Prnp-1* (GenBank accession number: NM001013297) and *Prnp-2* (GenBank accession number: NM001013298) DNA sequences were run on Ensembl. *Prnp-1* was found to be located on chromosome 10 at the location 15,938,997-15,942,796 and *Prnp-2* at position 2,825,253-2,832,937 on chromosome 25 in the zebrafish genome (Figure 4.1).

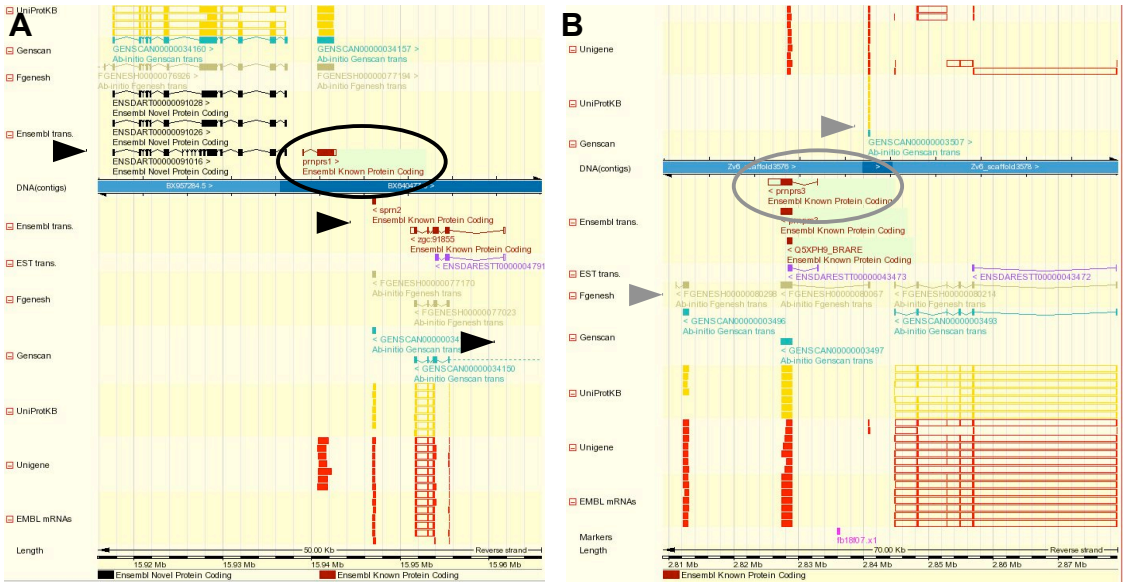


Figure 4.1: Screenshots of the zebrafish *Prnp-1* **(A)** and *Prnp-2* **(B)** gene loci from the Ensembl Web site. *Prnp-1* (*prnprs1*, black circle) was found to be located on chromosome 10 at location 15,938,997-15,942,796. The downstream gene adjacent to *Prnp-1* is *Sprn2*, coding for Shadoo2; upstream is an unknown gene (black arrows). *Prnp-2* (*prnprs3*, grey circle) was found on chromosome 25 at location 2,825,253-2,832,937. The genes adjacent to *Prnp-2* (grey arrows) are unknown loci (ENSEMBL DATABASE 2007).

The corresponding genomic sequences, as well as the sequences for the coding region and for the 5'- and 3'-untranslated regions (UTRs), were exported as FASTA format text files and the exon/intron structure of the *Prnp* genes was inferred from analysis of this data (Figure 4.2). The *Prnp-1* gene is composed of an 88 bp exon one and an 2322 bp exon two separated by a 1659 bp intron. *Prnp-2* exhibits a 3841 bp intron, which disjoins the 65 bp exon one from the 2411 bp exon two. The next step was the analysis of the genes adjacent to the *Prnp* genes, as the intergenic regions (as well as the introns) between *Prnp* and its neighboring genes are likely to contain the promoter and regulatory regions (1.3.1). For the *Prnp-1* locus, the upstream neighbor gene (ENSDART00000091016) is a predicted gene locus, coding for an unknown protein at location 15,917,590-15,937,218 (Figure 4.1 A). The 1779 bp intergenic sequence between these two genes, containing putative promoter and/or regulatory regions, was exported in the FASTA format directly from the Ensembl Web site. Similarly the 4476 bp region between *Prnp-1* and the downstream gene coding for Shadoo2 (Sho2; PrP-rel-1, GenBank accession number: NM_001007780) was located at position 15,946,865-15,947,272. The *Prnp-2* gene (Figure 4.1 B) was analyzed in the same way, and the 7750 bp and 12,202 bp intergenic regions between a predicted upstream gene locus (GENSCAN00000003507 at position 2,840,687-2,840,995), *Prnp-2*, and a predicted downstream gene locus (FGENESH00000080298 at position 2,811,060-2,813,051) were extracted from the Ensembl Web site. On the basis of these DNA sequences,

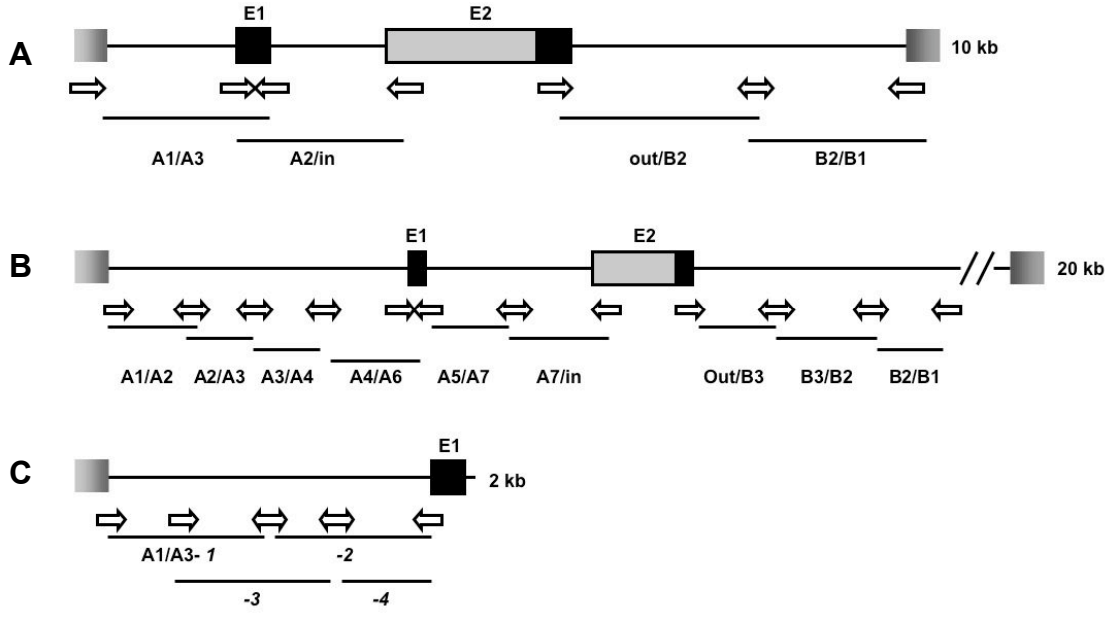


Figure 4.2: Schematic representation of the genomic loci, exon/intron structure of *Prnp-1* **(A, C)** and *-2* **(B)** and positions of designed primers. The PrP-1-fragment A1/A3 was subdivided into four smaller fragments (*1-4*) **(C)**. Graying boxes left and right of the genomic loci symbolize the adjacent genes. Black boxes are untranslated regions (UTRs), grey boxes indicate the coding region. Arrows symbolize selected primers and black bars the corresponding amplified DNA fragments. E1, exon one; E2, exon two.

specific primers were designed (2.1.1 and 3.1.1) to amplify the corresponding *Prnp* intergenic regions and intron fragments[5] using PCR, from whole genome zebrafish DNA (kindly provided by Dr. Málaga-Trillo). The fragments were chosen around 2000 bp since this fragment-size is still small enough to clone easily and long enough to not run the risk of interrupting putative promoter regions. The optimal primer annealing temperatures were determined by performing a gradient PCR with different annealing temperatures. Additionally, different dilutions of genomic DNA, 1:10, 1:50 and 1:100, were tested and the best amplification results were obtained with the 1:50 dilution (not shown). The PCR products were analyzed by agarose gel electrophoresis, extracted from the gel and initially cloned into a pCRII-Topo vector (Invitrogen) in order to maintain bacterial clones with the sequences, for subcloning (4.1.2) and sequencing. Sequencing was conducted with all obtained clones for the purpose of verifying the sequences of the PrP genomic fragments. Inconsistencies between the sequences provided by Ensembl and the fragments amplified were evident mainly for the *Prnp-2* gene locus, but also minor discrepancies for the *Prnp-1* gene locus were observed. The PrP2-A2/A3 and PrP2-A4/A6 genomic fragments (Figure 4.2), which were partially incomplete in the Ensembl database (identified as "N's"), were now completely sequenced (for the sequence see appendix 6.1.2). Due to the complexity of whole genome DNA amplification and to incorrectly assembled sequences in the Ensembl Web site (which inevitably led to incorrect primer design) only the most importantly, but not all PrP genomic fragments were amplified in this study. Table 4.1 presents an overview of the PrP genomic fragments identified, the predicted sizes calculated from the Ensembl sequences and the effectively obtained size.

DNA fragment	Predicted size [bp]	Amplified size [bp]
PrP1-A1/A3	1909	1905
PrP1-A2/in	1780	1841
PrP1-out/B2	2013	-
PrP1-B2/B1	1957	1575
PrP2-A1/A2	2268	-
PrP2-A2/A3	1948	1153
PrP2-A3/A4	1503	1506
PrP2-A4/A6	2025	1300
PrP2-A5/A7	1567	1543
PrP2-A7/in	2403	2028
PrP2-out/B3	2290	-
PrP2-B3/B2	2452	-
PrP2-B2/B1	1886	-

Table 4.1: PrP intergenic regions and intron fragments amplified with PCR from whole genome zebrafish DNA. Given are the predicted sizes calculated from the Ensembl Web site sequences and the effectively amplified sizes of the fragments in base pairs (bp). Bars indicate missing fragments.

[5] '*Prnp* intergenic region and intron fragments' will generally be termed 'PrP genomic fragments' in the following text.

As *Prnp-1* represents a much smaller gene locus than *Prnp-2*, only one fragment was amplified for each region of the *Prnp-1* locus: the upstream intergenic region (PrP1-A1/A3), the intron (PrP1-A2/in) and the second part of the downstream intergenic region (PrP1-B2/B1). For *Prnp-2*, three fragments of the upstream intergenic region (PrP2-A2/A3, -A3/A4, -A4/A6), two fragments of the intron sequence (PrP2-A5/A7 and -A7/in), but no fragments of the downstream region were amplified.

4.1.2 Cloning of PrP fragment-pEGFP constructs

In order to identify those PrP genomic fragments, that contain promoter and/or regulatory elements and thus are able to drive EGFP expression in zebrafish, the obtained fragments were subcloned from the pCRII-Topo vector into the pEGFP-C1 vector (BD Biosciences Clontech) (Figure 4.3). To this end, the vector's internal cytomegalovirus (CMV) promoter was first cut out of the vector using the restriction enzymes *Ase*I and *Nhe*I yielding a promoterless vector (pEGFP-C1ΔCMV) with the corresponding restriction ends. In order to ensure cloning of full-length fragments into the

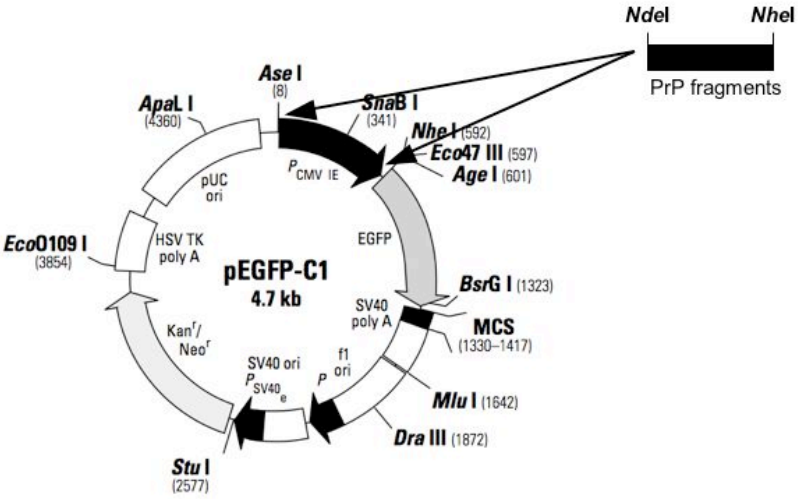


Figure 4.3: pEGFP-C1 vector (BD Biosciences Clontech) and schematically representation of the insertion site for the PrP intergenic regions and intron fragments instead of the cytomegalovirus (CMV) promoter in order to test their ability to drive EGFP expression.

pEGFP-C1ΔCMV vector, the sequences of the PrP genomic fragments were checked for internal *Ase*I and *Nhe*I restriction sites. Since *Ase*I sites appeared in every PrP-genomic fragment sequence, a different enzyme which produces compatible ends (*Nde*I) was chosen as an alternative. Thus, *Nde*I and *Nhe*I were used to design primers for subcloning into the corresponding restriction sites of the promoterless EGFP vector. Cloned upstream of EGFP, the PrP genomic fragments (containing putative promoter elements) should be able to drive EGFP expression in transgenic zebrafish. For the fragment PrP2-A2/A3, another cloning strategy was chosen since a *Nde*I site was included in the fragment's sequence and an *Ase*I site appeared once in the first ~400 nucleotides from the 5' end. Hence, the sequence was cut at the *Ase*I site regardless of the few 5' nucleotides that were lost with this restriction. All cloned PrP-genomic fragment constructs are listed in table 4.2 including their primers, target vectors, and fragment sizes. In the case of *Prnp-1*, a tandem construct composed of the intergenic fragment

PrP1-A1/A3 and the intron fragment PrP1-A2/in was cloned later (pEGFP-PrP1-A1/A3+A2/in) in order to test a combined regulation of these fragments (4.1.4.1). Additionally, the PrP1-A1/A3 fragment was subdivided into four smaller fragments in order to exclude inhibitory regulatory elements affecting promoter activity in this sequence (4.1.4.1) (pEGFP-PrP1-A1/A3-*1, -2, -3* and -*4*) (Figure 4.2; C).

Table 4.2: PrP-1 and -2 genomic fragments, cloned in a promoterless pEGFP-C1 (4731bp) vector in order to detect promoter activity. Shown are the primers used for amplifying the corresponding fragment, the target vector and the size of the fragment being cloned.

Construct	Primer	Target vector	Size [bp]
pEGFP-PrP1-A1/A3	PrP1.A1-NdeI-f PrP1.A3-NheI-r	pEGFP-C1ΔCMV	1905
pEGFP-PrP1-A2/in	PrP1.A2-NdeI-f PrP1.in-Nhe-r	pEGFP-C1ΔCMV	1841
pEGFP-PrP1-B2/B1	PrP1.B2-NdeI-f PrP1.B1-NheI-r	pEGFP-C1ΔCMV	1575
pEGFP-PrP1-A1/A3+A2/in	PrP1.A2-NheI-f PrP1.in-AgeI-r	pEGFP-PrP1-A1/A3	3752
pEGFP-PrP1-A1/A3-*1*	PrP1.A1-NdeI-f PrP1.A1/1-NheI-r	pEGFP-C1ΔCMV	996
pEGFP-PrP1-A1/A3-*2*	PrP1.2/A3-NdeI-f PrP1.A3-NheI-r	pEGFP-C1ΔCMV	931
pEGFP-PrP1-A1/A3-*3*	PrP1.A1/A3.mid-NdeI-f PrP1.A1/A3.mid-NheI-r	pEGFP-C1ΔCMV	808
pEGFP-PrP1-A1/A3-*4*	PrP1.4/A3-NdeI-f PrP1.A3-NheI-r	pEGFP-C1ΔCMV	515
pEGFP-PrP2-A2/A3	PrP2.A2-f PrP2.A3-NheI-r	pEGFP-C1ΔCMV	1153
pEGFP-PrP2-A3/A4	PrP2.A3-NdeI-f PrP2.A4-NheI-r	pEGFP-C1ΔCMV	1506
pEGFP-PrP2-A4/A6	PrP2.A4.2-NdeI-f PrP2.A6-NheI-r	pEGFP-C1ΔCMV	1373
pEGFP-PrP2-A5/A7	PrP2.A5-NdeI-f PrP2.A7-NheI-r	pEGFP-C1ΔCMV	1543
pEGFP-PrP2-A7/in.2	PrP2.A7-NdeI-f PrP2.in.2-NheI-r	pEGFP-C1ΔCMV	2028

4.1.3 Preliminary titration tests of DNA amounts for microinjection

In order to overexpress gene products in zebrafish embryos, a DNA construct encoding the gene of interest has to be microinjected at the one- to four-cell stage of the embryos. However, the injection of high amounts of circular DNA into zebrafish embryos often

produces abnormal or dead embryos. On the other hand, insufficient concentrations of DNA lead to embryos that express none or barely the transgenic protein. Therefore, the amount of DNA involves a trade-off between an effectively expressed transgene and the survival rate of embryos (NÜSSLEIN-VOLHARD and DAHM 2002). Published data concerning the amount of DNA used for microinjections varies widely, ranging from 2.5 pg/embryo (KOSTER and FRASER 2001) to 30 pg/embryo (INBAL *et al.* 2006), and 30 ng/µl (BERNARDOS and RAYMOND 2006) to 350 ng/µl (NÜSSLEIN-VOLHARD and DAHM 2002). In order to find the best concentration for the present experiments, the pEGFP-C1 vector and the pEGFP-zCR3 construct (4.2.2) were chosen as controls. Approximately 2 - 5 nl/embryo DNA solution were microinjected at different concentrations (ranging from 12 ng/µl to 330 ng/µl) into zebrafish embryos. Both constructs were separately microinjected into eggs from several zebra-fish pairs in order to exclude batch variations in egg quality. Embryos were allowed to develop until 24 hpf. Then, numbers of dead and living embryos from uninjected and injected embryos were counted and compared. The total number of surviving uninjected embryos was set at 100%. The results showed a decrease in the survival rate with an increase of the microinjected DNA amount. Based on this preliminary data, the lowest concentrations of microinjected DNA (12 ng/µl and 33 ng/µl) gave the highest survival rates (Figure 4.4).

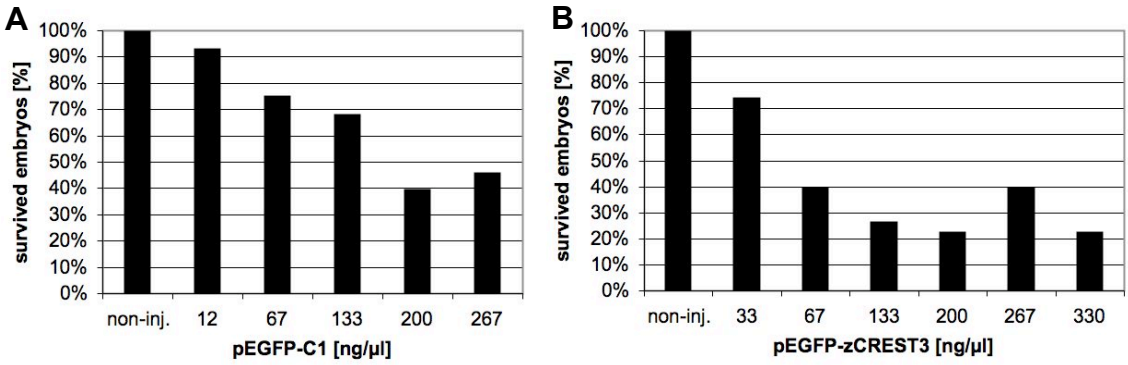


Figure 4.4: Diagrams of the zebrafish embryo survival rate at 24 hpf, microinjected with different concentrations of vector DNA. **(A)** pEGFP-C1, **(B)** pEGFP-zCREST3 microinjected embryos. The survival rate decreased in both experiments with an increase of the DNA amount. The total number of surviving uninjected (non-inj.) embryos was set at 100%.

In spite of these results, the best EGFP expression without high background staining and malformed embryos was obtained at a concentration of 67 ng/µl since with lower DNA amounts, embryos showed none or very little EGFP expression (not shown). Therefore, all subsequent experiments were performed using a DNA concentration of about 67 ng/µl.

4.1.4 *In vivo* expression of EGFP under the control of PrP genomic fragments

4.1.4.1 EGFP expression driven by PrP-1 genomic fragments

The three primarily cloned PrP-1 constructs (PrP1-A1/A3, -A2/in and -B2/B1) described in 4.1.2, were microinjected into zebrafish embryos at the one- to four-cell stage in order to monitor EGFP expression under the control of promoter and/or regulatory elements present in the cloned PrP genomic fragments. Embryos were allowed to develop until 48 hpf before they were fixed and analyzed under a fluorescence microscope. The pEGFP-C1 vector was microinjected as a positive control: Under the strong CMV promoter, EGFP was in a mosaic pattern and randomly expressed throughout the embryo (Figure 4.5-4.7; A). The religated promoterless pEGFP vector (pEGFP-C1ΔCMV) served as a negative control and showed weak, meander-shaped EGFP background expression in the brain (Figure 4.5 and 4.6; B) and expression in blood cells (Figure 4.7; B). For all microinjected PrP-1 fragments (Figure 4.5-4.7; C-E), only this sporadic EGFP background expression but no specific EGFP expression above background level by the PrP genomic fragments could be observed. The expression seemed to be rather unspecific, because the religated pEGFP-C1ΔCMV construct

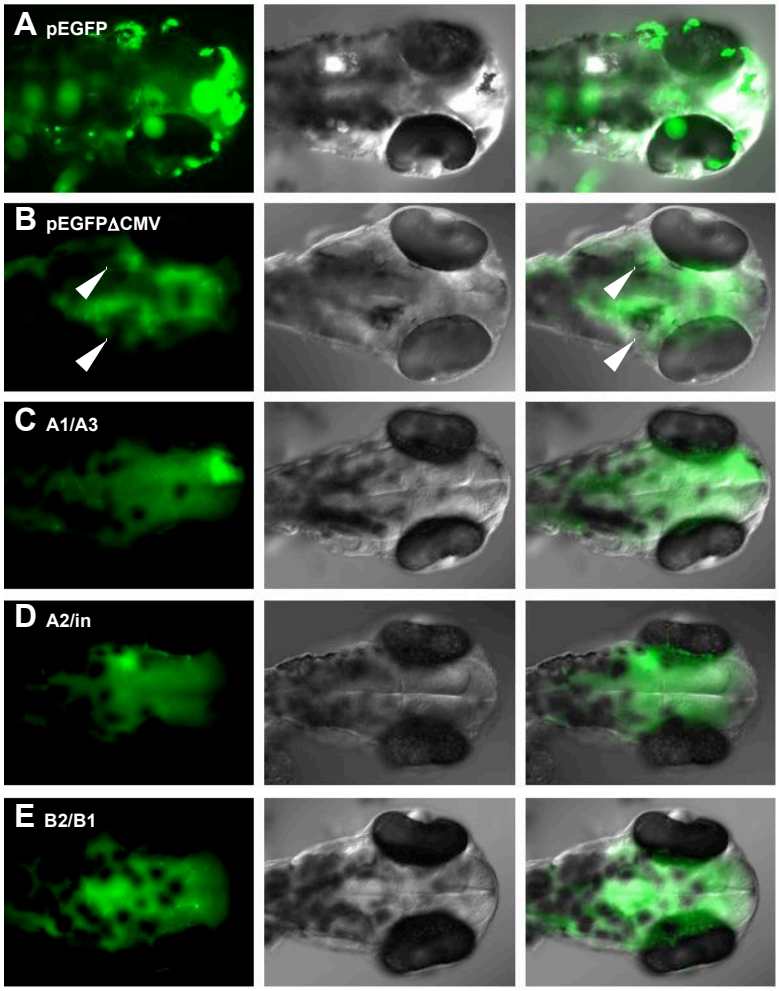


Figure 4.5: Dorsal view of the heads of zebrafish embryos at 48 hpf microinjected with the PrP-1 genomic fragment constructs. The pEGFP-C1 vector as positive control **(A)** showed strong and random EGFP expression whereas the negative control, the pEGFP vector without promoter (pEGFP-C1ΔCMV) **(B)** displayed only background expression (arrows). EGFP expression under the PrP1-fragments **(C-E)** was not observed and restricted to the background expression. The columns display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

produced similar EGFP expression patterns. The observed weak background expression suggested that the vector sequence itself harbors some promoter activity. Thus it was

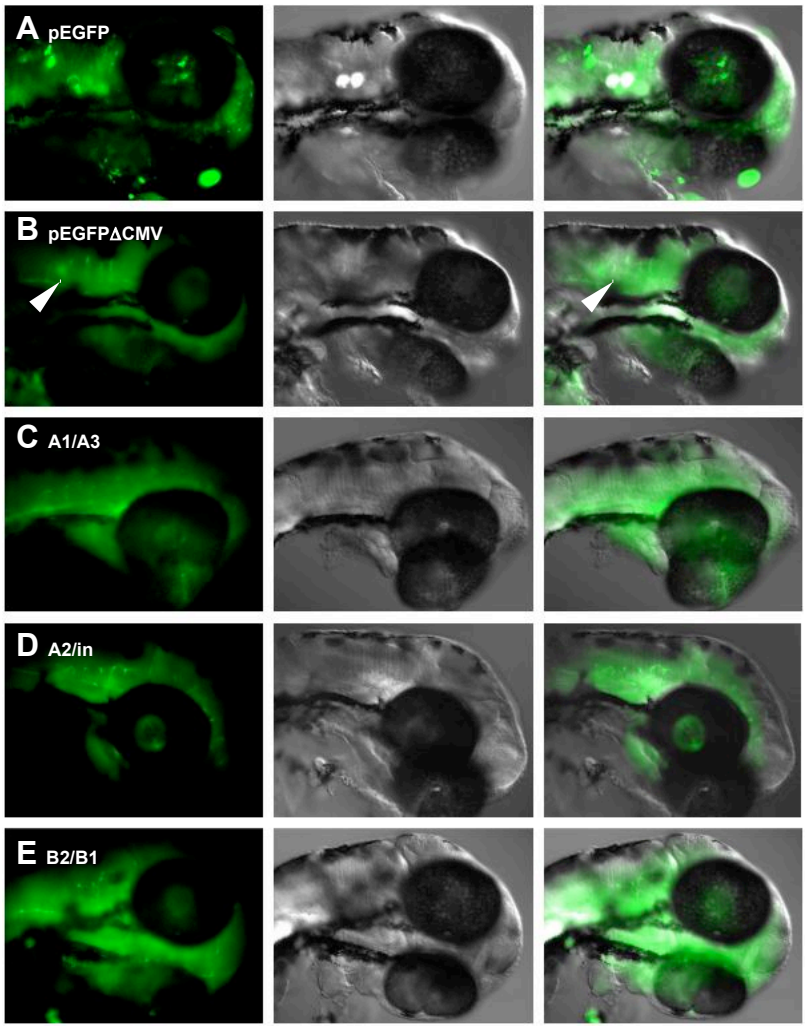


Figure 4.6: Lateral view of the heads from embryos at 48 hpf microinjected with the PrP-1 genomic fragments. The pEGFP-C1 control vector **(A)** showed strong and random EGFP expression whereas the pEGFP-C1ΔCMV construct **(B)**, displayed only background expression (arrow). EGFP expression under the PrP1-fragments **(C-E)** above background expression was not observed. The columns display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski

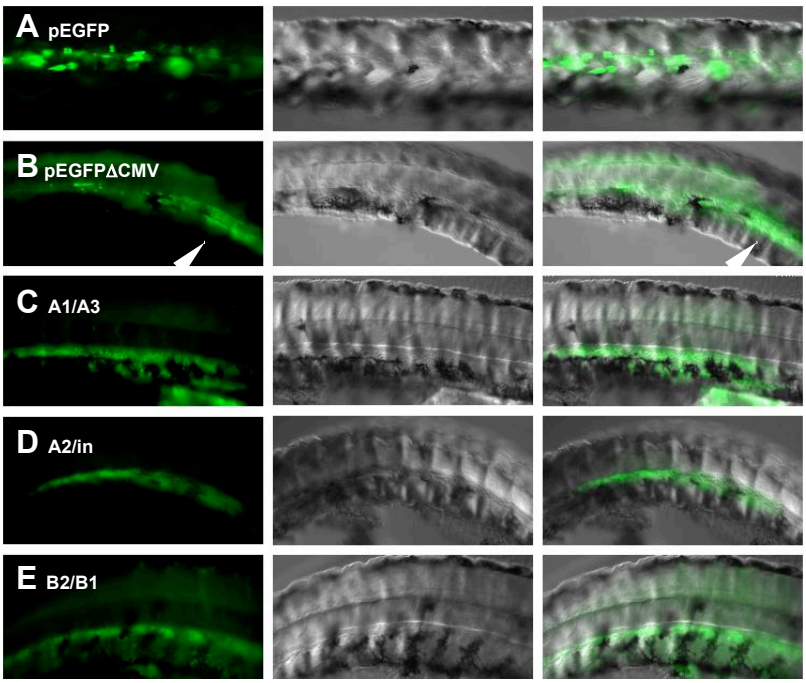


Figure 4.7: Lateral view of the trunks from embryos at 48 hpf. The pEGFP-C1 control vector **(A)** showed strong and random EGFP expression whereas the pEGFP-C1ΔCMV construct **(B)** displayed EGFP background expression in blood cells (arrow). EGFP expression under the PrP-1 genomic fragments **(C-E)** above background level was not observed For image columns see Figure 4.6.

not possible to identify significant promoter activity of the PrP-1 genomic fragments. Since previous experiments showed that PrP-1 is preferentially expressed at early embryonic stages (around 2.5 hpf) (1.3.3), it could be possible that the EGFP expression driven by the PrP-1 genomic fragments took place and ceased too early to be detected in our preliminary observations, usually carried out at 48 hpf. To test this scenario, embryos were monitored hourly for expression of the pEGFP-C1 control vector from 3 hpf until 12 hpf. As embryonic transcription in zebrafish does not start before 3 hpf (KANE and KIMMEL 1993; MARTIN *et al.* 2001), CMV promoter driven EGFP expression could be observed the earliest at 5 hpf, in 3% of the microinjected embryos. At 6 hpf, 25% of the microinjected embryos and at 7 hpf, nearly 50% of the microinjected embryos showed EGFP expression (not shown). In contrast, embryos microinjected with the PrP genomic

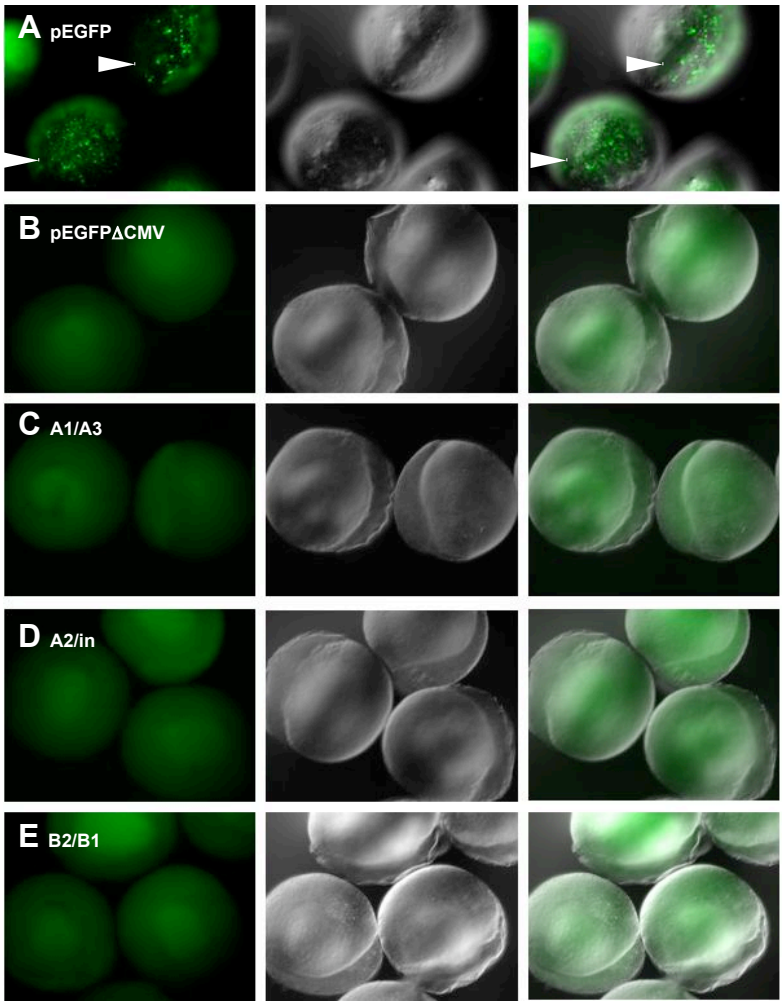


Figure 4.8: Images of zebrafish embryos at 8 hpf microinjected with the PrP-1 fragments. Strong EGFP expression was observed under the CMV promoter **(A)** (arrows), the negative control **(B)** (pEGFP-C1ΔCMV) and the PrP-1 fragments **(C-E)** showed no EGFP expression. The columns display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

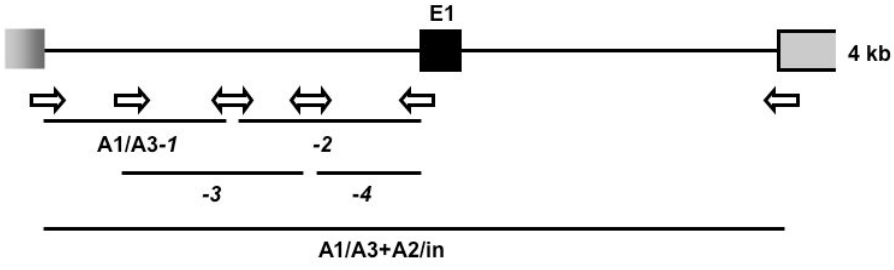


Figure 4.9: Schematic representation of the PrP-1 genomic regions chosen for the new five constructs to test expression ability.

PrP1-A1/A3 was subdivided into four smaller fragments (A1/A3-*1* to -*4*) and a tandem construct consisting of A1/A3 and the intron A2/in was built (PrP1-A1/A3+A2/in).

fragments showed no EGFP expression in the period between 3 hpf and 12 hpf. Representative embryos at 8 hpf were fixed and documented (Figure 4.8). The pEGFP-C1 microinjected embryos (Figure 4.8 A) showed strong expression in embryonic cells in contrast to the embryos injected with the PrP genomic fragments (Figure 4.8 C-E) and the promoterless control vector (Figure 4.8 B), which showed no EGFP expression. To further examine the PrP-1 genomic fragments for their ability to drive EGFP expression, five other constructs were cloned and tested. The PrP1-A1/A3 genomic fragment was subdivided into four smaller fragments and a tandem construct out of PrP1-A1/A3 and -A2/in was built (Figure 4.9). As others (FISCHER *et al.* 1996; INOUE *et al.* 1997) have reported that prion promoters are only active in association with the first intron, which probably contains regulatory elements (1.3.2), a new construct including the fragment upstream of the PrP-1 transcription start site and the intron was cloned and analyzed. Unfortunately, like the initially tested PrP genomic fragments, only background expression was detected in embryos at 48 hpf (Figure 4.10-4.12; C), as well as in embryos at

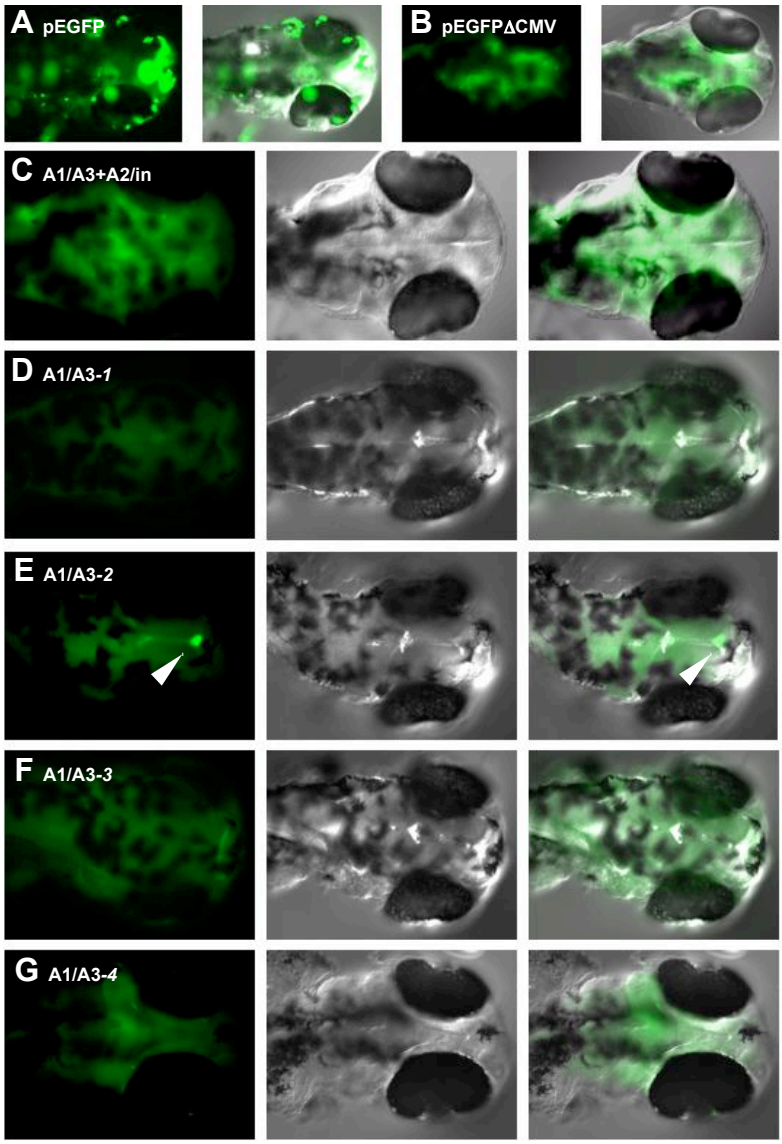


Figure 4.10: Dorsal view of the heads of zebrafish embryos at 48 hpf, microinjected with the pEGFP-C1 **(A)** and the pEGFPΔCMV **(B)** controls. pEGFPΔCMV microinjected embryos showed the same EGFP background expression as the PrP-1 tandem construct (consisting of the upstream region before the transcription start site (A1/A3) and the intron (A2/in)) **(C)** and the four small PrP1-A1/A3 fragments (PrP1-A1/A3-*1* to *4*) **(D-G)** injected embryos. EGFP expression was only observed in PrP-1-A1/A3-*2* and -*4* injected embryos in the brain (arrows) **(E, G)**. A-B, fluorescent (right) and overlay images (left); C-G, fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

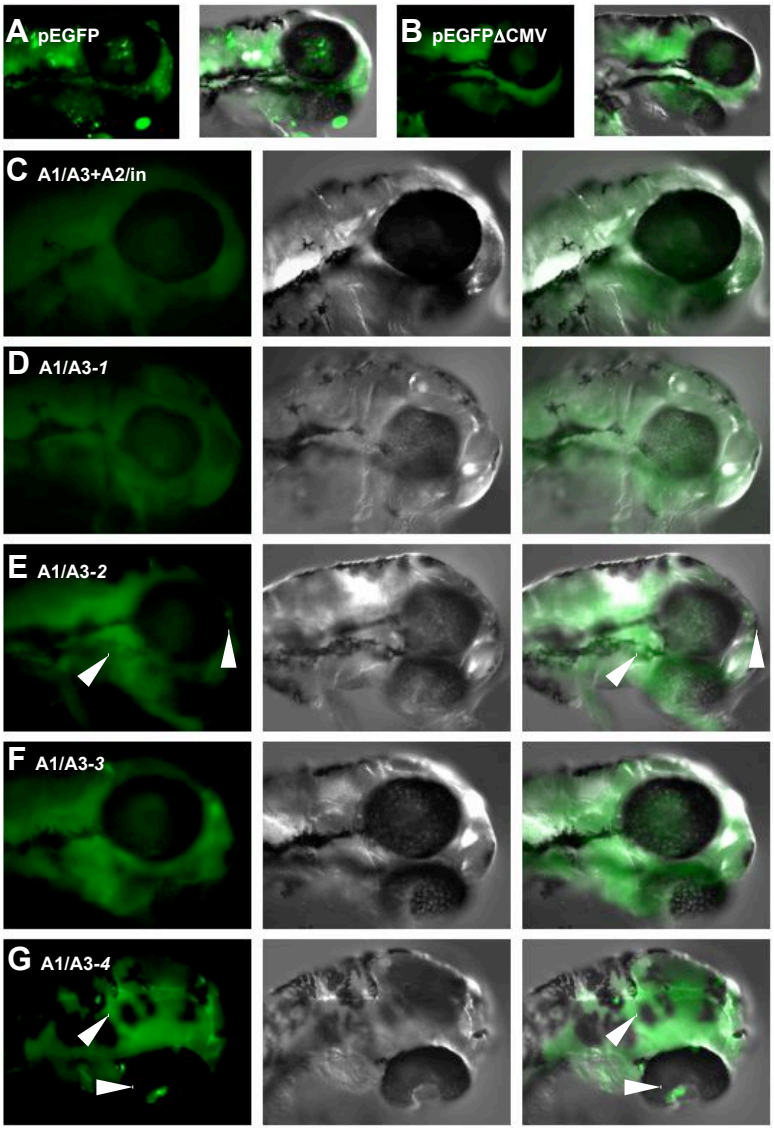


Figure 4.11: Lateral view of the heads of zebrafish embryos at 48 hfp microinjected with the pEGFP-C1 **(A)** and the pEGFPΔCMV **(B)** controls, the PrP-1 tandem construct **(C)** and the four small fragments of the PrP1-A1/A3 construct (PrP1-A1/A3-*1* to *4*) **(D-G)**. The tandem construct **(C)** and the PrP1-A1/A3-*1* **(D)** and -*3* **(F)** constructs exhibited only background expression as seen for pEGFP-C1ΔCMV **(B)**. Minor EGFP expression in the brain was seen in embryos injected with the PrP1-A1/A3-*2* **(E)** and -*4* **(G)** constructs (arrows). A-B, fluorescent (right) and overlay images (left); C-G, fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

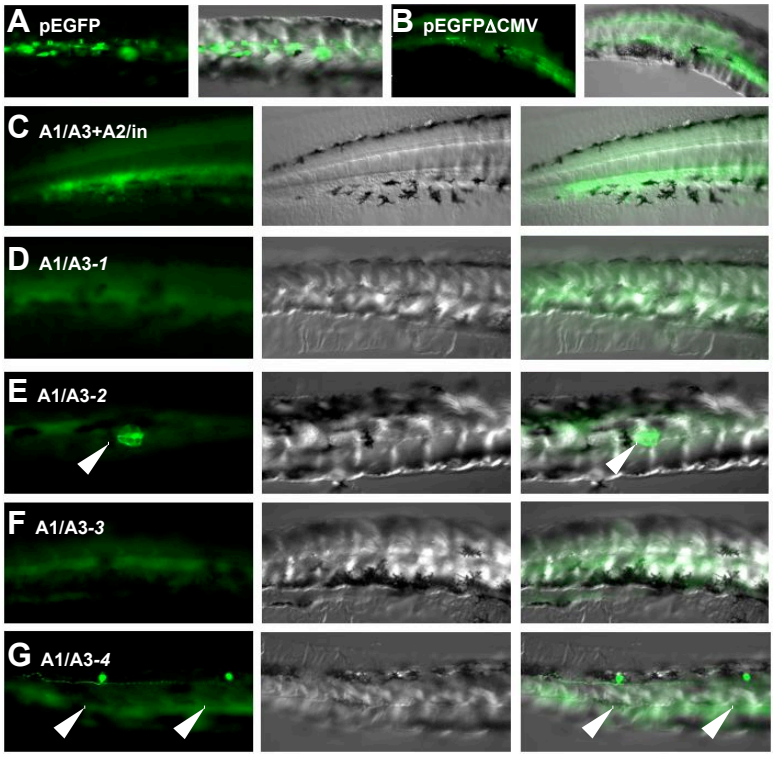


Figure 4.12: Lateral view of the trunks of zebrafish embryos at 48 hpf, microinjected with the pEGFP-C1 **(A)** and the pEGFPΔCMV **(B)** controls, which showed the same EGFP background expression in blood cells as embryos microinjected with the PrP-1 tandem construct **(C)** and the four small PrP1-A1/A3 fragments (PrP1-A1/A3-*1* to *4*) **(D-G)**. EGFP expression was observed in PrP-1-A1/A3-*2* and -*4* microinjected embryos in notochord cells (arrow) **(E)** and in Rohon-Beard neurons (arrow) **(G)**. For image columns see Figure 4.11.

8 hpf (Figure 4.13; C). Because the PrP-1 fragment upstream of the transcription start site (PrP1-A1/A3) contains nearly 1000 bp, it is likely that not only a promoter but also regulatory elements are present in this sequence. For instance, this fragment could harbor gene silencing binding motives, which would prevent EGFP expression in our assays. In order to sort out a possible promoter from silencing elements, the PrP1-A1/A3 fragment was subdivided into four (PrP1-A1/A3-*1* to -*4*) smaller fragments (Figure 4.9). These PrP genomic fragments were tested in embryos at 48 hpf (Figure 4.10-4.12; D-G), and 8 hpf (Figure 4.13; D-F) respectively. Interestingly, EGFP was not expressed in most embryos at 8 hpf (Figure 4.13; D-G) (except for the fragment PrP1-A1/A3-*4*) but slightly expressed in the telencephalon (Figure 4.10; E) and in notochord cells (Figure 4.12; E) of pEGFP-PrP1-A1/A3-*2* microinjected embryos at 48 hpf. In embryos at 48 hpf microinjected with the pEGFP-PrP1-A1/A3-*4* construct, EGFP expression was best observed in the brain (Figure 4.11; G) and Rohon-Beard neurons (Figure 4.12; G) and in embryos at 8 hpf randomly in embryonic cells (Figure 4.13; G, H). The two PrP genomic

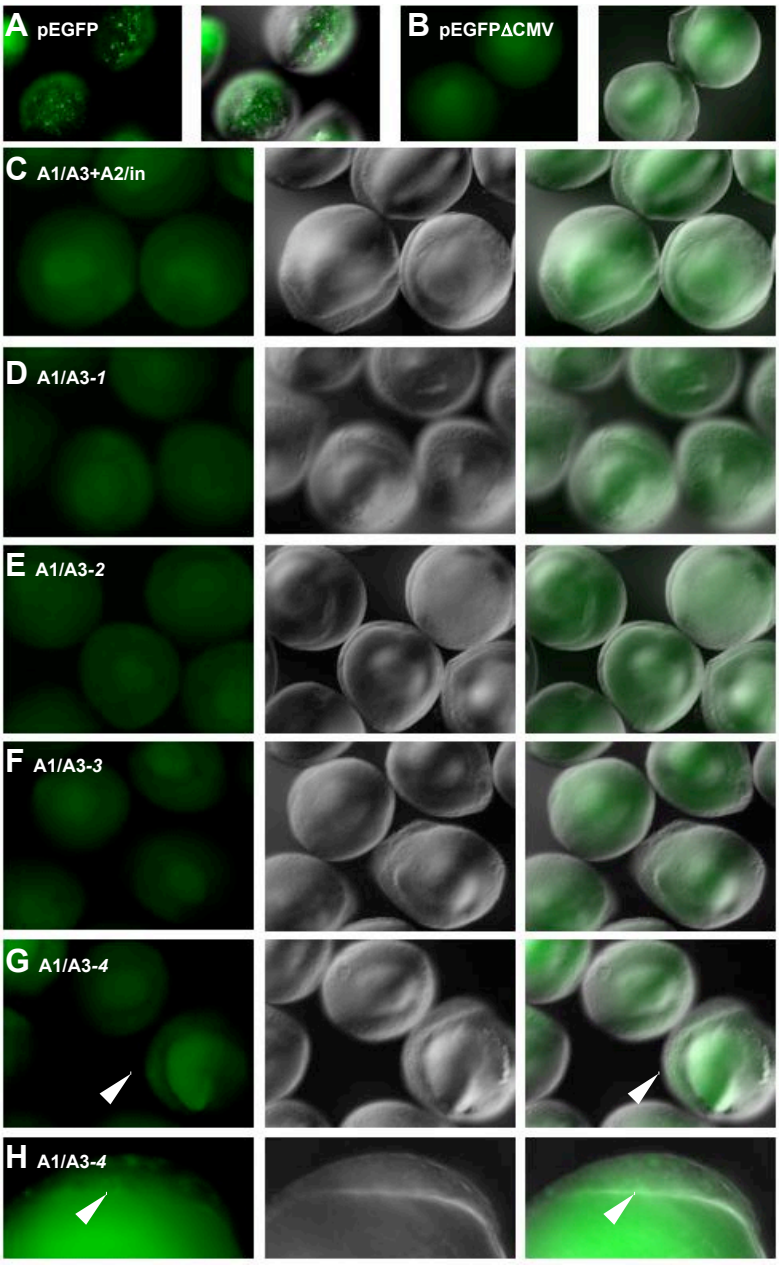


Figure 4.13: Images of zebrafish embryos at 8 hpf microinjected with the pEGFP-C1 vector **(A)** and the pEGFPΔCMV control **(B),** the PrP-1 tandem fragment **(C)** and the divided fragments of PrP1-A1/A3-*1* to -*4* **(D-H)**. EGFP expression was seen, although not as strong as in the positive control **(A)**, in embryos miccroinjected with the PrP1-A1/A3-*4* construct (arrows) **(G, H)**. A-B, fluorescent (right) and overlay images (left); C-G, fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

fragments further upstream (PrP1-A1/A3-*1* and -*3*) were neither able to drive expression in embryos at 8 hpf (Figure 4.13; D, F) nor in embryos at 48 hpf (Figure 4.10-4.12; D, F). Based on this data, it is likely that the promoter for PrP-1 is contained within the 500 bp sequence upstream of the transcription start site found in the PrP-genomic fragment PrP1-A1/A3-*4*. The PrP genomic fragment ~900 bp downstream of the transcription start site (PrP1-A1/A3-*2*) must contain regulatory elements like at least the promoter and one or part of one silencing binding motive that have to be examined (Figure 4.14).

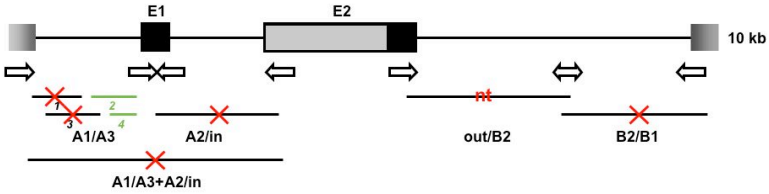


Figure 4.14: Schematic presentation of the *Prnp-1* gene locus and the regions found to contain regulatory regions (green; PrP1- A1/A3-*2* and -*4*). The other PrP-genomic sequences showed no promoter activity (crossed) or were not tested (nt).

4.1.4.2 EGFP expression under the control of PrP-2 genomic fragments

In order to test the intergenic sequences around the *Prnp-2* locus for promoter and/or regulatory elements, the same strategy as for *Prnp-1* was carried out. Since the *Prnp-2* (~40 kb) gene locus is almost twice the size as the *Prnp-1* locus, three constructs from the sequence upstream of the transcription start site and two constructs from the intron were obtained and tested in zebrafish embryos for their ability to drive EGFP expression. Embryos, microinjected with the PrP-2-intergenic fragment constructs, were allowed to develop until 48 hpf, fixed and analyzed. Immediately striking was the expression pattern observed in embryos, microinjected with the PrP2-A4/A6 construct (Figure 4.15, 4.17, 4.18; E) whose sequence lies directly upstream of the transcription start site; and embryos, microinjected with the PrP2-A5/A7 construct (first part of the intron) (Figure 4.15, 4.17, 4.18; F). Both showed conspicuous EGFP expression in contrast to the embryos microinjected with the other PrP-2 constructs, which exhibit only the meander-shaped EGFP background expression pattern in the brain (Figure 4.15 and 4.17; C, D, G) and EGFP background expression in blood cells (Figure 4.18; C, D, G) as seen for the PrP-1 genomic fragments and the promoterless control vector (Figure 4.15, 4.17, 4.18; B). Intense EGFP expression was observed for the PrP2-A4/A6-fragment in trigeminal ganglion neurons (Figure 4.15 and 4.17; E) and in Rohon-beard neurons (Figure 4.18; E). Embryos microinjected with the PrP2-A5/A7-fragment showed EGFP expression in the brain (Figure 4.15 and 4.17; F) and in a reticulated manner along the entire embryo (Figure 4.18; F). Therefore, the intron likely contains regulatory elements, which generate this expression pattern. Nevertheless, the PrP-2 promoter and regulatory elements sufficient to drive expression to neurons are present in the roughly 1400 bp sequence upstream of the transcription start. These results were verified with

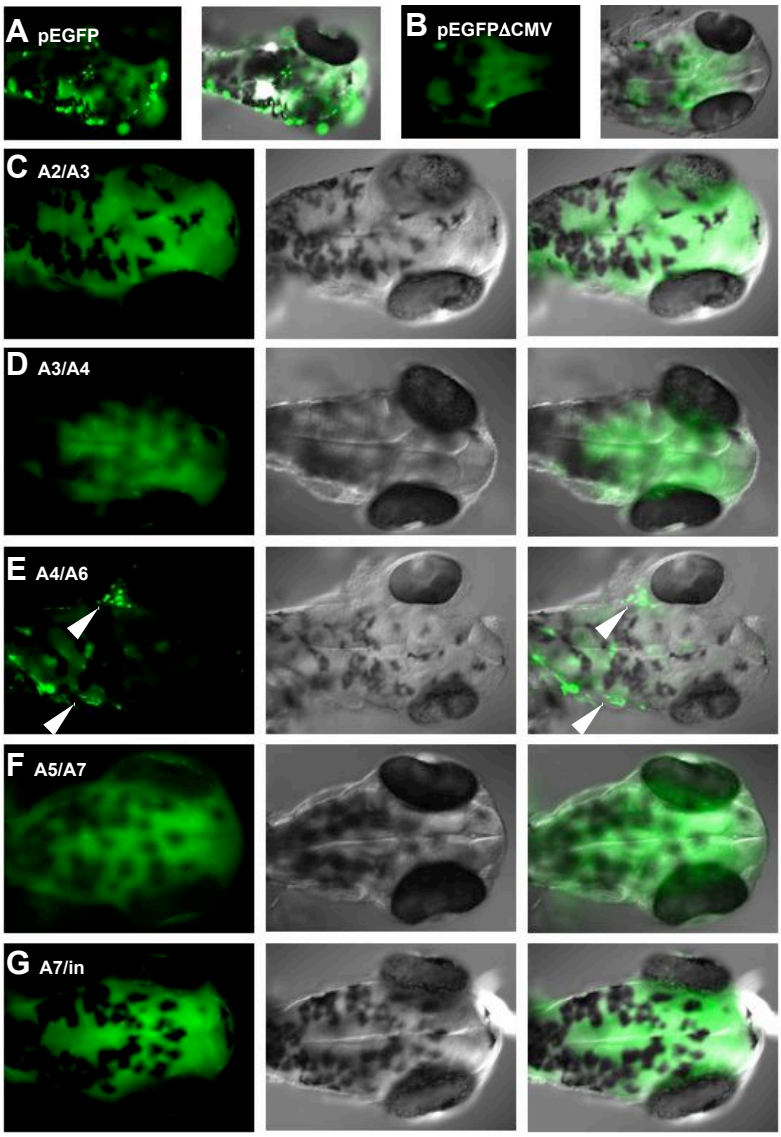


Figure 4.15: Dorsal view of the heads of zebrafish embryos at 48 hpf, microinjected with the PrP-2 genomic fragments. Most constructs **(C, D, G)** exhibited only background expression as seen in the pEGFPΔCMC control **(B)** but embryos microinjected with the PrP2-A4/A6 construct showed strong EGFP expression in trigeminal ganglion neurons (arrows) **(E)**. The pEGFP-C1 vector shows random expression **(A)**. A-B, fluorescent (right) and overlay images (left); C-G, fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

microinjections of the pEGFP-PrP2-A4/A6 construct into embryos at younger stages at 24 hpf (Figure 4.19) where the same expression pattern with expression in trigeminal ganglion cells (Figure 4.19; A, B) and Rohon-Beard neurons (Figure 4.19; C) was observed. These data demonstrate, that the ~1400 bp intergenic sequence, immediately upstream of the transcription start site of *Prnp-2* contains the promoter and an enhancer element which drives the expression in neural tissue like trigeminal- and Rohon-Beard neurons. The first part of the intron (~1500bp) probably contains regulatory elements that eventually modulated the background expression (Figure 4.16).

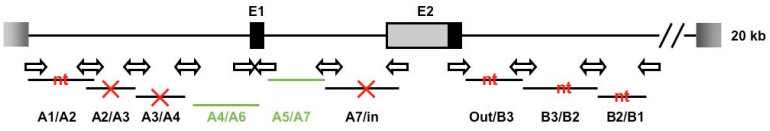


Figure 4.16: Schematic presentation of the *Prnp-2* gene locus and the regions found to contain regulatory regions (green; PrP2-A4/A6 and PrP2-A5/A7). The other PrP-2-genomic sequences showed no promoter activity (crossed) or were not tested (nt).

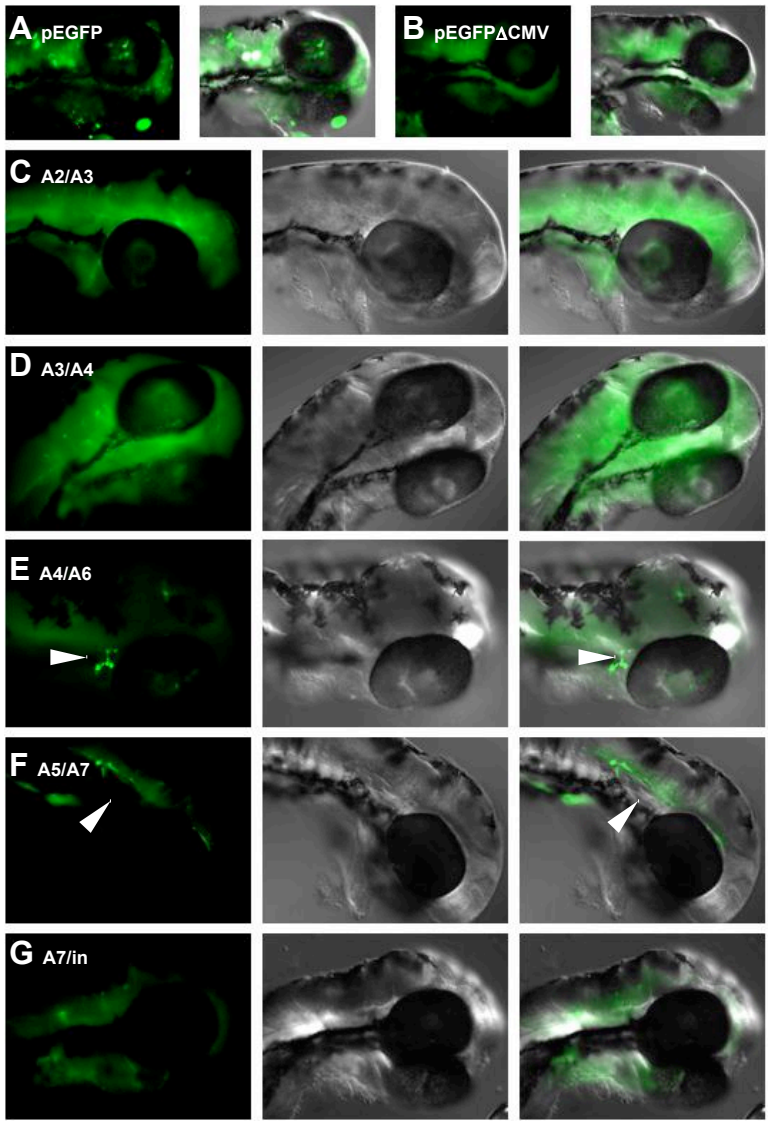


Figure 4.17: Lateral view of the heads of zebrafish embryos at 48 hpf microinjected with the PrP-2 genomic fragments. Most constructs **(C, D, G)** exhibited only background expression but embryos injected with the PrP2-A4/A6 construct showed strong EGFP expression in trigeminal ganglion neurons (arrow) **(E)** and in embryos microinjected with the first intron fragment, (PrP2-A5/A7) EGFP expression was observed in some structures in the brain (arrow) **(F)**. A-B, fluorescent (right) and overlay images (left); C-G, fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

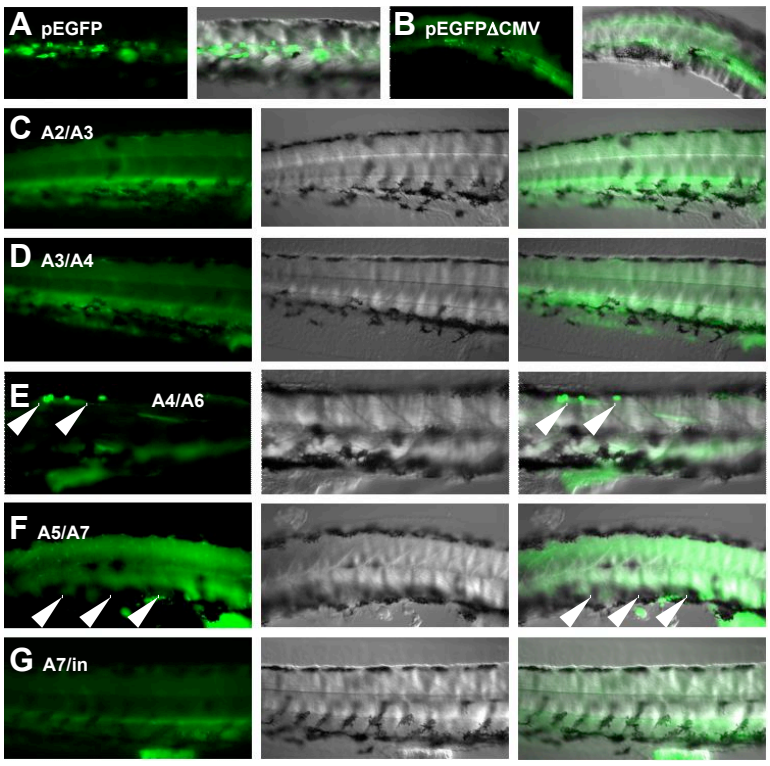


Figure 4.18: Lateral view of the trunks of zebrafish embryos at 48 hpf microinjected with the PrP-2 genomic fragments. In some embryos **(C, D, G)** only background expression was seen but EGFP was strongly expressed in Rohon-Beard neurons (arrows) in embryos microinjected with the PrP2-A4/A6 construct **(E)** and in a reticulated manner (arrows) in embryos microinjected with the first part of the intron (PrP2-A5/A7) **(F).** For image columns see figure 4.17.

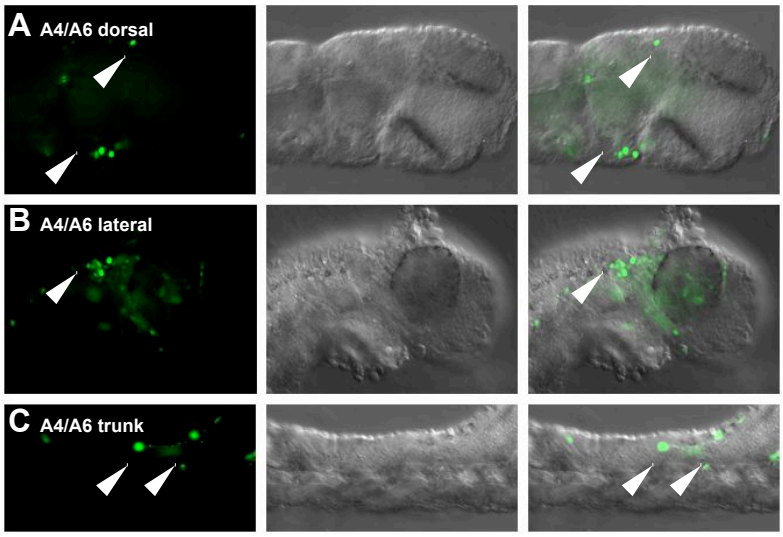


Figure 4.19: PrP2-A4/A6 microinjected embryos at 24 hpf. EGFP expression was seen in trigeminal ganglion neurons (arrows) in the dorsal **(A)** and lateral view **(B)** of the head and in Rohon-Beard neurons (arrows) in the trunk **(C)**. The columns display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

4.1.5 Expression of the pEGFP-PrP genomic fragments in N2a cells

In order to corroborate the results from the expression assays in zebrafish embryos, the same PrP-1 and -2 genomic fragment constructs were transiently transfected into mouse neuroblastoma N2a cells. As a transfection efficiency control, the pDsRed-monomer-N1 vector, which expresses the red fluorescent DsRed-monomer protein under the strong CMV promoter, was co-transfected. This positive control vector, mixed with the construct of interest prior to transfection, will enter the cell in similar amounts and thus can act as indicator for an effective transfection. The uniform expression of both constructs after transfection was controlled using fluorescence microscopy (not shown). Additionally, SDS-PAGE and Western blot analyses were performed using the monoclonal GFP antibody (2.2.4) against the 27 kDa EGFP and the polyclonal DsRed antibody (2.2.4) against the 27 kDa DsRed protein (Figure 4.20). As a positive control, the pEGFP-C1 vector was transfected, which showed a strong band at 27 kDa and smaller bands that present partial degradation of the protein in N2a cells. As expected, the pEGFP-C1 vector without the CMV promoter (pEGFP-C1ΔCMV), serving as negative control, showed no expression. In agreement with the results obtained using zebrafish embryos, none of the PrP-1 constructs exhibited EGFP expression (Figure 4.20; A). However, this result is not surprising, as PrP-1 seems to feature a more complex promoter structure than PrP-2 (4.1.1 and 4.1.4), which might be specific for expression in zebrafish and not functional in mouse cells. In contrast, N2a cells transfected with the pEGFP-PrP2-A4/A6 construct exhibit significant levels of expression (Figure 4.20; B). This result is consistent with the results from the zebrafish studies, where strong EGFP expression was also visible in pEGFP-PrP2-A4/A6 microinjected zebrafish embryos.

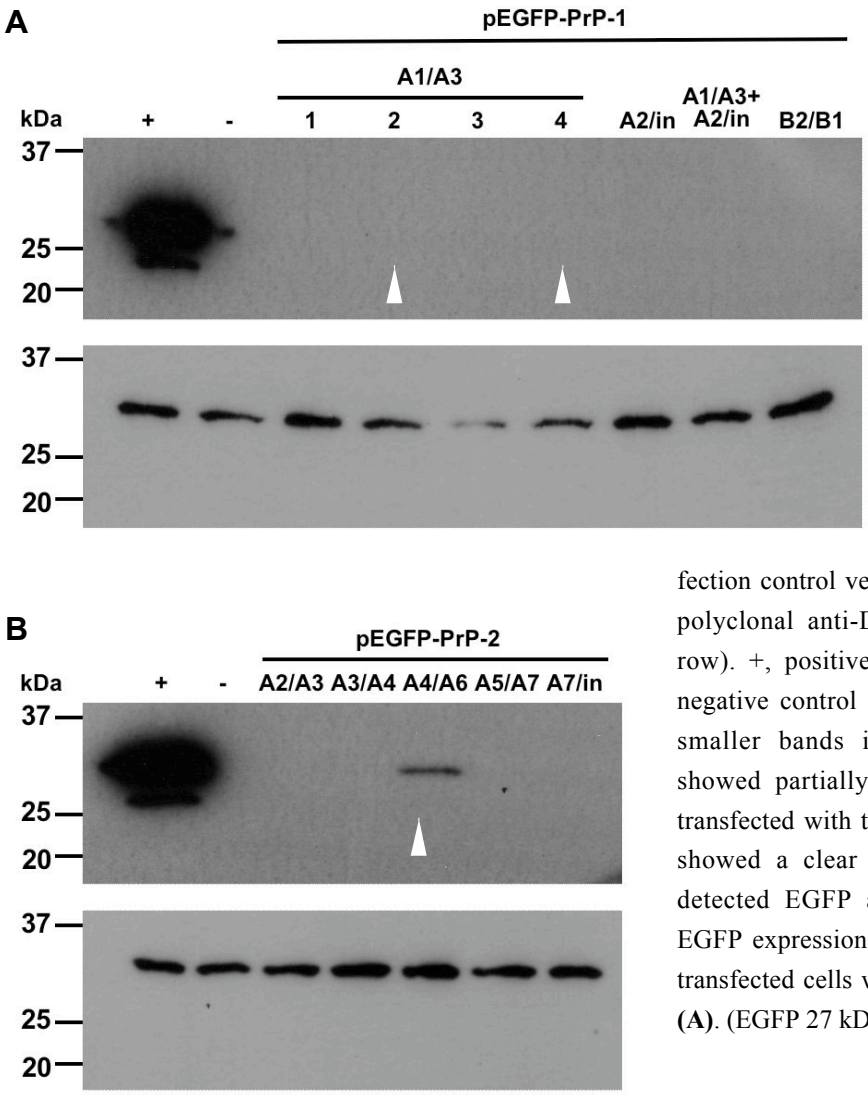


Figure 4.20: Western blot analyses of the pEGFP-PrP-1 **(A)** and pEGFP-PrP-2 **(B)** genomic fragments after transfection into N2a cells. Expression of EGFP through the fragments were detected using the monoclonal anti-GFP antibody (first row) and the CMV promoter driven DsRed cotransfection control vector expression using the polyclonal anti-DsRed antibody (second row). +, positive control (pEGFP-C1); -, negative control (pEGFP-C1ΔCMV). The smaller bands in the positive control showed partially degraded EGFP. Cells transfected with the PrP2-A4/A6 construct showed a clear band of expressed and detected EGFP at 27 kDa (arrow) **(B)**. EGFP expression of PrP1-A1/A3-*2* and -*4* transfected cells was not detected (arrows) **(A)**. (EGFP 27 kDa; DsRed 27 kDa)

4.1.6 Cloning of expression constructs with the zebrafish PrP-2 promoter

Having identified the zebrafish neuronal regulatory elements, it is now possible to generate transgenic fish that express EGFP-tagged prion proteins from different species under the zebrafish PrP promoters. To this end, zebrafish PrP-1, PrP-2 and mouse PrP were subcloned into the EGFP-PrP2-A4/A6 construct. Therefore, the entire EGFP was restricted (*Nhe*I/*Eco*RI) and replaced with previously cloned PrP-EGFP constructs

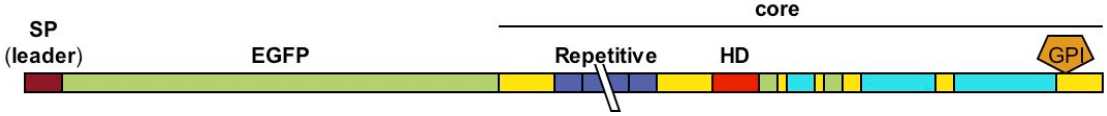


Figure 4.21: Schematic representation of the insertion site for EGFP between the signal peptide (SP, claret-red) and the PrP core protein including the repetitive domain (blue), the hydrophobic stretch (HD, red) and the GPI-anchor. Globular domain: green, β-sheets; cyan, α-helices. Modified from Rivera-Milla *et al.* (RIVERA-MILLA *et al.* 2006).

(SCHROCK, Diploma thesis 2006). These EGFP-PrP constructs contained EGFP inserted between the leader sequence (signal peptide), which is cut off post-translationally, and the core-GPI-anchor sequence (containing the repetitive domain, the hydrophobic stretch and the stable globular domain) of zebrafish and mouse PrPs (Figure 4.21).

Table 4.3: Cloned pEGFP constructs that drive EGFP-tagged PrP expression under the zebrafish PrP-2 promoter fragment (PrP2-A4/A6).

Construct	Previously cloned start vector	Target vector	Size [bp]
pEGFP-PrP2-A4/A6-PrP-1	pEGFP-PrP-1	pEGFPΔCMV-PrP2-A4/A6	856 (CR3), 146(L), 1677(C)
pEGFP-PrP2-A4/A6-PrP-2	pEGFP-PrP-2	pEGFPΔCMV-PrP2-A4/A6	856 (CR3), 221(L), 1485(C)
pEGFP-PrP2-A4/A6-PrP-Mo	PEGFP-PrP-Mo	pEGFPΔCMV-PrP2-A4/A6	1373 (A4/A6), 107 (L), 660 (C)

The constructs obtained (table 4.3) will now used for activity tests in zebrafish embryos and for the generation of stable transgenic zebrafish lines.

4.2 Targeted overexpression of PrP in zebrafish neurons

4.2.1 Amplification of the *Islet-1* enhancers

One of the major goals of our work is to understand the function of PrP in the nervous system, and whether this function (or the lack of it) is in any way related to the neuronal death observed during prion disease. Overexpressing EGFP-tagged PrP in zebrafish neurons *in vivo* can approach answering these questions. In order to target and overexpress the protein in neurons, three enhancer elements that normally regulate the expression of the zebrafish *Islet-1* transcription factor (1.4.2) in neurons, zCREST1, zCREST2 and zCREST3 (zebrafish conserved regulatory element for Isl1 1-3) (UEMURA *et al.* 2005) were chosen. Based on publicly available sequences (GenBank Accession numbers: AB158303, AB158304, AB158305), primers that include the appropriate restriction sites for sub-cloning (*Ase*I or *Nde*I) were designed (2.1.1 and 3.1.1) and the zCREST enhancer elements amplified using PCR from whole genomic zebrafish DNA. A gradient PCR was performed in order to determine the optimal primer annealing temperatures. Fortunately, all annealing temperatures tested, yielded comparable product amounts. Additionally, different dilutions of the genomic DNA, 1:10, 1:50 and 1:100 were tested and the clearest amplification results were obtained for the 1:50 dilution (Figure 4.22).

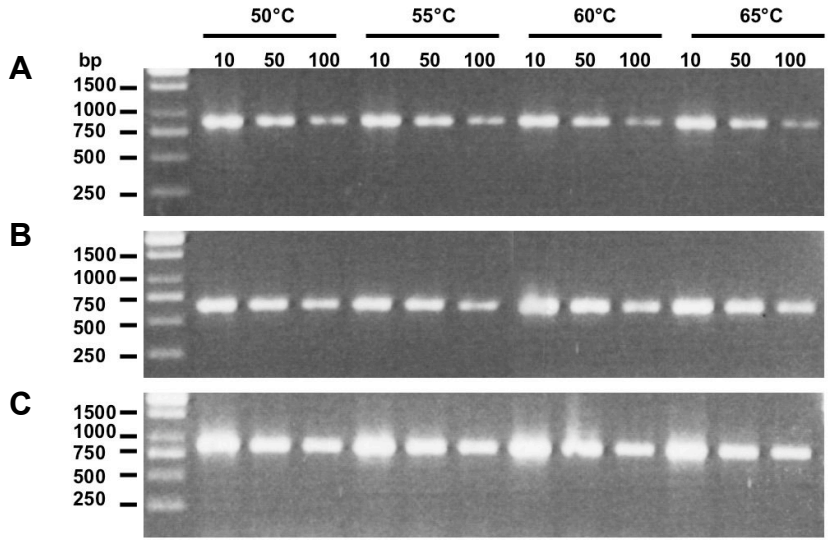


Figure 4.22: The gradient PCR for the amplification of **(A)** zCREST1, **(B)** zCREST2 and **(C)** zCREST3 was analyzed on a 1% (w/v) agarose gel. The primer annealing temperatures were chosen at 50°C, 55°C, 60°C and 65°C and yielded in comparable product amounts. Different dilutions of the genomic DNA (10, 1:10; 50, 1:50; 100, 1:100) represented increasing amounts of products. Predicted sizes: zCREST1, 886 bp; zCREST2, 683 bp; zCREST3: 856 bp.

To maintain the amplified zCREST sequences for subcloning (4.2.2) and sequencing, the PCR products were analyzed by agarose gel electrophoresis, extracted from the gel and primarily cloned into a pCRII-Topo vector (Invitrogen). The cloned enhancer sequences were verified by shotgun sequencing.

4.2.2 Cloning of pEGFP-zCREST constructs

In order to drive the expression of EGFP (and later PrP-EGFP) in neurons, the zCREST1, -2 and -3 sequences were subcloned upstream of the cytomegalovirus (CMV) promoter (*Ase*I restriction site) in the pEGFP-C1 vector (BD Biosciences Clontech) (Figure 4.23).

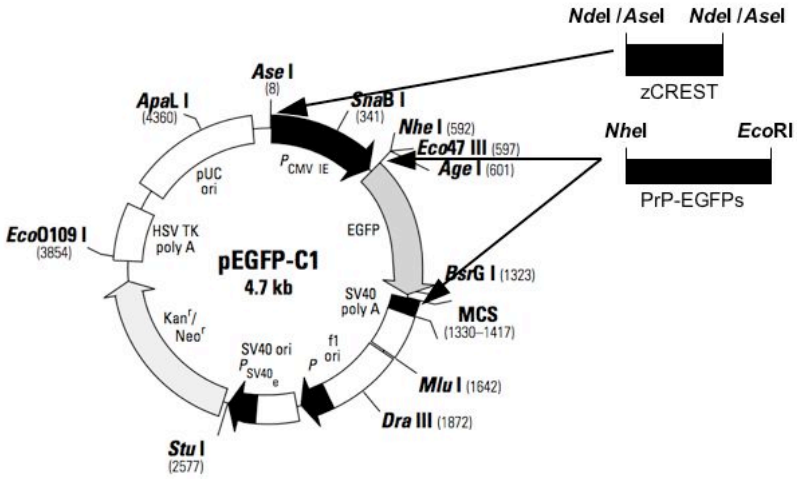


Figure 4.23: pEGFP-C1 vector (BD Biosciences Clontech) with representation of the insertion site for the zCREST enhancer sequences upstream of the cytomegalovirus (CMV) promoter (*Ase*I restriction site) and the insertion site for the PrP-EGFP fusion constructs.

Because all enhancers drive expression into different subsets of neurons (1.4.2), a tandem construct composed of zCREST2 and 3 was also cloned (zCREST2/3). For an overview, all pEGFP-zCREST constructs are listed in table 4.4. In addition, zCREST3 was subcloned into the pDsRed-monomer-N1 vector (BD Biosciences Clontech), which was later introduced as a control (4.2.5).

Table 4.4: pEGFP constructs with the *Islet-1* enhancers used in order to target the CMV promoter driven EGFP expression specifically to the corresponding neuronal cells. Shown are the primers used for amplification of the appropriate enhancer, the target vector and the size of the enhancer being cloned.

Construct	Primer	Target vector	Size [bp]
pEGFP-zCR1	zCREST1-AseI-f zCREST1-AseI-r	pEGFP-C1	886
pEGFP-zCR2	zCREST2-AseI-f zCREST2-AseI-r	pEGFP-C1	638
pEGFP-zCR3	zCREST3-NdeI-f zCREST3-NdeI-r	pEGFP-C1	856
pEGFP-zCR2/3	zCREST2-NdeI-f zCREST3-NdeI-r	pEGFP-C1	1494
pDsRed-monomer-N1-zCR3	zCREST3-NdeI-f zCREST3-NdeI-r	pDsRed-monomer-N1	856

4.2.3 Expression pattern studies of the pEGFP-zCREST constructs in zebrafish embryos

The pEGFP-zCREST (pEGFP-zCR) constructs were microinjected into zebrafish embryos and EGFP expression was monitored using a fluorescence stereomicroscope. As expression could be observed for all constructs, embryos were fixed and embedded for more detailed analyses. When solely under the control of the CMV promoter, strong, random and mosaic EGFP expression was seen throughout the embryo at 24 hpf (Figure 4.24 and 4.25; A). In contrast, under the regulation of zCREST1 (zCR1), expression of EGFP was enhanced in the forebrain, midbrain and hindbrain (Figure 4.24; B), possibly in cranial motor neurons as described by Uemura *et al*. (UEMURA *et al.* 2005). Lateral views of the trunk showed expression in the spinal chord and in motor neurons (Figure 4.25; B). On the other hand, injections of the pEGFP-zCREST3 (pEGFP-zCR3) construct resulted in strong expression in trigeminal ganglion neurons, their axons (Figure 4.24; D and 4.26; B) and Rohon-Beard neurons and their axons, dorsal to the spinal cord (Figure 4.25; D and 4.26; C). These results fit perfectly with published data, where zCR3 driven expression was also observed in trigeminal

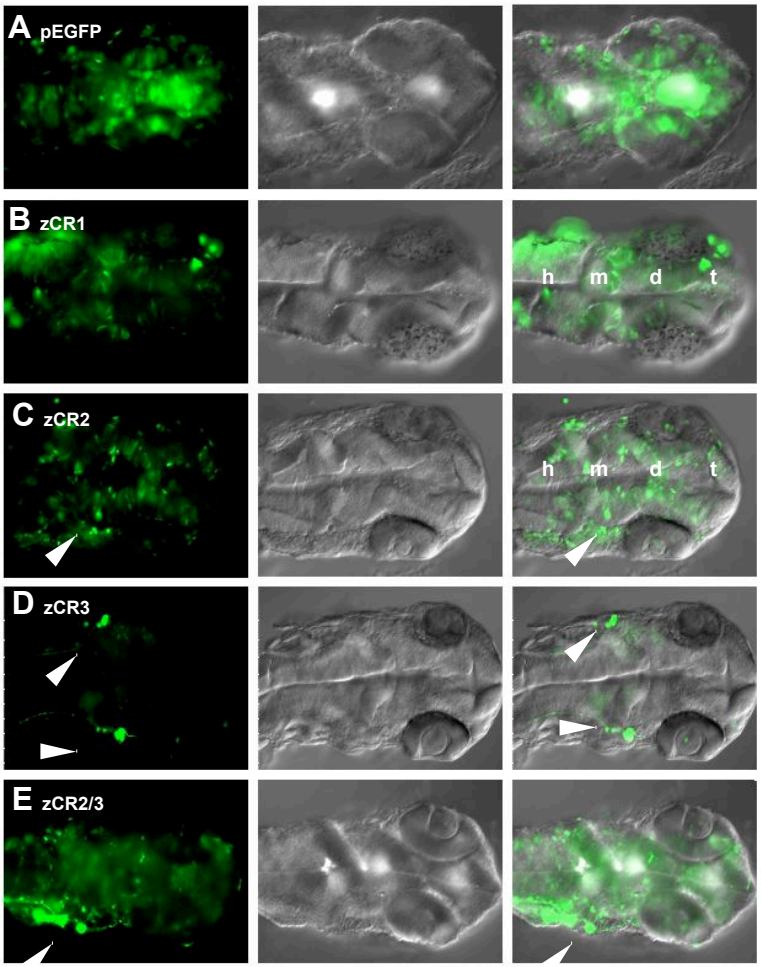


Figure 4.24: Dorsal views of the heads of zebrafish embryos at 24 hpf, microinjected with the pEGFP-zCR constructs. The pEGFP control **(A)** shows random expression. pEGFP-zCR1 microinjected embryos **(B)** showed strong expression in the fore- (telen- and diencephalon), mid- and hindbrain. In the pEGFP-zCR2 microinjected embryos **(C)**, expression was seen in the brain as well as in trigeminal ganglion neurons (arrow) and in pEGFP-zCR3 microinjected embryos **(D)** only in trigeminal ganglion neurons (arrows). The tandem-construct pEGFP-zCR2/3 **(E)** showed an expression pattern comparable to that of pEGFP-zCR2 solely. d, diencephalon; h, hindbrain; m, midbrain; t, telencephalon; tg, trigeminal ganglion neurons. The columns display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

ganglion neurons and Rohon-Beard neurons (UEMURA *et al.* 2005). Also consistent with the data from Uemura *et al.* is the expression pattern observed for the zCREST2 (zCR2) construct, which drove EGFP expression in both, sensory neurons (such as trigeminal ganglion neurons) and cranial motor neurons in the fore-, mid- and hindbrain (Figure 4.24; C). A more detailed view of the cranial motor neurons at 24 hpf is shown in figure 4.26 (A), where expression of the zCR2 construct can be seen in the facial (VII), trigeminal (V), trochlear (IV) and oculomotor (III) nerve neurons. Lateral views of the trunk display EGFP expression in notochord cells and Rohon-Beard neurons (Figure 4.25; C). As expected, the tandem construct including zCR2 and -3 showed expression

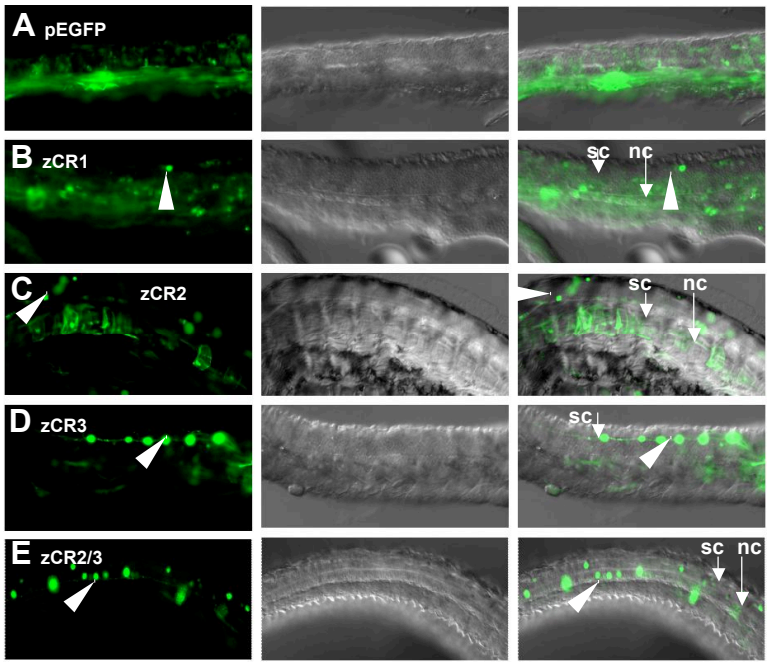


Figure 4.25: pEGFP-zCR transgenic zebrafish embryos at 24 hpf. The pictures show lateral views of the trunk. The control vector pEGFP **(A)** showed random expression. pEGFP-zCR1 microinjected embryos **(B)** displayed expression in the spinal chord (sc), in the notochord (nc) and in motor neurons (arrow). In embryos microinjected with pEGFP-zCR2 **(C)**, expression was observed in notochord cells (nc) and Rohon-Beard neurons (arrow). The pEGFP-zCR3 **(D)** and the tandem construct **(E)** also drove expression into Rohon-Beard neurons (arrows). The columns display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

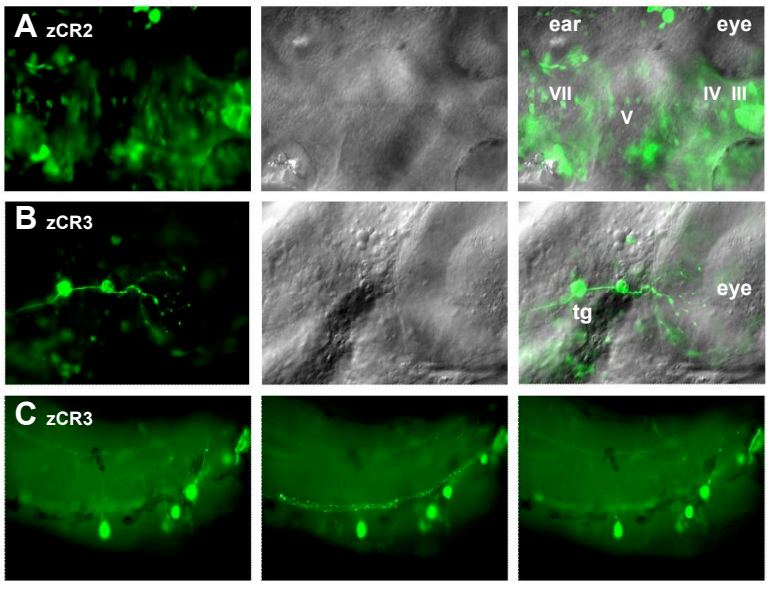


Figure 4.26: Detailed views of the cranial motor neurons of a zCR2 microinjected embryo at 24 hpf **(A)**, the trigeminal ganglion neurons **(B)** (tg) and Rohon-Beard neurons **(C)** after injection of zCR3 into embryos at 24 hpf. VII, facial nerve; V, trigeminal nerve; IV, trochlear nerve; III, oculomotor nerve. A, B display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right); C shows z-stack fluorescent images.

patterns comparable to zCR2 with EGFP expression in the brain, trigeminal ganglion cells (Figure 4.24; E) and Rohon-Beard neurons (Figure 4.25; E). Experiments with embryos at 48 hpf represented the same expression patterns for the zCR constructs as seen at 24 hpf (not shown). These experiments showed, that pEGFP-zCR3 worked best and drove EGFP expression into neurons similar to those where zebrafish PrP-2 is naturally expressed (trigeminal ganglion neurons, Rohon-beard neurons) (MÁLAGA-TRILLO *et al*. in review by Cell-press). Thus, all of the following steps were conducted with this construct.

4.2.4 Cloning of pEGFP-zCREST-PrP constructs

To examine the consequences of targeted overexpression of EGFP-tagged PrPs in zebrafish neurons, the PrP sequences from representative vertebrate species were subcloned into the pEGFP-zCR3 vector. The *Isl-1* zCR3 enhancer drives protein expression directly in sensory neurons such as trigeminal ganglion and Rohon-Beard neurons (4.2.3). Therefore, the pEGFP-zCR3 construct was used to target the overexpression of EGFP-tagged PrPs. The pEGFP-PrP constructs, where EGFP is in between the leader sequence and the core-GPI-anchor sequence of PrPs (4.1.6), were previously cloned in this lab (Figure 4.27) (SCHROCK, Diploma thesis 2006).

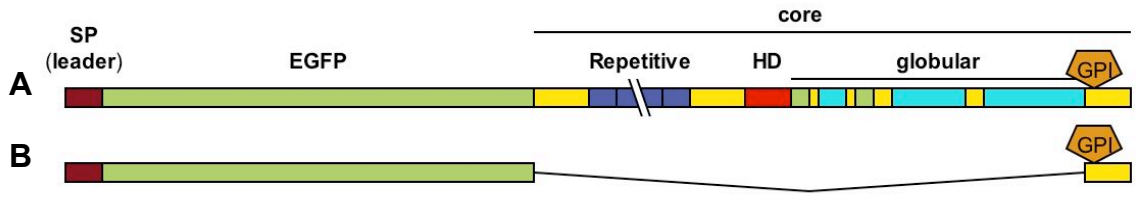


Figure 4.27: (A) Schematic representation of the insertion site for EGFP (green) between the signal peptide (SP) and the core protein (including the repetitive domain (blue) and the hydrophobic stretch (HD, red)). **(B)** In the PrP2(L+GPI) construct, the core sequence up to the GPI-anchor is absent. Globular domain: green, β-sheets; cyan, α-helices. Modified from Rivera-Milla *et al.* (RIVERA-MILLA *et al.* 2006).

For subcloning, the entire 'PrP-leader-EGFP-core-GPI' sequence was cut out of the pEGFP vector using *Nhe*I and *Eco*RI and inserted into the appropriate restriction sites in the pEGFP-zCR3 construct. The PrP sequences for zebrafish (PrP-1 and -2), mouse (PrP-mo), chicken (PrP-chk) and *Xenopus laevis* (PrP-xe) were subcloned into pEGFP-zCR3. As a negative control, a pEGFP-zCR3-PrP2 construct (pEGFP-zCR3-PrP2(L+GPI)) (Figure 4.27; B), featuring only the leader sequence (L) and the GPI anchor for PrP-2 without the core sequence was subcloned from a previously cloned construct (SCHROCK, Diploma thesis 2006). Three constructs with pEGFP-zCR2/3 and zebrafish PrP-1, -2 and mouse PrP were also cloned, but not further analyzed because the zCR3-PrP constructs gave optimal results (Table 4.5).

Table 4.5: Constructs for targeted overexpression of PrP in zebrafish neurons. Previously cloned pEGFP-PrP constructs with PrP in between the leader (L) and the core (C) sequence (SCHROCK, Diploma thesis 2006) were subcloned into pEGFP-zCREST enhancer constructs. Shown are the start and target vectors and the size of inserts.

Construct	Previously cloned start vector	Target vector	Size [bp]
pEGFP-zCR3-PrP-1	pEGFP-PrP-1	pEGFP-zCR3	856 (CR3), 146(L), 1677(C)
pEGFP-zCR3-PrP-2	pEGFP-PrP-2	pEGFP-zCR3	856 (CR3), 221(L), 1485(C)
pEGFP-zCR3-PrP-mo	pEGFP-PrP-m	pEGFP-zCR3	856 (CR3), 107(L), 660(C)
pEGFP-zCR3-PrP-chk	pEGFP-PrP-chk	pEGFP-zCR3	856 (CR3), 129(L), 713(C)
pEGFP-zCR3-PrP-xe	pEGFP-PrP-xe	pEGFP-zCR3	856 (CR3), 126(L), 547(C)
pEGFP-zCR3-PrP2(L+GPI)	pEGFP-PrP-2 GPI only	pEGFP-zCR3	856 (CR3), 221(L), 183(GPI)
pEGFP-zCR2/3-PrP-1	pEGFP-PrP-1	pEGFP-zCR2/3	1494 (CR2/3), 146(L), 1677(C)
pEGFP-zCR2/3-PrP-2	pEGFP-PrP-2	pEGFP-zCR2/3	1494 (CR2/3), 221(L), 1485(C)
pEGFP-zCR2/3-PrP-mo	pEGFP-PrP-m	pEGFP-zCR2/3	1494 (CR2/3), 107(L), 660(C)

4.2.5 Expression studies of the pEGFP-zCREST3-PrP constructs in zebrafish embryos

In order to analyze the effect of overexpressed PrP in neurons, the cloned constructs (4.2.4) were microinjected into zebrafish embryos at the one- to four-cell stage and embryos were allowed to develop until 24 and 48 hpf. The phenotype of zebrafish embryos at 48 hpf, microinjected with the different pEGFP-zCR3-PrP constructs was rather striking. As expected, the control zCR3 transgenic fish without tagged PrP strongly expressed EGFP in trigeminal ganglion neurons (Figure 4.28; A), as well as in Rohon-Beard neurons and their axons (Figure 4.30; A). In contrast, embryos microinjected with the pEGFP-zCR3-PrP constructs for zebrafish PrP-1 (Figure 4.28 and 4.30; C), zebrafish PrP-2 (Figure 4.28 and 4.30; D), mouse (Figure 4.28 and 4.30; E), chicken (Figure 4.28 and 4.30; F), and *Xenopus* (Figure 4.28 and 4.30; G) PrP consistently lacked PrP-EGFP expression in neurons. As weak background PrP-EGFP expression could be seen in blood-, myoseptum- and epithelia cells (Figure 4.28-4.30), these results hinted at possible neurotoxic effects of enhanced PrP expression in these neurons. Background expression in blood cells (Figure 4.30) was probably due to a promoter activity of the pEGFP vector (4.1.4). Alternatively, PrP overexpression might be not tolerated in neurons leading to an inhibition of expression or protein degradation. In order to control if the observed phenotypes exist only because of the neuronal overexpressed PrPs and to exclude expression inhibiting effects of the tagged EGFP, the PrP-2 construct containing only the leader and GPI-anchor sequence without the PrP-2 core (pEGFP-zCR3-PrP2(L+GPI)) (Figure 4.27;B), was also microinjected. Since this construct was expressed, even if weaker, in the expected neurons (Figure 4.28 and 4.30; B), an impairment of the correct biosynthesis pathway and processing of the EGFP-tagged PrPs can be ruled out.

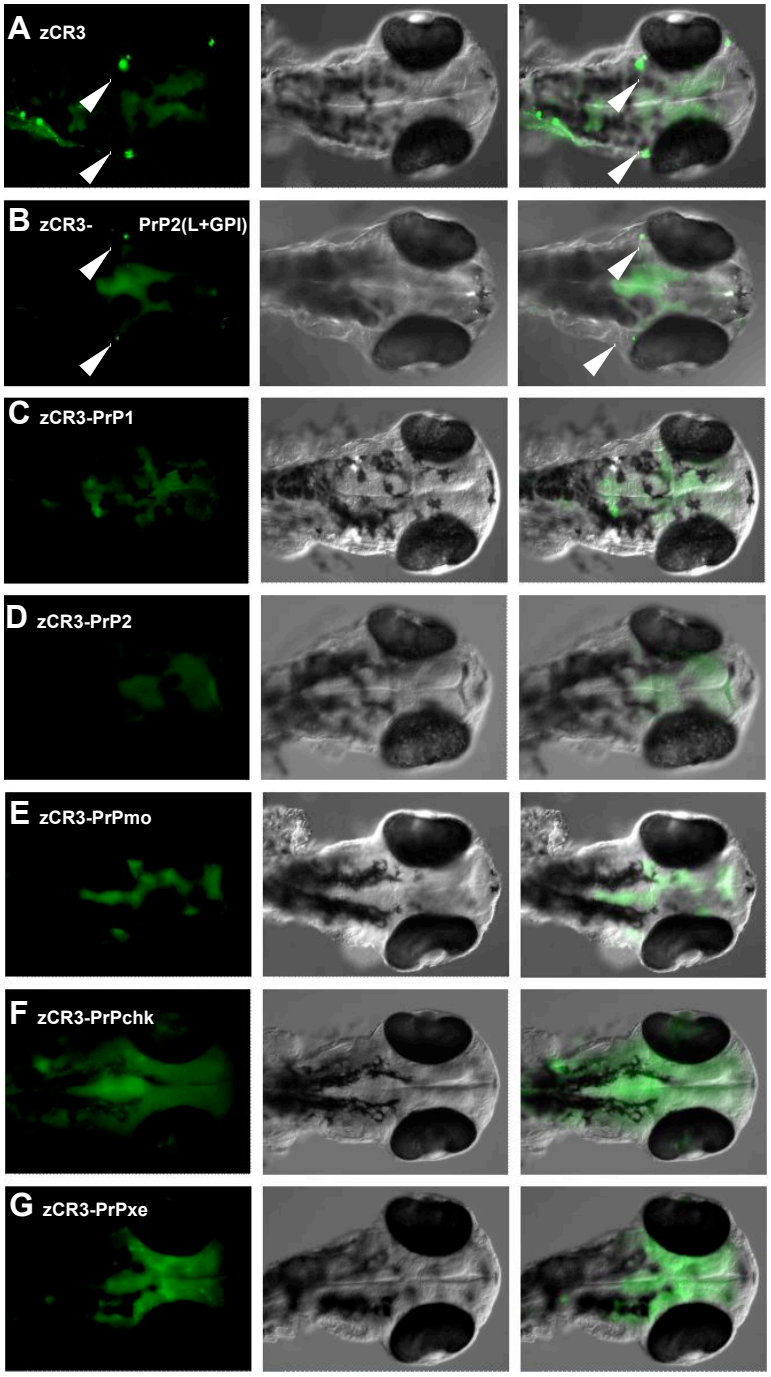


Figure 4.28: EGFP-tagged PrP transgenic zebrafish at 48 hpf. Dorsal pictures of the heads display in the controls: pEGFP-zCR3 microinjected zebrafish **(A)** and PrP-2 transgenic fish without the PrP core sequence (pEGFP-PrP2(L+GPI) **(B)**; a clear EGFP expression in trigeminal ganglion neurons (arrows). The EGFP-tagged PrP transgenic fish **(C-G)** lacked EGFP expression in the expected neurons. The columns display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

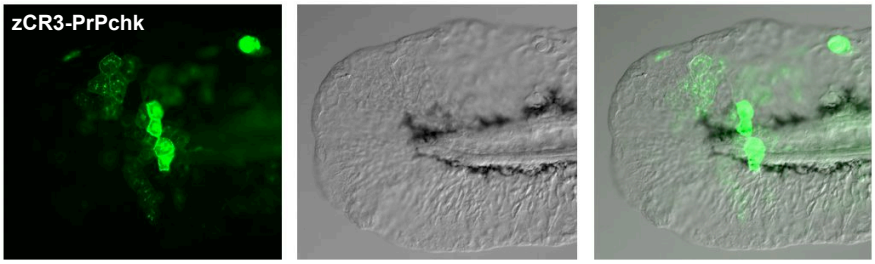


Figure 4.29: EGFP-tagged PrP chicken, expressed at the plasma membrane of epithelia cells in the caudal fin of a transgenic zebrafish at 48 hpf. Fluorescence image (left), Normarski image (center) and the overlay of fluorescence and Normaski image (right).

In order to further examine the assumption of possible toxic effects of PrP overexpression in neurons, co-injections of the pEGFP-zCR3-PrP constructs together with a pDsRed-monomer-zCR3 construct, used as an expression control, were conducted. Both constructs were mixed together at the same concentration and microinjected into early zebrafish embryos. In theory, both constructs reach the same nucleus and thus are expressed in a similar manner in the same cells. If DsRed is not expressed in EGFP-PrP coinjected embryos in trigeminal ganglion- and Rohon-Beard neurons, a loss or reduction of these neuronal structures as a result of PrP toxicity could have occurred. On the other hand, pDsRed expression in neuronal structures of EGFP-PrP coinjected embryos could showed that neurons were not impaired because of PrP overexpression and PrP expression is rather shut down. To test the specificity of the pDsRed-zCR3 control construct to target DsRed expression into neurons, the pDsRed vector and the "neuronal" pDsRed-zCR3 construct were microinjected into zebrafish embryos. Neuronal expression in trigeminal ganglion cells and Rohon-Beard neurons was clearly seen in pDsRed-zCR3 transgenic zebrafish (Figure 4.31 and 4.32; B), whereas the CMV promoter driven pDsRed control vector shows strong and random DsRed expression in epithelia and notochord cells among others (Figure 4.31 and 4.32; A). Because the injection of many different constructs into zebrafish embryos is very time-consuming, all the following experiments

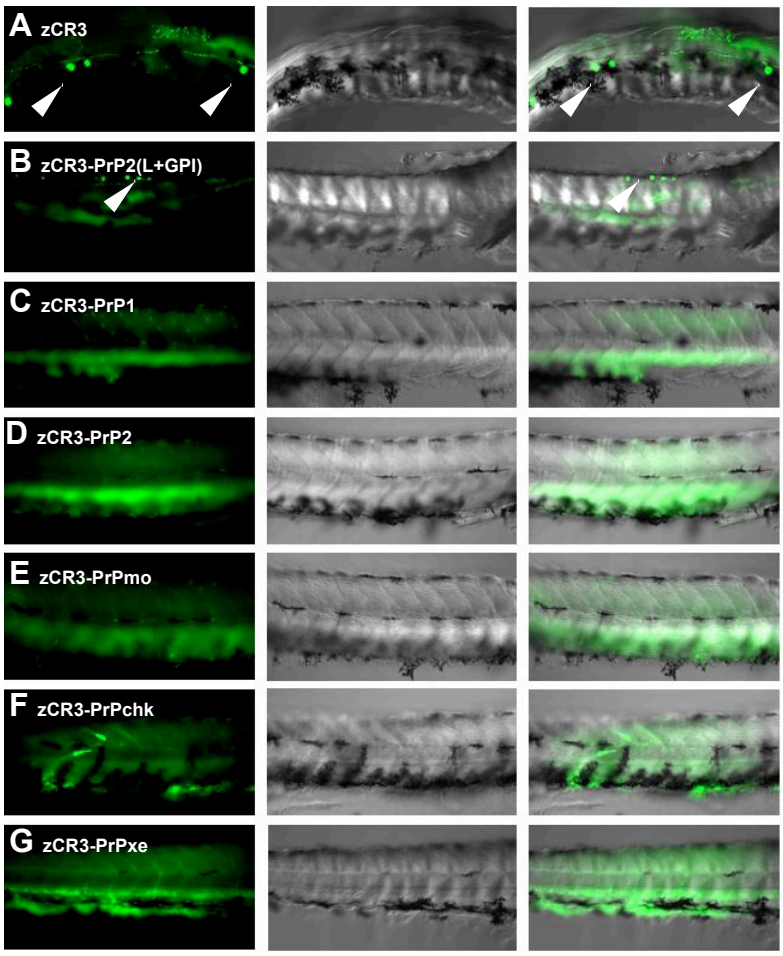


Figure 4.30: Lateral views of the trunk (ex. (A), dorsal view) of pEGFP-zCR3-PrP microinjected zebrafish at 48 hpf. The controls pEGFP-zCR3 **(A)** and pEGFP-zCR3-PrP-2(L+GPI) **(B)** microinjected embryos displayed strong EGFP expression in Rohon-Beard neurons (arrows). In contrast, pEGFP-zCR3-PrP microinjected embryos **(C-G)** showed no detectable neuronal EGFP expression above background level. The columns display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

were restricted to the most interesting constructs, which were zebrafish PrP-1, -2 and mouse PrP. As before, no EGFP-PrP expression could be observed in EGFP-tagged PrP and DsRed coinjected zebrafish (Figure 4.31 and 4.32; C-E), except for the mouse PrP transgenic fish, which showed little expression in a trigeminal ganglion (Figure 4.31; E). Remarkably, the coinjected embryos indeed showed strong DsRed expression in trigeminal ganglion neurons and Rohon-Beard neurons, indicating that the zCR3-PrP targeted neurons are still present and not lacked due to toxic overexpression of PrPs, but do not express the targeted EGFP-tagged PrPs. Since the EGFP and DsRed constructs without PrP could be successfully targeted and expressed in neurons, these results suggest that overexpression of the PrP-core sequences is specifically inhibited in these neurons. In view of the fact that weak expression could be observed in the mouse PrP microinjected embryos, PrPs from different species may not equally affect neurons or PrP indeed is expressed and expression is shut down after a certain time. Our analyses showed that targeted PrP expression was absent at 48 hpf, raising the question whether PrP expression

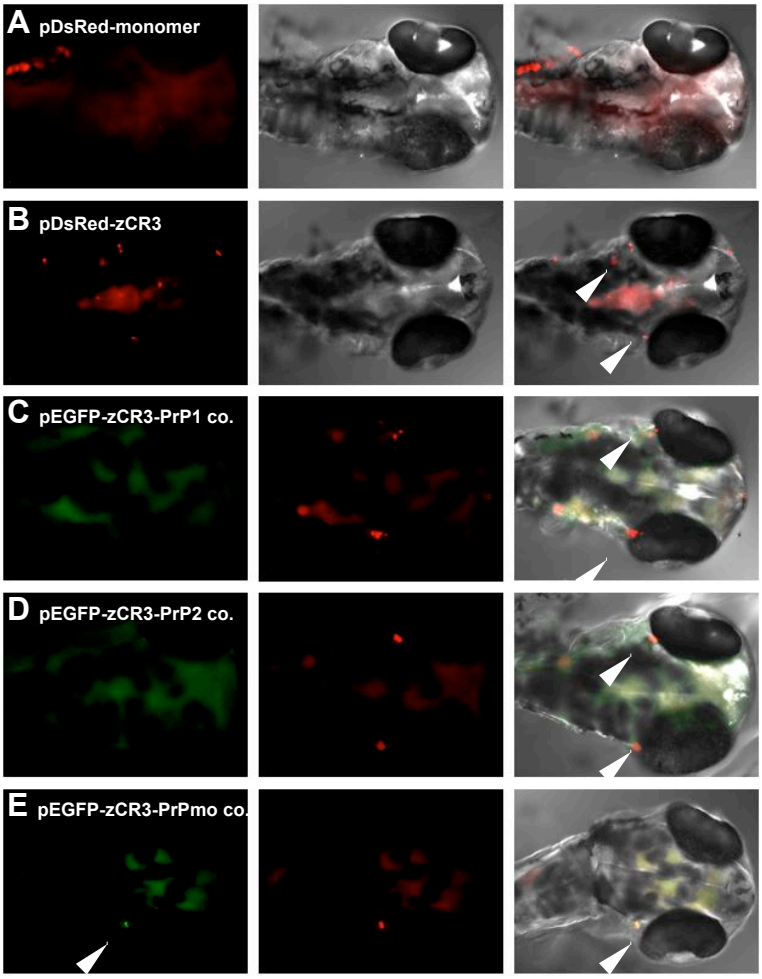


Figure 4.31: Dorsal view of the heads of zebrafish embryos at 48 hpf coinjected with pEGFP-zCR3-PrPs and pDsRed-zCR3. The microinjected control pDsRed vector **(A)** showed random expression. DsRed expression was directed by zCR3 to trigeminal ganglion neurons (arrows) **(B)**. **(C-E)** show the coinjected embryos. No EGFP expression was seen in PrP-1 **(C)** and PrP-2 **(D)** microinjected embryos whereas weak expression was detected in the trigeminal ganglion neurons of mouse PrP microinjected embryos **(E)**. In contrast, zCR3 driven DsRed expression was displayed in all coinjected embryos in the trigeminal ganglion neurons (arrows). co., coinjection with pDsRed-zCR3. The columns display in A and B fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right), C-E represent the EGFP fluorescence image (left), the pDsRed fluorescence image (center) and the overlay of both fluorescence and Normaski images (right).

took place at earlier stages and was then inhibited or it never took place. To distinguish between these two scenarios, the coinjection experiments were repeated and younger embryos at 24 hpf were analyzed. Interestingly, zebrafish PrP-2 and mouse PrP transgenic embryos at 24 hpf exhibit EGFP-tagged PrP expression in trigeminal ganglion neurons (Figure 4.33; E, F) and Rohon-Beard neurons (Figure 4.34; E, F) similar to the zCR3 and PrP2(L+GPI) microinjected controls (Figure 4.33, 4.34; B, C). Furthermore, expression of EGFP and DsRed is mostly observed in the same cells. Notably, embryos microinjected with the PrP-1 construct lacked EGFP expression although they expressed the pDsRed construct (Figure 4.33, 4.34; D). These results suggest that neuronally targeted PrP overexpression is developmentally downregulated. The reasons for this remain unclear but could be related to regulatory processes specific to neuronal differentiation, and do not exclude the possibility of PrP overexpression being neurotoxic at these stages. However, since PrP was successfully overexpressed in neurons despite others having reported that PrP overexpression is pathogenic (WESTAWAY *et al.* 1994b), further studies with neuronal markers were carried out to analyze the morphology of PrP overexpressing neurons (4.2.6).

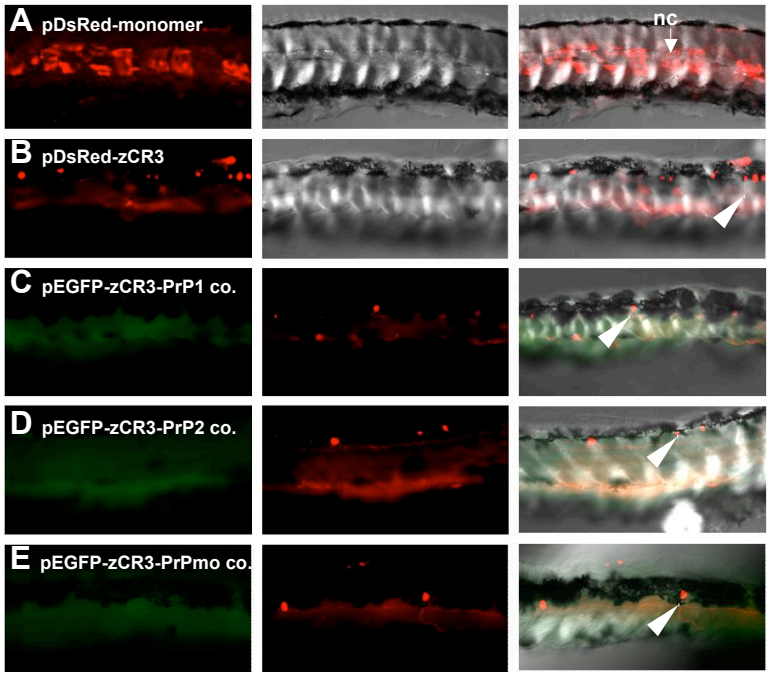


Figure 4.32: Lateral view of the trunks of coinjected embryos at 24 hpf. The pDsRed microinjected control **(A)** showed random DsRed expression mainly in notochord cells (nc). In contrast, pDsRed-zCR3 **(B)** microinjected embryos showed expression mainly in Rohon-Beard neurons (arrows). The pDsRed-zCR3 and pEGFP-zCR3-PrP coinjected embryos **(C-E)** showed DsRed expression in Rohon-Beard neurons (arrows) but no EGFP-tagged PrP expression. co., coinjection with pDsRed-zCR3. The columns display in A and B fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right), C-E represent the EGFP fluorescence image (left), the pDsRed fluorescence image (center) and the overlay of both fluorescence and Normaski images (right).

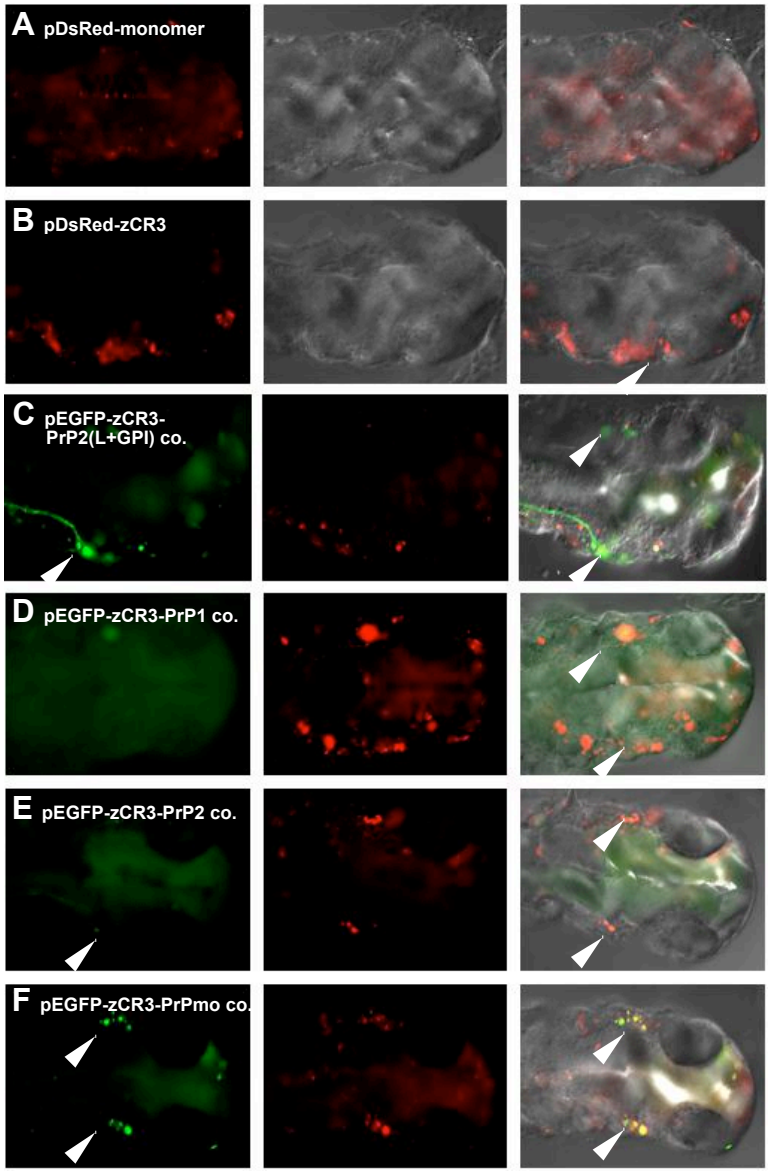


Figure 4.33: Dorsal view of the heads of zebrafish embryos at 24 hpf. **(A)** pDsRed control. The pDsRed-zCR3 control **(B)** displayed expression of DsRed in trigeminal ganglion neurons (arrows). **(C-F)** show the coinjected embryos. Unlike embryos at 48 hpf (Figure 4.31), these embryos indeed showed EGFP-PrP expression in trigeminal ganglion neurons (arrows; **E, F**) except for PrP-1 **(D)**, which showed no expression. The control construct PrP-2(L+GPI) **(C)** showed DsRed and EGFP expression in trigeminal ganglion neurons. The columns display in A and B fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right), C-F represent the EGFP fluorescence image (left), the pDsRed fluorescence image (center) and the overlay of both fluorescence and Normaski images (right).

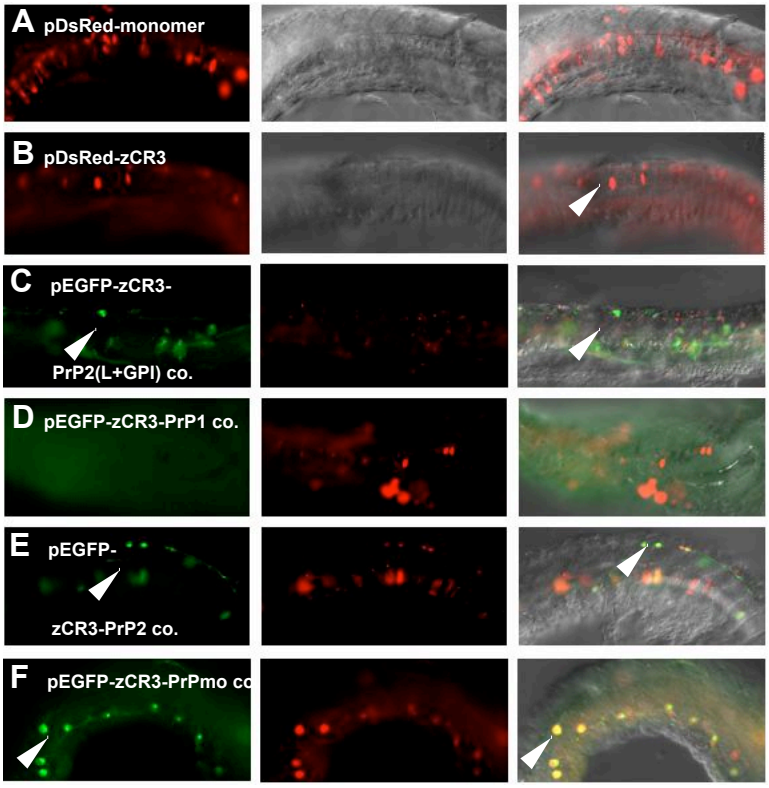


Figure 4.34: Lateral view (B, F dorsal) of the trunks of coinjected embryos at 24 hpf. The pDsRed control **(A)** showed random DsRed expression mainly in notochord cells (nc). In contrast, pDsRed-zCR3 microinjected embryos **(B)** showed expression mainly in Rohon-Beard neurons (arrow). The pDsRed-zCR3 and pEGFP-zCR3-PrP coinjected embryos **(C-F)** showed DsRed and EGFP-tagged PrP expression in Rohon-Beard neurons (arrows), except for PrP-1 **(D)**, which showed no EGFP expression. For image columns see figure 4.35.

4.2.6 Neuronal staining of EGFP-tagged PrP overexpressing transgenic zebrafish

The complex formation and connection of axon tracts in the zebrafish can easily be visualized using either antibodies against axonal cell-surface proteins or cytoskeletal proteins like acetylated tubulin, or through labeling axons by injection of horseradish peroxidase (HRP), and have been described extensively (CHITNIS and KUWADA 1990; ROSS *et al.* 1992; WILSON *et al.* 1990). Microtubules are the first cytoskeletal elements

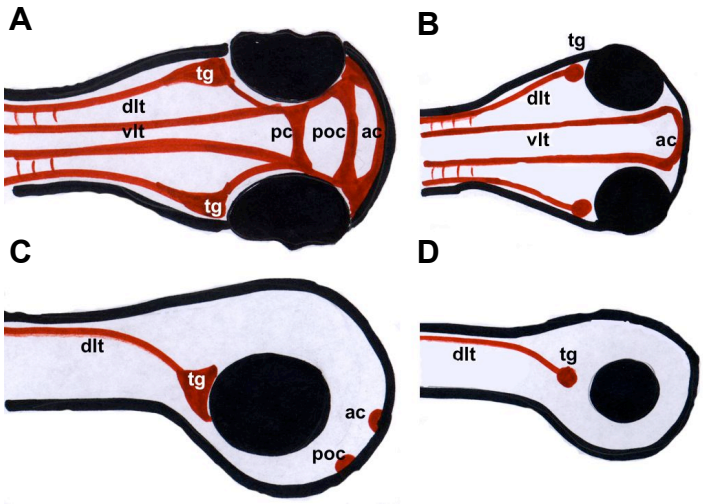


Figure 4.35: Schematic representation of axon tracts in embryos 48 hpf **(A; C)** and 24 hpf **(B; D)** in dorsal **(A; B)** and lateral **(C; D)** view. ac, anterior commissure; dlt, dorsal longitudinal tract; pc, posterior commissure; poc, postoptic commissure; tg, trigeminal ganglion neurons.

that appear in all neurons, and are made up of α- and β-tubulin. In contrast to non-neuronal tissue, neuronal α-tubulin is posttranslationally modified with acetyl groups (CAMBRAY-DEAKIN and BURGOYNE 1987). Since a neurotoxic effect of PrP overexpression on neurons cannot be excluded, the appearance and formation of axon tracts in zebrafish embryos that overexpress EGFP-tagged PrP compared to wild type (wt) embryos were analyzed using a monoclonal antibody against acetylated α-tubulin (PIPERNO and FULLER 1985) and a fluorescent secondary antibody (Cy3) (2.2.4). As a control, uninjected wt embryos that develop normally were used to identify variances in axon tracts or neuronal development compared to transgenic fish. In order to exclude neuronal impairments due to microinjection or the strong expression of membrane targeted proteins, pEGFP-zCR3 and PrP2(L+GPI) acted as additional controls. All main axon tracts (bundle of axons coursing together in the central nervous system) were stained in 24 hpf and 48 hpf embryos using the antibody mentioned above. The same tracts present after 24 hpf were also visible after 48 hpf but much broader and accompanied by additional new tracts (Figure 4.35). The axons of zCR3 expressing neurons (trigeminal ganglion neurons, Rohon-Beard neurons) were also stained. The immuno stained embryos in these experiments showed the same EGFP-PrP expression patterns

as mentioned in 4.2.5. At 24 hpf, embryos showed EGFP-PrP2 and EGFP-PrP-mo expression in trigeminal ganglion neurons (Figure 4.36 and 4.37; E, F) and Rohon-Beard neurons (Figure 4.38; E, F) similar to the controls (pEGFP-zCR3 and pEGFP-PrP2(L+GPI); Figure 4.36 - 4.38; B, C). PEGFP-zCR3-PrP1 microinjected embryos showed no (Figure 4.36 and 4.37; D) or minimal expression (Figure 4.38; D) in the expected neurons. Embryos at 48 hpf showed no EGFP-PrP expression (Figure 4.39-4.41; D-F). In contrast, the control embryos, microinjected either with pEGFP-zCR3 or pEGFP-PrP2(L+GPI) showed EGFP expression in trigeminal ganglion neurons (4.39 and 4.40; B, C) and Rohon-Beard neurons (4.41; B, C), although much weaker in PrP2(L+GPI) than in zCR3 transgenic embryos. For both stages, 24 and 48 hpf, no conspicuous differences between the main axon tracts of wt and transgenic embryos could be observed. In PrP overexpressing embryos, all axon tracts exist in the same manner as in the controls. Little variations are due to monitoring technique and to normally occurring differences in developing embryos. Therefore, neuronally targeted PrP

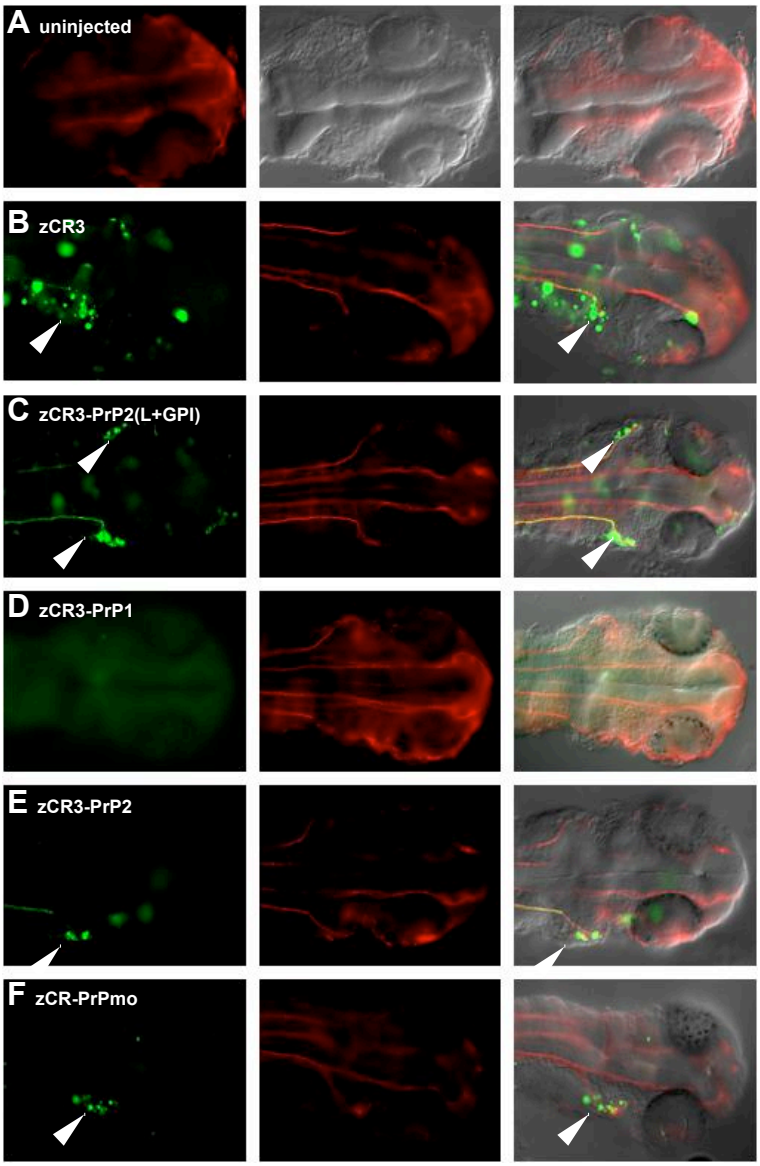


Figure 4.36: Dorsal views of the heads of pEGFP-zCR3-PrP microinjected zebrafish embryos at 24 hpf stained with antibodies against acetylated tubulin. The uninjected **(A)** control embryos, the pEGFP-zCR3 **(B)** and PrP-2(L+GPI) **(C)** microinjected embryos showed no differences in the main axon tracts compared to PrP microinjected embryos **(D-F)**. EGFP-PrP microinjected embryos showed expression in trigeminal ganglion cells (arrows), except for PrP-1 **(D)**. The columns in A display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right), B-F represent the EGFP fluorescence image (left), the pDsRed fluorescence image (center) and the overlay of both fluorescence and Normaski images (right).

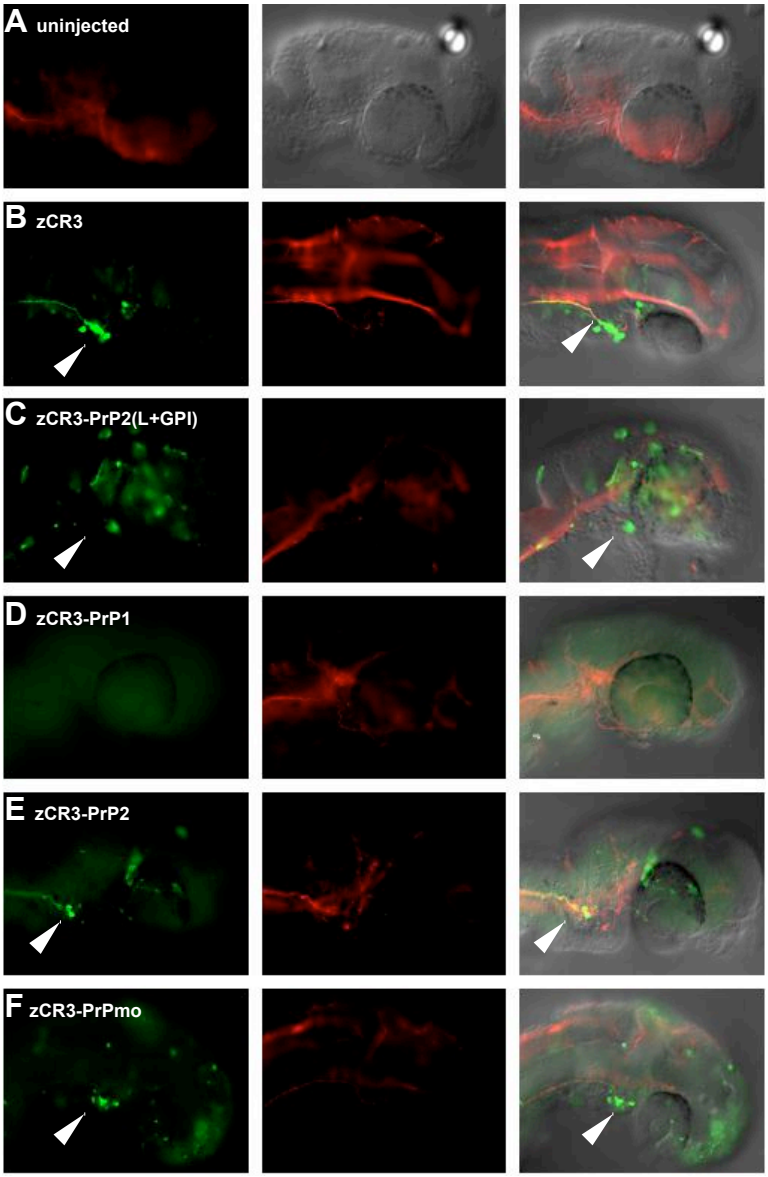


Figure 4.37: Lateral views of the heads of pEGFP-zCR3-PrP microinjected zebrafish embryos at 24 hpf stained with antibodies against acetylated tubulin. The uninjected **(A)** control embryos, the pEGFP-zCR3 **(B)** and PrP-2(L+GPI) **(C)** microinjected embryos showed no differences in the main axon tracts compared to PrP microinjected embryos **(D-F)**. EGFP-PrP microinjected embryos showed expression in trigeminal ganglion cells (arrows), except for PrP-1 **(D)**. The columns in A display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right), B-F represent the EGFP fluorescence image (left), the pDsRed fluorescence image (center) and the overlay of both fluorescence and Normaski images (right).

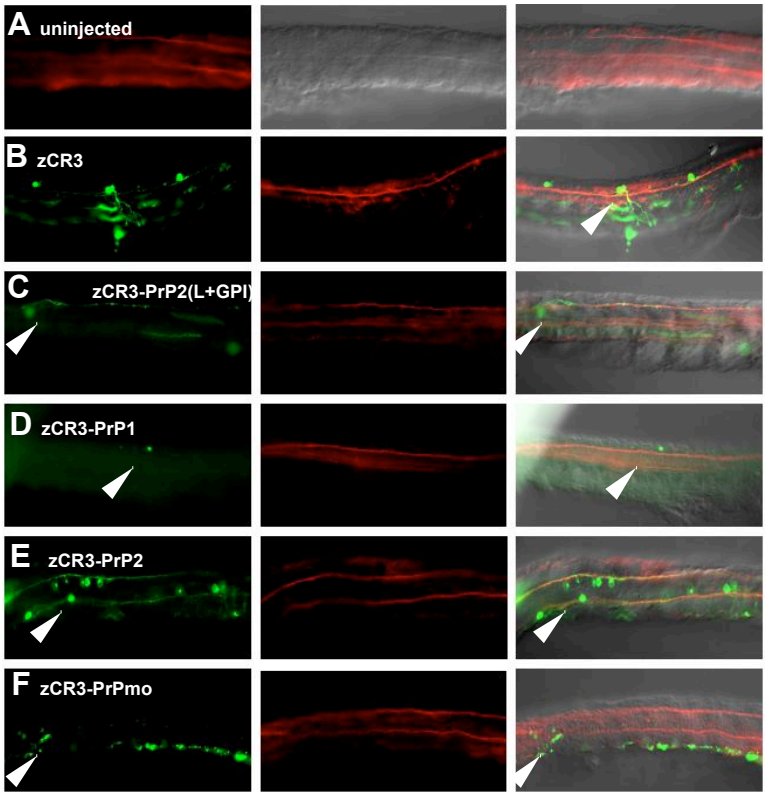


Figure 4.38: Lateral views of the trunks of pEGFP-zCR3-PrP microinjected zebrafish embryos at 24 hpf stained with antibodies against acetylated tubulin. The uninjected **(A)** control embryos, the pEGFP-zCR3 **(B)** and PrP-2(L+GPI) **(C)** microinjected embryos showed no differences in the main axon tracts compared to PrP microinjected embryos **(D-F)**. EGFP-PrP microinjected embryos showed expression in Rohon-Beard neurons (arrows), except for PrP-1 **(D)**. For image columns see figure 4.33.

overexpression does not appear to affect axogenesis. Similar *in vivo* PrP overexpression experiments have been carried out in *Xenopus* pituitary cells and no mutated cell structure or malfunctioning could be observed (VAN ROSMALEN and MARTENS 2006a). Furthermore, overexpression of PrP in a stroke rat model showed a neuroprotective function of PrP rather than a neurotoxic one (SHYU *et al.* 2005) and mice motorneurons that overexpress PrP displayed better survival after facial nerve section than wt mice (COULPIER *et al.* 2006). Nevertheless, it is remarkable that embryos after 24 hpf expressed EGFP-tagged PrP and embryos at 48 hpf did not.

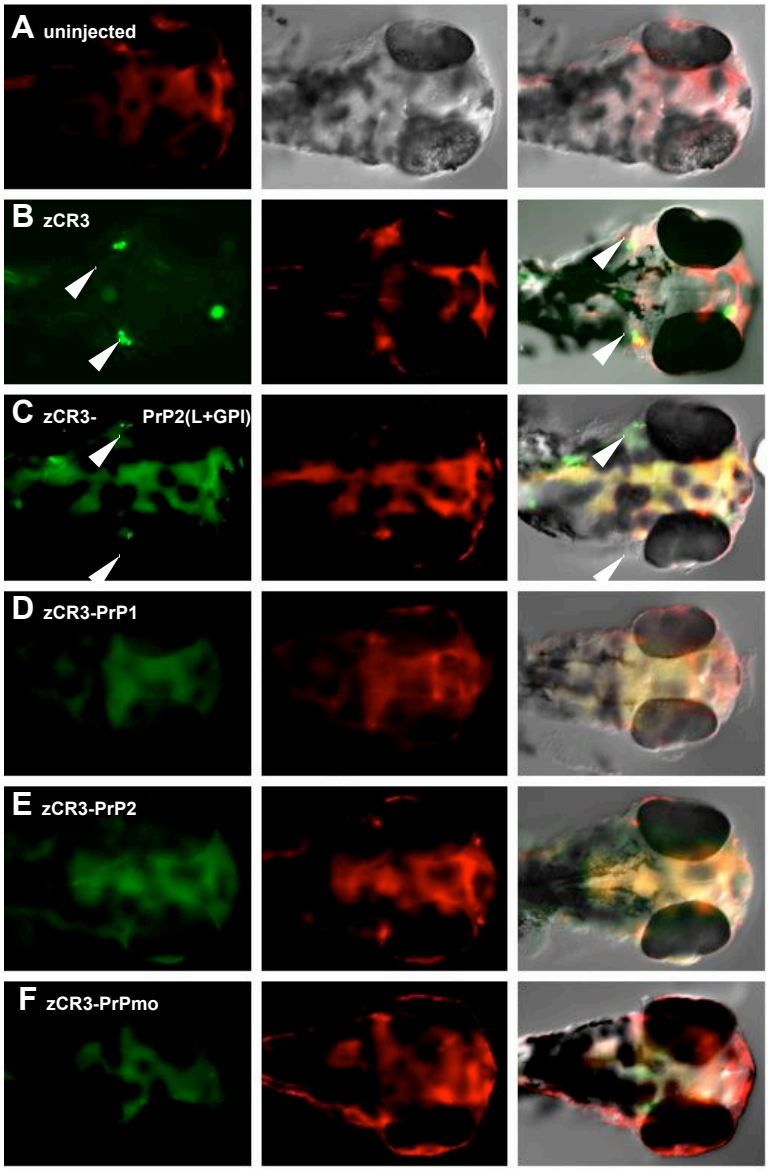


Figure 4.39: Dorsal views of the heads of pEGFP-zCR3-PrP microinjected zebrafish embryos at 48 hpf stained with antibodies against acetylated tubulin. The uninjected **(A)** control embryos, the pEGFP-zCR3 **(B)** and PrP-2(L+GPI) **(C)** microinjected embryos showed no differences in the main axon tracts compared to PrP microinjected embryos **(D-F)**. No EGFP-PrP expression was detected in PrP microinjected embryos but in trigeminal ganglion neurons of zCR3 (arrows) **(B)** and PrP2(L+GPI) (arrows) **(C)** transgenic embryos. The columns in A display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right), B-F represent the EGFP fluorescence image (left), the pDsRed fluorescence image (center) and the overlay of both fluorescence and Normaski images (right).

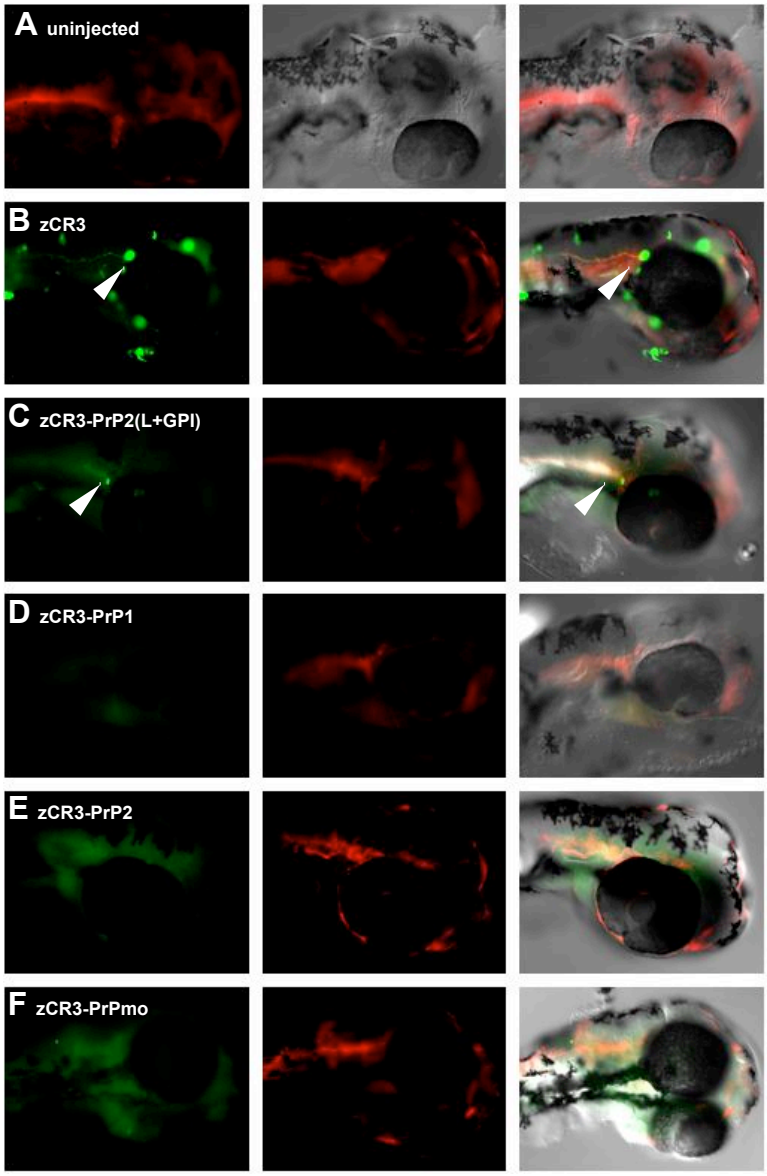


Figure 4.40: Lateral views of the heads of pEGFP-zCR3-PrP microinjected zebrafish embryos at 48 hpf stained with antibodies against acetylated tubulin. The uninjected control embryos **(A)**, the pEGFP-zCR3 **(B)** and PrP-2(L+GPI) **(C)** microinjected embryos showed no differences in the main axon tracts compared to PrP microinjected embryos **(C-F)**. No EGFP-PrP expression was detected in PrP microinjected embryos **(D-F)** but in trigeminal ganglion neurons of zCR3 (arrows) **(B)** and PrP2(L+GPI) arrows) **(C)** transgenic embryos. The columns in A display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right), C-F represent the EGFP fluorescence image (left), the pDsRed fluorescence image (center) and the overlay of both fluorescence and Normaski images (right).

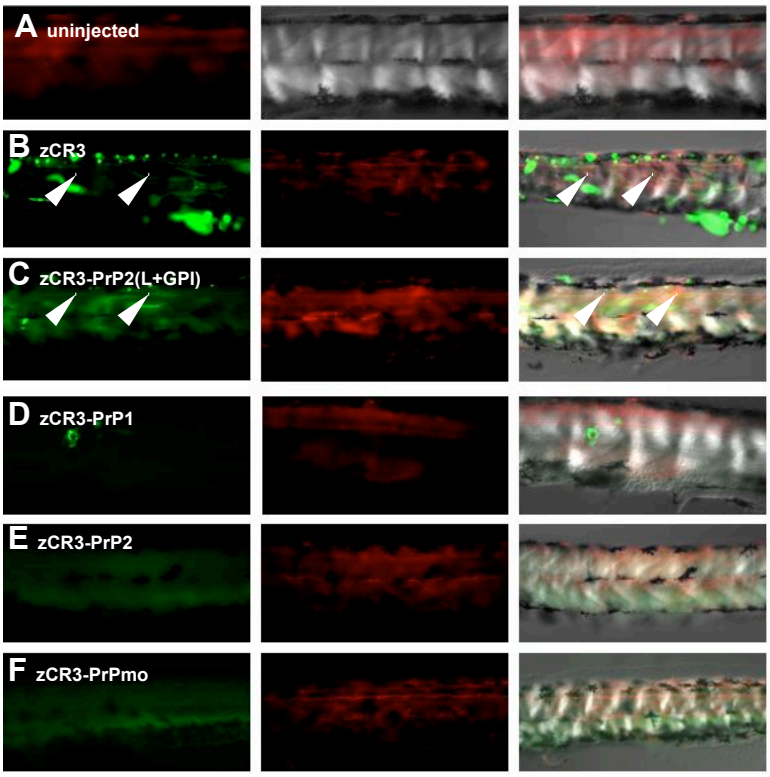


Figure 4.41: Lateral views of the trunks of pEGFP-zCR3-PrP microinjected zebrafish embryos at 48 hpf stained with antibodies against acetylated tubulin. The uninjected **(A)** control embryos, the pEGFP-zCR3 **(B)** and PrP-2(L+GPI) **(C)** microinjected embryos showed no differences in the main axon tracts compared to PrP microinjected embryos **(D-F)**. No EGFP-PrP expression was detected in PrP microinjected embryos but in Rohon-Beard neurons of zCR3 (arrows) **(B)** and PrP2(L+GPI) (arrows) **(C)** transgenic embryos. For image columns see figure 4.36.

4.2.8 Amplification of the zebrafish Hsp70 promoter

Because the developmental expression of zebrafish PrPs is not only spatially but also temporally regulated (natural occurring PrP-2 has a expression peak at 30 hpf (1.3.3)) the zebrafish heat shock protein 70 (Hsp70) promoter was cloned as a heat inducible promoter for a temporally regulated expression of the pEGFP-zCR3-PrP constructs.

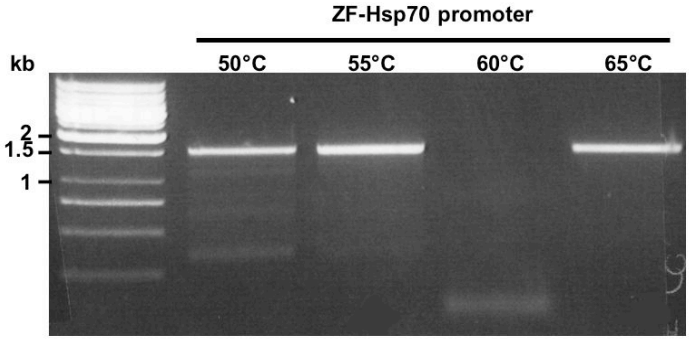


Figure 4.42: The gradient PCR of the amplification of the ZF-Hsp70 promoter was analyzed on a 1% (w/v) agarose gel. The clearest band was obtained for an annealing temperature of 65°C. Predicted size: 1591 bp.

Furthermore, the zebrafish Hsp70 promoter is more likely to exhibit less background staining compared to the strong viral CMV promoter. Primers with the appropriate restriction sites (*Nde*I and *Nhe*I) for cloning into the pEGFP-C1ΔCMV vector were designed on the basis of available sequences (HALLORAN *et al.* 2000) (GenBank Accession number: AF158020). The Hsp70 promoter was amplified by means of gradient PCR (Figure 4.42) as described (4.1.2; 4.2.1) and cloned into the pCRII-Topo vector.

Table 4.6: Instead of the CMV promoter, the temporally adjustable Hsp70 promoter was cloned into the pEGFP-C1 vector. Shown are the corresponding primers to amplify the promoter, the target vector and the size of the insert.

Construct	Primer	Target vector	Size [bp]
pEGFP-ZF-Hsp70Prom	ZF-Hsp70-Chr8-NdeI-f ZF-Hsp70-Chr8-NheI-r	pEGFP-C1ΔCMV	1591

For EGFP expression, the vector was subcloned into the pEGFP-C1ΔCMV vector as described (4.1.2). Table 4.6 displays the data for this construct.

4.2.9 GFP expression through the heat-inducible Hsp70 promoter in zebrafish

The family of heat shock proteins comprises proteins that are expressed in all organisms under normal conditions, often acting as chaperones. Some members of this family are specialized for expression under stress conditions, as for instance the Hsp70 family, which is highly expressed during thermal stress (KREGEL 2002). To test the cloned Hsp70 promoter for its ability to express the EGFP, zebrafish embryos were microinjected with the pEGFP-ZF-Hsp70Prom construct and allowed to develop at 28°C

before applying for one hour a 37°C heat shock at 30 hpf. It was recently reported that the *Hsp70* gene is expressed naturally during normal development of zebrafish in the lens from 28 to 38 hpf (BLECHINGER *et al.* 2002). In order to see this natural expression, zebrafish embryos were chosen at 30 hpf. At this time, one half of the embryos were heat shocked for one hour at 37°C in order to initiate EGFP expression, whereas the other half was further incubated at 28°C as a control. Blechinger *et al.* described that the *Hsp70* gene is expressed throughout the embryo after one hour of heat shock at 37°C (BLECHINGER *et al.* 2002). But the preliminary results were inconsistent with these data. No EGFP expression was visible in the lens at 31 hpf without heat shock (Figure 4.43; A, C, E) and only weak expression was seen immediately after one hour at 37°C (Figure 4.43; B, D, F). Since no data were available about the time that is necessary to visualize expression of EGFP after heat shock, additional rounds of experiments with time series and with older embryos were performed. After 46 hpf at 28°C without heat shock, the embryos clearly showed natural EGFP expression in the lens (Figure 4.44; A). After one hour heat shock at 37°C with embryos at 50 hpf, the embryos were kept again at 28°C and EGFP expression was monitored at 51 hpf (not shown), 52, 53, 55 and 72 hpf. Consistent with the previous data, no EGFP expression was visible after one hour heat shock (not shown), and no up regulation was

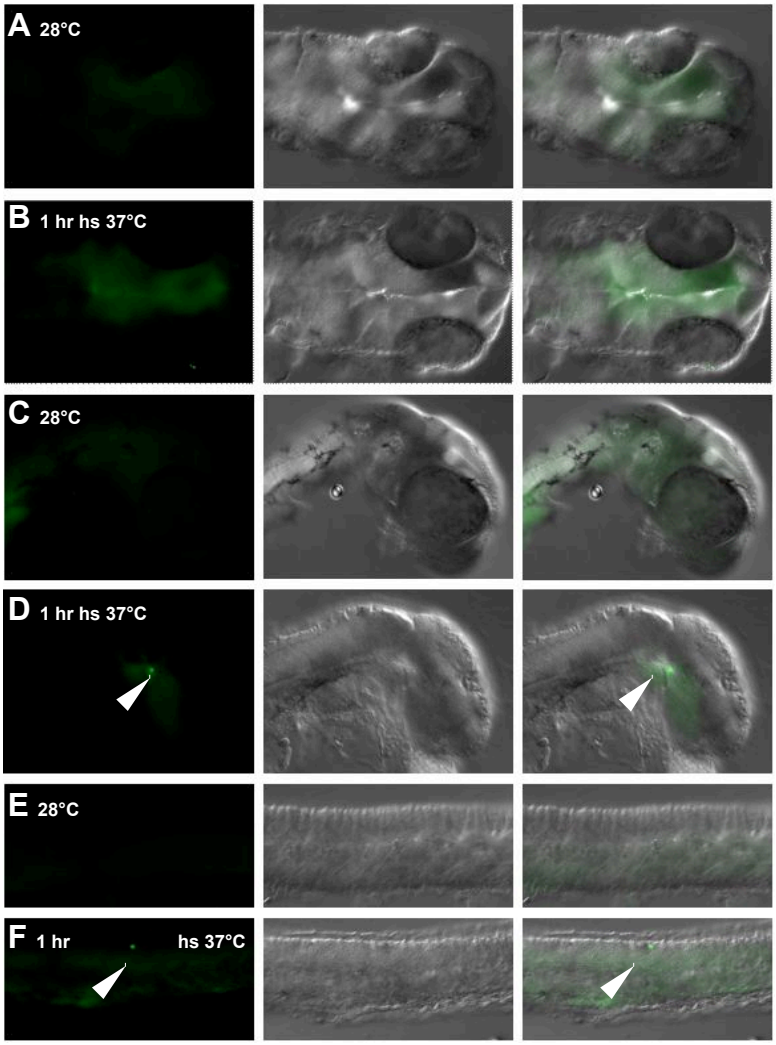


Figure 4.43: pEGFP-ZF-Hsp70 promoter microinjected zebrafish embryos at 31 hpf. Embyos were rised together at 28°C until 30 hpf. Half of the embryos stayed at 28°C (**A**, head dorsal view; **C**, head lateral view; **E** trunk) whereas the other half was heat shocked for one hour at 37°C (**B**, head dorsal view; **D**, head lateral view; **F** trunk). Only weak EGFP expression was observed after heat shock (arrows). hr, hour hs, heat shock. The columns display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

visible at one hour post heat shock (hphs) (Figure 4.44; B). However, 2 hphs, increased EGFP expression throughout the whole embryo were seen, which were stable until 72 hpf (Figure 4.44; C, D, E). Remarkably, no mosaic pattern, as normal for DNA injection (3.2.3.3) was observed in these embryos. In order to use this promoter for PrP expression, additional rounds of experiments to optimize the heat shock procedure and to establish the entire system needs to be performed.

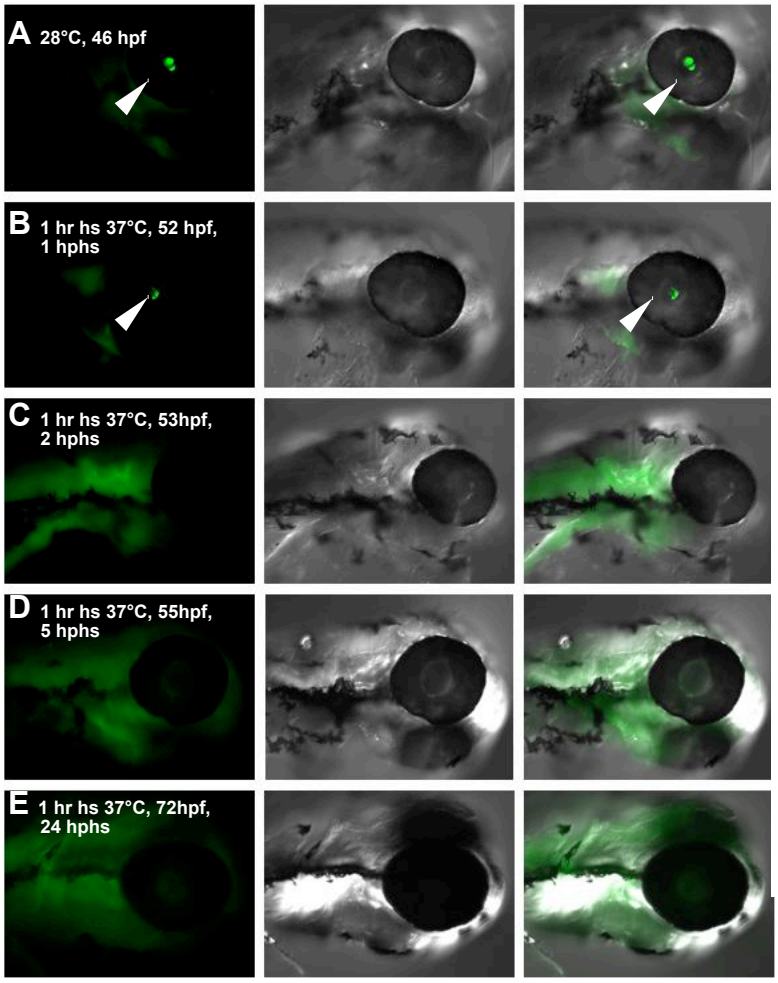


Figure 4.44: Images of the heads (lateral view) from pEGFP-ZF-Hsp70 promoter microinjected zebrafish embryos. **(A)** 46 hpf, rised at 28°C without heat shock, **(B)** 52 hpf, one hour post heat shock (hphs) at 37°C, **(C)** 53 hpf, 2 hphs at 37°C, **(D)** 55 hpf, 5 hphs at 37°C, **(E)** 72 hpf, 24 hphs at 37°C. EGFP expression was observed in the lens of 46 hpf and 52 hpf embryos (arrows) and up regulated expression was seen from 53 hpf until 72 hpf in the entire embryo. The columns display fluorescence images (left), Normarski images (center) and the overlay of fluorescence and Normaski images (right).

5 Discussion and perspectives

During this diploma thesis, fundamental steps towards the generation of PrP transgenic zebrafish were undertaken by analysis, including the cloning and testing, of the promoter/regulatory regions of zebrafish PrP-1 and -2. The intergenic regions of both genes were found to contain a common regulatory element (core promoter), which drives expression to trigeminal ganglion and Rohon-Beard neurons. For PrP-1, finer dissection of this fragment showed that it likely contains an additional repressor or silencing element, which restricts endogenous PrP-1 expression to very early stages.

In addition, zebrafish neuronal enhancers (zCREST) were successfully cloned and used to examine the effect of targeted EGFP-PrP transgene expression in zebrafish neurons and to gain insight into the developmental function of PrP in the CNS. Interestingly, EGFP-PrPs were efficiently overexpressed in zebrafish neurons at 24 hpf but this expression was apparently lost by 48 hpf. Co-injections and immunohistochemical analysis showed no evidence of major morphological defects in these neurons. Therefore, a negative effect, which leads to the downregulation of neuronal PrP expression in older embryos cannot be excluded. Whether such effect is indicative of PrP neurotoxicity remains to be further investigated.

5.1 Identification and use of the zebrafish prion protein promoters

This study constitutes the first functional characterization of the zebrafish PrP-1 and -2 promoter/enhancer regions. A BLAST search in the Ensembl database discloses a location for *Prnp-1* on chromosome 10 and for *Prnp-2* on chromosome 25, both gene loci being composed of two exons. The *Prnp-1* and *-2* intergenic and intronic regions were amplified, cloned into a promoterless pEGFP vector and characterized.

5.1.1 Regulation of the PrP-1 gene

For the *Prnp-1* gene locus, one fragment upstream of the transcription start site, the intron and a downstream fragment were analyzed. The sequence able to drive EGFP expression in zebrafish embryos is contained within the ~900 bp directly upstream of the transcription start site. Two sub-fragments of this sequence produced EGFP expression in embryos at 48 hpf. The shorter 515 bp fragment (PrP1-A1/A3-*4*) drove EGFP expression in the brain, in trigeminal ganglion- and in Rohon-Beard neurons. Additionally, this fragment was able to drive expression in embryos in a mosaic pattern at only 8 hpf (75%-epiboly stage). A longer fragment containing the 515 bp plus an additional

416 bp more of the upstream sequence (PrP1-A1/A2-*2*) showed minor EGFP expression restricted to notochord cells of embryos at 48 hpf. Interestingly, these expression assays did not recapitulate the endogenous expression pattern of PrP-1, which is expressed ubiquitously at high levels around 2.5 hpf (Málaga-Trillo *et al.* in review by Cell-press). However, early or maternal gene expression cannot easily be assayed using DNA microinjection because transcription in the zebrafish embryo does not start before 3 hpf (KANE and KIMMEL 1993). Additionally, physically microinjected DNA has to enter the nucleus and to integrate into the zebrafish genome before transcription is able to start. On the other hand, the expression patterns obtained after microinjection of PrP-1 constructs are consistent with the endogenous expression pattern of PrP-2, which is detected in the brain, in trigeminal ganglion neurons, neuromeres, lateral line ganglia and Rohon-Beard neurons (Málaga-Trillo *et al.* in review by Cell-press). Therefore, PrP-1 and PrP-2 probably contain similar promoter regions which led to these similar expression patterns.

5.1.2 Regulation of the PrP-2 gene

From the *Prnp-2* gene locus, three fragments upstream of the transcription start site and two fragments of the intron were analyzed. The intergenic fragment PrP2-A4/A6, which contained the 1373 bp directly upstream of the transcription start site, was found to be capable of driving EGFP expression in trigeminal ganglion neurons and Rohon-Beard neurons of zebrafish embryos at 24 and 48 hpf. This expression pattern is consistent with the natural distribution of PrP-2 transcripts during development, implying that this fragment already contains a core promoter together with enhancer elements for neuronal expression. The ability of the PrP2-A4/A6 construct to drive EGFP expression was also verified by transient transfection into N2a cells. Additionally, weak EGFP expression was seen in embryos microinjected with the construct containing the first part of the intron (PrP2-A5/A7), hence it is likely that intronic enhancer elements contribute to the transcriptional regulation of PrP-2 as described by similar studies in mammals (FISCHER *et al.* 1996, INOUE *et al.* 1997). To find out whether the PrP-2 intron contains regulatory sequences, it would be necessary to fuse it to a promoter sequence, as done for PrP-1. However, until now it has not been possible to clone this construct. For the bovine intron one, the presence of a TATA box has been recently described (HAIGH *et al.* 2007) and preliminary bioinformatics analyses with the PROSCANv1.7 database predicted also a region of promoter activity by the presence of a TATA box within the intron fragment of PrP-2 (not shown).

These results showed that the promoter/enhancer sequences of both zebrafish *Prnp* genes are able to drive EGFP expression in similar neuronal structures even though they display distinct natural expression patterns. Therefore, both *Prnp-1* and *-2* regulatory

regions contain neuronal enhancers, which nevertheless result in restricted expression patterns possibly due to the presence of additional *cis*-elements. This is in agreement with the idea that the two proteins share the same function but under different regulations since they are both able to rescue the PrP-1 knockdown phenotype (Málaga-Trillo *et al.* in review by Cell-press). Since zebrafish *Prnp-1* and *-2* originated in an ancient genome duplication event, it stands to reason that they once shared the same genomic structure, including promoter/enhancer regions. However, after multiple gene rearrangements (RIVERA-MILLA *et al.* 2006) it is conceivable, that the promoter/enhancer regions of *Prnp-1* incorporated changes during evolution which resulted in a divergent expression pattern. For instance, an additional repressor or silencing element could be responsible for the early-restricted expression of PrP-1. This would explain why, although no EGFP expression was observed after injection of the construct containing the full 1905 bp fragment (PrP1-A1/A3) upstream of the transcription start site, the smaller 515 bp (PrP1-A1/A3-*4*) and even the 931 bp (PrP1-A1/A3-*2*) sub-constructs were able to drive EGFP expression. However, to test the hypothesis of the existence of an additional repressor or silencing element, it would be helpful to clone the genomic fragment, which contains the putative PrP-1 repressor/silencing-element, upstream of the CMV or PrP-2 promoter in order to test this construct for expression in zebrafish embryos. In addition, bioinformatics analyses of the sequences for known motifs could be done.

5.1.3 Towards the generation of stable PrP zebrafish lines

Having cloned and characterized the promoter/enhancer sequences of zebrafish *Prnp-1* and *-2*, it is now possible to generate stable transgenic zebrafish lines. The constructs required for this, expressing mouse PrP and zebrafish PrP-1 and -2, have already been cloned. Transgenic zebrafish expressing reporter genes (such as EGFP or *lacz*) under the control of tissue specific promoters/enhancers are often used to simplify the observation of given cells and tissues during development. In our lab, two stable transgenic lines were previously used to visualize neuronal development upon PrP morpholino antisense RNA treatment (LUNCZ, diploma thesis 2006). The *Islet-1* transgenic line, expressing GFP stable under a the *Islet-1* promoter/enhancer in cranial motorneurons (HIGASHIJIMA *et al.* 2000); and the POU transgenic line, that expresses GFP stable in retinal ganglion cells and mechano-sensory hair cells of the lateral line and inner ear (XIAO *et al.* 2005). Stable transgenic zebrafish that express a reporter gene under the PrP promoter/enhancer would allow the investigation of the exact expression pattern of PrP during development and into adulthood. Furthermore, the specific effect of genetic manipulations, e.g. morpholino knock down on neurons, could be observed live without additional staining procedures on fixed embryos. Another great advantage of generating stable PrP transgenic fish would be the overexpression of mammalian PrPs. Since until now only mammals are known to be affected by prion disorders, overexpression of

mammalian PrPs, as for instance mouse PrP in zebrafish, under the regulation of a zebrafish promoter/enhancer, would allow further investigations of the mechanisms that lead to disease. In addition, the verification of the prion hypothesis would be possible: since the zebrafish, as a non-mammalian vertebrate should not be accessible to prion infection, a stable transgenic zebrafish that expresses mouse PrP could be infected with PrP^{Sc}. Moreover, transgenic expression of PrP-1 under the promoter/enhancer of PrP-2 would answer the question as to whether PrP-1 and -2 share the same function under restricted regulation or if they adopt different functions.

5.2 Neuronal targeted overexpression of prion proteins from different species in zebrafish

Most of the current work in our group is aimed at analyzing PrP loss- and gain of function phenotypes in zebrafish. Due to the technical limitation of having to microinject zebrafish embryos in the one- to four-cell stage, it was not yet possible to produce a PrP-2 gain of function phenotype of specific late and neuronal overexpressed PrP. Therefore, the goal of this subproject was to produce zebrafish that *in vivo* overexpress EGFP-fused PrP in zebrafish neurons in order to understand the function of PrP-2 in the nervous system. To this end, specific enhancer elements, which normally regulate the neuronal expression of the *Islet-1* gene in zebrafish, were amplified, cloned and tested.

5.2.1 Analysis of the zCREST neuronal enhancers

Three different enhancers (zCREST (zCR) 1, -2 and -3) of the *Islet-1* gene, which drive expression in different subsets of neurons have already been described (HIGASHIJIMA *et al*. 2000; UEMURA *et al.* 2005). Here, they were preliminary cloned upstream of the CMV promoter to regulate EGFP expression after microinjection into zebrafish embryos at 24 hpf. The expression patterns obtained were largely consistent with the published data (UEMURA *et al.* 2005). EGFP expression driven by zCR1 was seen within the brain and attributed to cranial motor neurons, while zCR2 drove expression into cranial motorneurons and sensory neurons like trigeminal ganglion neurons. The expression pattern achieved with the zCR3 enhancer, which shows strong expression mainly in trigeminal ganglion- and Rohon-Beard neurons, correlated at best with the PrP-2 expression pattern known from previous experiments (1.3.3; Málaga-Trillo *et al.* in review by Cell-press). Thus, constructs that express EGFP-PrP fusion proteins from different species, driven by the zCR3 enhancer, were cloned.

5.2.2 Targeted overexpression of PrPs in developing neurons

In order to generate zebrafish embryos that neuronal overexpress PrP, driven by the zCR3 enhancer, the EGFP-PrP constructs were microinjected into zebrafish embryos.

Preliminary results from embryos at 48 hpf, microinjected with the EGFP-tagged PrPs showed no expression in the expected structures. This observation raised the possibility that the constructs used were not efficiently expressed or processed. However, these EGFP-tagged PrPs were previously cloned in our lab and successfully used in expression studies with *Drosophila* Schneider-2 (S2) cells (SCHROCK, Diploma thesis 2006). In addition, control embryos microinjected with either only zCR3 driven EGFP or a EGFP-PrP construct without the PrP-core (pEGFP-zCR3-PrP2(L+GPI)),exhibited EGFP expression in the expected subsets of neurons (trigeminal ganglion- and Rohon-Beard neurons). Since overexpression of wt PrP in transgenic mice has been shown to be pathogenic and leads to several typical symptoms of prion diseases, e.g. brain vacuolization (WESTAWAY *et al.* 1994b), the first explanation of the lack of EGFP-PrP expression in transient transgenic zebrafish was that PrP overexpression might be neurotoxic and that the affected neuronal structures failed to develop. Another explanation considered was that the expression of EGFP-PrPs was developmentally downregulated or stopped either initially or after a certain time during development of the zebrafish. It could also be possible that EGFP-PrP was indeed overexpressed but the protein was rapidly degraded. With coinjection experiments in embryos at 48 hpf, where the EGFP-tagged PrPs were microinjected together with a zCR3 driven red fluorescence vector (pDsRed), clarity was brought to the status of the targeted neurons. Both constructs were driven by the same regulatory elements (zCR3-CMV promoter) to the same neuronal structures. Thus, since DsRed was expressed it could be concluded that these neurons were not depleted. The control constructs (zCR3 only and PrP2(L+GPI)) showed that both vectors, pEGFP and pDsRed, were expressed in similar cells at 48 hpf. Embryos coinjected with zCR3 driven EGFP-PrP and DsRed constructs indeed showed the expression of the DsRed protein in trigeminal ganglion- and Rohon-Beard neurons but no expression of the EGFP-tagged PrPs at 48 hpf. This result implies that the targeted neurons are not eliminated due to the PrP overexpression but that EGFP-PrP expression is developmentally lost after 48 hrs. This conclusion is supported by the fact that the PrP2(L+GPI) construct was successfully expressed in zebrafish neurons at 48 hpf, which also indicates that the downregulation effect is directly related to the expression of the PrP core. Another explanation for the lack of EGFP-PrP expression is that the targeted neurons inactivated the transcription of the constructs after early, neurotoxic overexpression. In order to find out whether expression is developmentally downregulated, co-injected embryos at 24 hpf were analyzed. Interestingly, EGFP-tagged PrP-2 and mouse PrP could be detected co-expressed with DsRed in trigeminal ganglion- and Rohon-Beard neurons. These results strongly suggest that neuronally targeted PrP expression is indeed downregulated. Nevertheless, expression of EGFP-tagged PrP-1 could not be observed, even in younger embryos. This could mean that the early expressed protein is degraded after a certain time; however no labeled vesicles indicative of EGFP-PrP degradation could be observed. To clarify the dynamics and mechanisms

involved in the developmental downregulation of EGFP-tagged PrPs, further experiments are required such as time series and *in situ* hybridization to monitor transcription of the PrP transgene.

5.2.3 Morphological analyses by antibody staining of PrP overexpressing neurons

PrP transgene expression in neurons appears to be developmentally downregulated. Since these neurons normally express PrP at 24 hpf, this result would suggest that PrP overexpression is detrimental to neurons. Therefore, to examine the morphology and development of axon tracts and neurons, PrP transgenic and wt embryos were immunohistochemically stained with an antibody against acetylated α-tubulin. Again, expression of EGFP-tagged PrP was observed in trigeminal ganglion- and Rohon-Beard neurons in embryos at 24 hpf, microinjected with either zebrafish PrP-2 or mouse PrP. However, expression could neither be seen in PrP-1 transgenic zebrafish at 24 hpf nor in all microinjected embryos at 48 hpf, excluding the controls, the zCREST3 and PrP2(L+GPI) microinjected embryos. These results confirmed the co-injection experiments. However, staining of the main axon tracts with antibodies against acetylated α-tubulin in 24 hpf and 48 hpf embryos showed no major morphological differences between neurons and axons of PrP overexpressing and control embryos. The lack of PrP-1 expression is remarkable and hints at a very early downregulation of its transgene expression, much like it was observed for zebrafish PrP-2 and mouse PrP but at even earlier stages, in correlation with its endogenous expression pattern (at 2.5 hpf; 1.3.3). However, potential negative effects of PrP overexpression might not be easy to detect at the morphological level. For instance, PrP overexpression might cause progressive neuronal apoptosis, which could be measured using more sensitive methods such as the TdT-UTP nick-end labeling (TUNEL) apoptosis assay. Investigations of *in vivo* targeted overexpression of PrP in *Xenopus* neuroendocrine pituitary cells have shown similar results: changes in neither overall cell structure nor function of these cells could be observed (VAN ROSMALEN and MARTENS 2006a). Nevertheless, the authors later published that transgene expression of PrP in *Xenopus* pituitary cells affected the secretory pathway and induced crinophagy (VAN ROSMALEN and MARTENS 2006b). It will therefore be interesting to look not only at the formation of axons and neurons and apoptosis but also at cellular behavior and the functioning of signaling pathways.

5.2.4 Expression through the Hsp70 promoter

Later-induced PrP overexpression (around the time of naturally occurring PrP-2 expression; ~30 hpf) could exhibit a more definite phenotype which is not perturbed by initial PrP overexpression during early zebrafish development. To this end, the zebrafish Hsp70 promoter was amplified and cloned upstream of EGFP in the pEGFP vector in-

stead of the strong virulent CMV promoter. Since it was described that Hsp70 is naturally expressed without heat shock in the developing zebrafish lens between 28 and 38 hpf (BLECHINGER *et al.* 2002), microinjected embryos were monitored at 30 hpf in order to test the suitability of the Hsp70 promoter. However, neither expression in the lens nor expression elsewhere in the embryo could be observed directly after one hour heat shock at 37°C or one hour after the heat shock. It therefore seemed, that more time is required after heat shock to see EGFP expression and that embryos at 30 hpf were too young to develop natural expression. Accordingly, embryos monitored at 46 hpf showed strong expression of EGFP in the lens. A time series of these embryos after a one hour heat shock at 37°C showed the first visible EGFP expression after 3 hours, which remained stable until 72 hpf (24 hphs). These preliminary assays were successful but have to be optimized before the zebrafish Hsp70 promoter is cloned instead of the CMV promoter between the zCREST3 enhancer and EGFP-tagged PrPs to drive PrP overexpression under the Hsp70 promoter live in zebrafish embryos.

5.3 Perspectives

Having identified the regulatory regions of zebrafish *Prnp-1* and *-2*, it is now feasible to produce stable PrP transgenic zebrafish lines in order to, for instance, overexpress mammalian PrPs. Additionally, the regions found to control the *Prnp-1* gene expression needs to be further analyzed in order to detect potential silencing or additional regulatory elements. For this reason, such fragments could be cloned upstream of a core promoter (CMV, Hsp70) and tested for their ability to regulate EGFP expression. Likewise, the intron of *Prnp-2* could be tested for its ability to regulate the EGFP expression of a core promoter. For both genes, the intergenic and intronic sequences have to be bioinformatically screened for known regulatory motifs in order to correlate them with the i*n vivo* expression results.

In order to further investigate the effect of targeted overexpression of PrP in zebrafish neurons, the time point at which EGFP-PrP expression is shut down has to be examined in a time series. Also, the mechanism by which the protein is potentially switched off has to be determined. With mRNA *in situ* hybridization it would be possible to distinguish between transcriptional regulation and a regulation at protein level. Furthermore, possible PrP neurotoxic effects could be further analyzed by visualizing apoptotic cells using a TUNEL assay or testing normal cellular functions such as signaling pathways.

6 Appendix

6.1 Zebrafish sequences

Bold sections are primers used for amplifying the fragments; grey letters display the beginning or end of the next adjacent gene and orange letters show the 5'- and 3' UTR, respectively. Green letters are the coding sequence for PrP and underlined parts are unamplified, hence unsequenced stretches whose sequence is exported from the Ensembl Web site.

6.1.1 PrP-1

CGGATGTTGTATGTGTGTCTTCAGGTGTTAGAGGAAGACCTGGCTCGCAGACGAAGAGAAAAAGAAGGGACCAGGAGACGAGGGAGTG
AGCCGGAAAACAGATTTGCAGCTCGACGCCCAAAGTTCTTCCATAATCTAAGCTTCTCCTGACAAAGTTAAGAAATACTTTCAACTCC
AATGTCAATTATATACAGTATATGATGCTGTCTTTCAACGTTTTGGGGTAAGAAAAGTTAAATAAAGGAAATAATTTTCAGAAATATT
ATAATGAATTATATTAAAATATAAAACTTTGTATTTATTCTACAATGAAATTTAATATTCAAATTATTTAAATGACAATATTTTGATT
TGAGATTTGGATGAAATGTTAGCAGATTTTTATAGAATAAAACAAATATTATAATTTTGCTCAAACTCACACCTAATAAATCAGTGAG
ATCATTCTCATGTTGTCTTATAATTCGTTTTTGCATAGCTGTACATATAGATACATTGTATAAGCAACAAATAATAACTTGACTTCCT
GTTAATCATTTGGAAAAGTGGTTAAAGGTAGTAACATCAGTCATGTTGGGGTGGAGGAAAAATCATGGTTAAGGG**GTTCAGTTACATT**
CAGTTTGGGGATGTGCGAGAGATCTGCAGAGTTGATGGCAACATCCACAGCCTGCGGTATCAAAATATTTGTGCTGCCCTTTGCATTA
CAAACCACAGAGAGGGCAAATTCTTTAGCACGATAGCGCTCCTTCTCACACTTCAGCCTTACATCAAAGTTCCTGAAAGCAAAGAAGG
TCATGGTGCTCCAGGATTGGCCAGCCCAGTCACCAAACATGAACATTATTGAGCATGTCTGGGGTAAGATGGAGGAGGCATTGAAGAT
AAATCCAAAGAATCTTGATGAAGTCTGTGAGTCCTGCAAGAAAGCTTTCCTTGCAATTCCAGTTGACTTTATTAATGAGTTGTTTGAG
TCGTT**GCAGAGATGTATGGATGCAGTC**CTCCAAGGTCATGGGGGTCATACTCAATATTCGTTCTTTTTCCACTGCACCATGACTTTAT
ATTCTATACTCTACATTATTTCTGTTAAGTGACAAGACTTTTATCTAAGCAAAGCTGTCCTAATTAAATAATTAAAATCAAGGCATGA
CCATATATTATTTTGGTAAACAAGCAACATCTAGAGCCTTTGCCTTTCAAGCACTTCTGATACCAAATGATCAACTAGAAGTCAAGTT
TTATTTGTATATCTTAAAACTTGGATAGACTACAAGACTTTGTCAGAATTTGATCAATAGTATTATAACTTACAATATTAAATTTTAA
TAATTAATTTAATAATAATTCACAGCTGTTTTATTACTTATTTTAATTTCCAAATAGATTAAGTAAAA**GACAGCTTATGACAACTCAT**
CTTTTTCCCCCTTAGCAAACTGTATAGTGATTATAAAATGTAAAGTAGTTTTTATAGAAATTGATGTATATATGCATAATAAAAAAGC
TTACCCTAAGAAAAATTAAAACAACACATACATATACTCTATATGTTGACATTTGGGTTTTAGTGTTGAAAATATTTTGCCTGGAGTT
TATGTATTTTATTTTATTTTTTGTAAATATGTTTAAAATATCTGCTTAGATTCCTTTGTAAGAAAGAAAAGAATTCATGTGCGAATAA
ATTTTCTAACCATTTCGGACAAAGAGTCTATTCAGTGGTTGTTTATTCCTTCTGCTAGATGTGTTTGAAGCTTTTAATAGTCACGCAC
ACAGAAACAGTACCCCAGCCTTATTAGCTTTCTGTAAGAACATTATTTCATGAAGCCACACCCAGACCACTATCTGTCCCGACTAC**AG**
TCAGCGTGATCTCTGTTGAGCAGAGAGTGC**TGCGGGAGAGACGCTTCTTCAACCT**TTTTATGGTAAGACATTACCAAACTATCATATT
GCTCACCGTAGAGACATATATAACATCTTAAATGTGACATTTAAATTAAACAAACATACTATAAAAGGTTCTTTACGATGTTATTATT
TGTATGAAGGACCTTGATTTGAGATTGTTGGTTTTGTAATGTCATTTATATGCATATCTATGAATGTGTCTGTCTAGCCATTACTTAT
TTACCACATTTAAATTTGTTTACTTAGCAGACATTCTACCTTTAACTACTTGGCTAAAATATAAACAAAAATAGTCACACTGCTGTAT
TATTTAAATTATATTTGCTGAAAATATCTCATAATAACAATTTATTCAGCACAATTGCTGCCAATTAAAGCATATTAAAGTTAATCGC
CCCAAGAGGAATCTTGAATTTTGCATGATAAACACTTTCTATCAAATTGTAATCTCTGACTGTATGGATTCTGGTTTGCTGAAGAGCA
ACTCTATA**TAGCCTTATCCTTCGTGGCATAC**ATAAAAGGGTTAGTTCACTCAAAATTTAAATTATGCTATTAATAACTTGCCCTAGTG
TCATTAAAAACCCATGAAACCTTTGTCCATCTTCAGAACACAATTTAAGATGAAATCCAAGAGTTCCCTCATACTCCATAGACAGAAA
CAGTCCTCAGATGTTTAAAGTCAGAAAAGAAACCAAAAACATTGTCAAAAGATTTAGTGTGACATCAGTGGACAAACTATAATCATAC
GAAGCTACAAAAACACTTTTGTTTGTGCAAAAAAAAAAAAAAAAAAAACTGTAAAAATGACTCAATATGCTTACTTCTCTCCCTTAAA
ATATTGCGTGGGGGCGCTGTTTATCTTTGCACATTATTCCCTGGAGGCAATCGAACAGTAAGGGTGGCTTGGTTAAGTATATTGGGGC
TGAAGCTGTCTAACTGATGTCAACAGAGAAGGACTGCTACTGCAAACACATGAGCAAATGGCCAAACTTTAAAGAGATCTAAAACTTT
TTTTTTCACTGGACTAACTTAGATTAATTGTTCACCTAAAGAATAATGATGTGCAGATTTAATCTAAAATAACTTAATTTGTGTTCCC
AAGATGAACAAAAGTCTGGGGGGATTAAAATGACATGAGACTGAGGCATTAATAACACAAATTTCATTTTGTGGCGAACAAAACATCTT
GATTAAGTGTTAATGCAATTATTGTTGATTTTGTTTTTCTTGGAATGTTTTATTGAAGGATAGTGAAAATGCATTGATAATATCTTAA
GGATCATGCTTTAGGCTTTTAATGTGAAGATGATTTTATGGGTATTAATCTCATACGCTATAATGGACAAAAAATTGATAAGCTGAAA
GTGCTGTAGAGCCATATTACTCTATAGAAATGCTTTCATGTCCATTACTGTCTTGTCTCCTCTCTGTGCTGTTTCTGAATACTAAAAG
CATTCTCCATTAGACCTGTGGTAAACACTAACTCTGCTGGCCTTTCCGAACAAGATGCTGTTTACTAAAGCCTTGTTTAGGAATGTTT
TTACTGTGTTCTCTCCTGTATTAAGTATCAGTTTGACTCTTGTTTTGTATGAAGTAAAATCAGTGAATTTCATTAAGCTTACTTTCTG

TGTCTTTACCTCCTTGATCATTGTCAGGTCTGCACTAATTCACTGTGTTTTTTGTTAGGACCAAA*ATG*GGGGAGTTATGCAAACTTCT
AGCCATCGTTTTGGTTTTGATGGCAGTGTTACATTATACATCAGGAGGAAAGAA**AGGCACTGTAAGCAAGACTGTTCCC**AAGAAGCCA
TCCGAAAAAACATCACAAGGGACCAAGACCCAACCTAACTATCCCAGACAACCCTCCAATCCTGGTGCAGGAAGTAACCCAGGCTACC
CTAACCAGCAGTATCCTGGGAGAGGTGGATCTAGTCCATCAGGGTATCCCAACCAGAATCCTGGGGCAGGCAGCTATCCAGCTGGAGG
CAGCTATCCGTATCCTGGAAGAGGTGGCTCCAGCCCTGGAGGGTATCCCAACCAAAATCCTGGAGCAGGTTCTTATCCATCTGGAGGC
AGCTATCCCTCAGCTGGTGGAAATCCCAATCAGTATCCTGGGAGAGGTGGCTACAACCCTGGAGGGTATCCCAACCAGAATCCTGGAG
CAGGTTCTTATCCAGCAGGAGGCAGCTATCCTTCTGCTGGTGGAAATCCCAATCAGTATCCTGGAAGAGGTGGCACCAGCCCCGCAGG
GTATCCCAACCAAAATCCTGGAGCAGGTTCTTATCCAGCAGGAGGCAGCTATCCTTCTGCTGGTGGAAATCCCAATCAGTATCCTGGG
AGAGGTGGCTCCAACCCTGGAGGGTATCCCAACCAGAATCCTGGAGCAGGTTCTTATCCAGCAGGAGGCAGCTATCCTTCAGCTGGTG
GAAATCCCAATCAGTATCCTGGAAGAGGCGGCTCCAGCCCCGGAGGGAATCCCAACCAGAATCCTGGAGCAGGTACCTATGCAGGTGG
TGGCTACCCCAATCAGTATCCTGGGGGAGGGGGTTACTCTAACCAGAACCCTGGTAGAAGTGGATACAGCCCTGGTGGGTATCCAGGA
GCTGGATCTTACCCAGTGAGAAATGCAGGGCAGCCTGGAGTTTACCCAGGAGCACACCTTCTGCTGGAGGAGGATATCCCAACTGGAA
CCCCAACAATCAGATTCTCAGTCCACGATATGGAGGCTCATTTGGAGGTGGTGGGTTTGGGACGGGTGGCTCCCCTTTCTCACAATCA
GTGAAAAACATGGGCTACAACCCTTCTGCAAAATCCAAAGGCTTCGGGAAAAAGGCAGTGGTGGCTGCAGGAGTTGGTGCAATGGCAG
GAATGGCTATAGGCTATGGAATAGGAAACTTTCAACGCCCCAACTTCCAGTTCAGAAGCCCACAGGAGGAACGCTACTACAACAACCA
TATGTACCAACGTCAGGACACACGTTTTAAAGATGGAGGTAACAAGAAAAAACCCAGTCACACCGCAGGAACCGACAACACATCAAAG
CTACCAGCTCAAGCTCAGTCATACGACAATTTCATGAAGACATGCATGAAGCGAAATAATCTTTTAAGAGACCAAGACTCAAAAGAAT
CTGCAGGTAACAGCAATATTCAAAGAGATGAACCAATGAGTGATTTTCCAGGTTTGAATGAAGAATCCTCACAGAAAGGTGGGCCAGA
GAAGAATGACACCACATCTAATACAACCACCCGTGATTATCCAAAGGCTGCTGACATTCATAAGAATGAGGATGAGGAAACAGTGAGC
ATCATGCAGATTGGTTATCCGGCACTTATTGAGCAGATGAAATCTCGAAAGTGTGTTGAGCTGTACATTGTTTACTCAGAGAGTCAGA
GAGATGATAGAATGGAAAAAAGAGAAGGGAAAATTCCCAGAAGTTCCAATAACCATAATGGACACAGCATCTCCGAAGTGCTCCTGTT
GCTCACTGCTTCCACTATGCTTTTTAGTACCACT*TAA*TGGAACTATAACAATGAACATATAATGTATTAAGTAATGGAATTACATTTC
CTTTCCACTCTATAGGAATCCAAATAACAGCACTTATATTGCCATTTGCATTTTAATGAATGAATTTTGTTATGATAAATCATCATCA
TACAATAATATTGTTACTTTTACAACTGTATAATAAAAATGTAAATACCTTAATACAATTTTTATGTGTTCACTTCGTTTATGCATTT
ATATTTTCAAGGATAATATTTAATCACATTTTTGCCTTATACATAAATAAATAAAATACTAAAAGATTCGTTTATTTTGATTACTTAT
GTTACTTTCATTCATACCTTTCGGCAAAATCTCTGCAGTTCGTTTGCACAGGTACAGTACATATAAACAAGAGAACAAATATGTTTTC
AATGTTTATTCATTTTCTTTTCTTCTCTTTTTGCCACAGTGGAATGAACCGCCAACTTATCCAG**CATATGTTTTACACAACAGATGCC**
CTTTCAGCCAC<u>AACCTCAAACTGGGAAACACCCTTATACTCTTGCTTTCACACTCATACACTAAGCCCAATTTAGCTTATTAATTTCA
CCTGTACCACACGTCTTTGGACTGTGGGGGAAACCGGAGCACCCGGACGAAACCCACGCCAACATGGGGAGAACATGCAGACTTCACA
CAGAAATACCTACTGTCACAGCTGGGGCTTGAACCAGCGACCTTCTTGCTGTGAGGAGATCATGCTACCCACTGCGCCACCGTGAAGC
CCACGTTTTCAATATTTTATAGACAATGGCTGGTCTGTAAAACAAAATGAAGCTCACTGCCATTGTAAGATCACAGAGCGACATACCC
AGGCCTTCACATGATACACGTTGCTAGGAGACCAGGGCATTCAGCAAACCAACGTCACAATGAGAAAGCAATCTGTCAGTTCAGCAAA
CAACATGGACGGCAATAAGCAGAGTCAATTATTTATTCATTTAAACAGTACAAGAAGCAAATAGTTACATGGTTATAAACACAAGCCA
AAACACACATCAAGCCCTAAAATAAGAGTATTAACTGATGCCAATGTGCTAAAGAAATGGCAAAAGCTAAATCTGTTTTCTCTTTTAT
TAATTTTGCCCATCAGAGTACATCGGCTCACATGACTTGCAAAAAAATGACTCAGTTAAATATTAGCCCTTAAATTAAAGCTGGTTCT
TTGTTAATATACACAACAACCAAAACTGAGGTTATATGATTGCTGAATTATCGGTCTTTAGCAACACATCATGCTTAAATGTTTAACA
TTCATCACAAGTGAAAAAAAAAACCATTTGGATATACATTTCATATTCAAATCAACACACAACCTAAATGCTTTTTCTTACTTTGGAA
CTGTGAACAACATACAATTGTGTTGTTTCCATAGAAAAAAATGCTGTTGTGTAAAAAAAAATTATTTTGTAAAGAAACAATTGTTGAT
TTGGATGTCAAAAACTTACCTAAAAAATGAGTACATTAAACCAATGCATTAAATATTGTAAAACCCTAATTTGTGTCATGTAAATGAA
CAGTCAACACACATTCAAAGCTTTTACATTTTTAGTTAAAATCCTACAGTTAAGGTTTTTAACTAAACTCAACTAAAAACCTAATAAT
TAAAAAGCATTTTAAGGGCCACAACTTTTAAAAACTAGTTTTATAATAAAAGTCTGTGCATGTGCTCAGATATGTAGTGTTTCTTCAC
AAAGTGGAATTGCCAACTGAAGGCCTGGCAGTGTTTTTTAATTGTTTTTTTTGTGAAATGGGGATCATTTAAAAAAAAATGTCTGTACC
CTAAACATTTTACAAATGAATTTGTCCTTTTGTCCTAAAATGAACACTAAAATAAAAATTGACAAATAATAGTAATTATAATAATTAT
TATTATTATTAAAAAGATAAAAATCCTACAGATTAGGGCTGCACAATATTGGAAAAATCTGATATTACGATAAATATTGTGAAATGAA
TACAGTTTTAGAATATGGTTTTGAATAGCTCTATTTGTCGGTTTTTAGGGGAGTCTAACAGTATTTTGGTGCAAAAATTAAATAATCA
CAATGCAAAAAAAGATATATATACTTTTATTACTTTGTTTTGTCTTATTTCTAGTCCGAATATAAAAAAAAATCTTAGACCAAGTAAA
AATTTTGTTTAGTTTTCACCGTCAAAAAAAAAAATTATAATAATTGAAAATTAAGAGTTTTTTTTTTGCTTGTTTTAAGAAAATTATC
TGCCAGTGGGGTAAGTGAAATAATCTTTTTTTCGCCTAAAAATGTAGATATTTGAAAATTATAATTTTTTGCATAAATCATATAAATT
CCATATACAGTAATTAAATAAAAATACAAAGCTCCTGGTCAACTATAATTCAGACTGGACATTGCATATCTTTGCGATGTGACTATTG
TGATACTGATGCTGTAACAATATACTGT**GCAGCCCTACTACAGATACAGCACC**GACACTAATCCATTAATCATGCCATTGACTAAACT
TTACCTTTGATGAACTGTTTATAATAAGAATATCTGCATTTAAGGTGCTTAATCAGATAAACAATTATATTTTTTGCTCCCCTAACAT
TCCTGCATTGTTCCAGATTAGGTATTATTTTGTGAACCAGATAAAAAGGTTCTAGGATCATTCACTCTATGTTGTTGTTGTTTTTTGT
TTGTTTGAACAACTCATAGAGATCCTAAAAGACAACAATAGGCCTACATAAATGTTCTGCTGATTATATTTGCTAATATTTTATTTGC
TATTAGATGAATGACCATAGAGCTGGGCCAATAAAATGATACTATAACAATTTGCAATAAAATGTATGTCAATAACAATGATAAGCTC
TGGGCTGTTTTATTCTACAGTGACCCAATCTCACAGCAGAAATATGCAACAATGGGAATTTAATGGACCCTCTAATTTTAGTGGCTTA
AGTTACTCTTTTAAATTTTACTCAGATTTAAATCTTAACACCTGATACCAAAATATATACATCGCTATCGTTCAATATGGAAAATATT
TATCGAGATTGCATTTTTGCCAAATCGCCCAGCTCTAACTGTCCAACAGAACCAAAAACTGTTAGCTGGGAAGGTACTTGTGGAATAT
TTGGCAGTAAAAGTTTCCACCCCCCTCCCCATCAGATTAACCATGCTTTTATTGTTATTAAGCCTAAATGTGTATGGTCGTTTGGTGT
ATTGATTAACAACAGACATCTGTAACACATTTTGCCTACAAACAAGGTTAATTTGTGGTTAAAATAGCTACAACTTTTCTTTTTTTTT
TTGAAAGAAAACTCCACATAAAACATTTTCTTCACACAGCATACTGTGTTTTCTCACCCTCTTTGAATTGACAGAACATATTAAGCCC

TGATCAGTGAAAATAGCTTTCATCAGCTAGTGATGAACTCTATAAGAAATACACCTGCAGATTCTATGAACACATCAATTTACCATCA
TTTTAGTAAAACGGTACATCAATAATTTATGTTTTTACGTCGCTGTAAGGGGTCTAGTGTGGGGGTGGGTAGTAATTAAACACAATGT
AATGAGTTATATAATAAACAATTCACGTTAACTTCCGACCAAAGTTGTACCAGATCCCAGATTGAG**GCACCAATCTGGGTCCTGGAGC**
CGGTGTCCCTGCAGGGTTCAGCTCCAACTTGCCTCAACACACCTGCCTGAATGTTTCAAGCATACCTAGAGAGACCTTGATTAGCTTG
TCCAGGTGTGTTTGATTAGGGTTGGAGCTAAAATCTGCAGGACACCGGCCCTCCCAGGGAAACCAATTTGGTTATAGCAACAACTCAA
AAACACAACAAAAAAGGTCTTCTTTAGCCTACATTTTATCAAACAGCATACTCGATAAATGATTGCATCTTCCTTTTTAAAAACTTTC
TTATTGTTTCAAATAATAAATTGTCTTTCATTCAAAAGAAATGCCTCAAGGCTAAAGCTGCCCATGTGCTCTGAGCTGCCCGTGCTCC
CGTAATACCCACGGTGTGACTGGTTGTATACGGGCTGGTGATAGCCGTCAGAGTAGCCGTTGTGGCCGTACCTCGGTCTCCCCAGAGA
GCCC

6.1.2 PrP-2

Because the downstream sequence of PrP-2 was not amplified in this study, the sequence displayed here ends after the 5'UTR.

CGCGTGGCCGGGTTGCGCTCATTGCCGAACGCGCCGCCGCGGGTCTCGTTCTCCTTGAGACGCGTGAAGTACTGCAGGGCGAACTCCA
GCAGATCCGCCGGCTGGTTCCTCAGGACCTCCACGGTAAAGCTCTGCAGAAGCTCCGTCAAGCCCTCGGGGATTTCTATACTCATTTC
CTGACGGTGAAATGTGCGCAAAAATTCGGCCCGATCTCTCGCGCGTTACAACAGTCCGTTTTTTTTTTTAATCGCCGCGTCAGCTGAG
AAGGAATGACCCGCGCACGCGTCAGAGGGAACGCGACGGATTTTCTC**GCTATTCCACCATCTGCGTCTGTTC**CCCGACCGCGTCCTCT
AATTCGCGGGCGTAAAAATGGCAGGGAATTCAAGAGCTGCTGGCATCTTTTGTTTTTTTTTTCTGTCCTGCGCTGCTGCTGCTGCTGC
TGGCTACATCAGAGCCGCGCATGCGATGCGGGAAACCATGGCAACCAGCATCCCGGGATTTGCGCAGGAGGAACGCGACGCATTGGAA
CGCGACGCAAGCGCTTAAATATCCAATGAGATGTTTGTATAATTTCACACGTCGTCGTACGAAGATTTATGACACCTACTGTTAATAT
CTGTTATTTAACCTGTGTATGTCTTTAAATAGCTTTGTTTGGGGCAACATAGGGGCACAGGGGTGTATGAATATGGATATCATTCAGG
ACTTTTTAATATATATTTTACTCTATATTGTATAATCACAGGAGCTATATGTTAAATTACTGATTGTAATTTAATAATGTGCACATTA
AAGCTAATGGTCATCAAAATTCTAAAGAAAATTGTATAAAATTGTTATATATATATATATATATATATATATATATATATATATATAT
ATATATATATGTGTGTGTGTGTGTGTGTGTGTGTATGTGTGTGTGTGTGTATGTATATATGTATATATGTATGTATGTATGTATGT
ATATATATATGTGTGTGTATATATGTATGTATATATATATATGTATATATATATANNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTAATCTTGGGTCTCTATCA
GTAGCTTGGGTGCTGTGGTTTCCTTGAACATTACTGCTGAACATTCAAGTAGATTAACAAACGAGCTTGGCACTTTGCTAAATCATTG
ACCTAGGGACTTTGGCACTGGATGATCAGCAGAAGGCAATGTATAACGCCTTATTGTTCGCATATGTAATAATGTTATGCCTTTGGGA
TGTTGTGAATGTTCATGTGTGTGTAATGTTACCATCACTGGGATTAAAGTGATTAAATGCACCTTAGTATTGTGTCTGCCTATTTTAC
TTGGGTATTTTTAGAAACCATAATCTAGAATGGGGCTTTTTGGTCTTCGCTTTGAAACAAAACTGTGCCAGATAATAGGATGTGACCC
ACTTGCATAGTTCCGTAGCTTTAACACACTATATTTGGCCTAGTGTTTATCTAATATCATTCATTCATTTTCCTTTGGCTTAGTCACT
TTATTCATCAGGGGTTGCTACAGTGGAATGAACCACCAACTTATCCAACATATGTTTTACACAGTGGATGCCCTTTCAGCTGCAACCC
AACACTGGGAAACATCCATACACACTCAACGGCCAGTTTAGTTTTTTCAGTTATCCTATAGCGCATGTGTTTGGACAGTGGGGGAAAC
CGGCGCACCCGGAGGAAACCCACACCAACATGGAAAGAACATGCAAACTCCACACAGAAATGCCAACTGACCCAGCCGGGACTTGAAT
CAGCAGCCTTATTGCTGTGAGTTGACAGTGCTAACCACTGAGCCACTGTGTCGCNNNNNNNNNNNNNNCTAATCGATTCATTGGGTAA
CGCCCTATGTGTATATATGTATGTCATTTAAAATGTACTGGTGTAGTTTCACGGCGAGTTTAGAATGAGAATGAAAATAAGAATGTAG
ATGTGCATGATTAATGGCTAGATTGTTGCTATTTATCATTCAGACATTCACAAAACCAAAAGATTCACAGGTCTGTTAGATCTGATCA
CATTTAAAATCTGCATTTGGCCCAGATATTTATAATTATGATGAAACCTTTTTTTTTTTCTCAGATACACCATTTCTATACATTTCTT
TTTCAGAAGGTCTAATTATTAATTAAAGTGCTAGGATAACCGTTTACAGGTTGGATGTAGATGGATATTAATGGCAACTATAATGTTT
GTTTGTAGTTACAATTAATGTTATTTTCCATCTGTGTGCAGTAAGGGTGGTTTATTTTGCTTTTAATTACTTTAACTTGGGTATAATA
AGCCAAAGCCATTGCAATACATACCATTGTTTACAGTAAGGGCGTACTTTTTATTTTTAAATTTTTTTTATTATTATTTTTTGTCATC
TGAAATGCATCAAATAAGGTACTTTTTAAATTAATTTTTGTTTATTTAATACCCATGGATACAAACAAAGATTTAATTGTTCATCAGA
A**CCACTTCATGTGATGGAGCACAATGC**TTTAAAACAGACCCCCAAACAACATTGAATCAAACTTTGCCATATACAATCATAGCCAGTA
TCCCACACCAAGAAGTGCGAGTTTATTTTGCTAGATCTACATTAGAGTTGCACAACACTGGGAACATATGTGAGATGTGCTTTAATAG
TTGAATACTGCAGTAATTTATTTAAATTTTTAGCCATTTAAAAACATTTATGTAATGATCAAATCTAAAATAAACCAGTAAAATCTAA
ACAAAATAAATTGGGTTAAAGAAAATATTCCATATAAGTAAGGCCCCATCTGATTTGTCAAATTTGGGTAAAGAACCTATGCATTTAA
TATGCACATATACAACAAACACAAATGTAATTCATTGCACATCTCTCCGATATGAATATTAAATATTT*ATTAAT*TCATTTATTTTTCT
TTAGCTTAGTCCCTTTACTCAACACAGCAAAATGAACCACCAACTTATCCAGCATATGTTTTACACAGAGGCTGCCTTTCCAGCTGTA
ACCCACTACTGCGAAACACCCATACACTCTTACACACATACACTACGGCCAGTTTAGTTGTTTCAATTCACCTATAGCGCATGTGTTT
GGACGGTGAAGGAAACTGGAGCAGACGAGTTAAATTCATGCCAACATGCCAACATGGGAGAACATGCAAACCATGTCGCCCGTATAGA
TAGATAGATGGATGGATGGATGGATAGATAGATAAATGGATGGGCAGACGGATGGATAGATAGATAGACAGACAGATAGATAGA
TGGACGGATAGATGATAGATGGACGGATAGATAGATGGACAGATGGATGGATGGACGGATAGATAGATGGATGGACGGATAGATAGAT
GGATGGACGGATAGATAGATGGACGGATGGATGGATGGACGGATAGATAGACAGACGGATAGATGGATGGATGGATAGATAGATAGAT
GGATAGATGGACGGATAGATGGATGGATAGATGGACGGATAGATAGATGGATGGATGGATGGACGGATAGATGGATAGATGGGCGGAT
AGATGGACGGATAGATGGATGGACGGATAGATAGACAGACAGACGGATAGATAGATGGATAGATGGACGGATAAATGGATAGATGGAC
AGATAGATGGATGGATGGATAGATGGACGGATAGATAGACAGACGGATAGATAGACGGATAGATAGACGGATAGATAGATGGATGGAT
GGATAGATAGATAGTTAGATGGATGGATGGACGGACGGATAGATAGATAGATAGACAGACAGACGGACGGATGGATAGACAGACAGAC

GGATAGATGGATGGATGGATAGATAGATTGATAGATGGATGGACGGATAGATAGATAGATAGATAGATAGACAGACGGATAGATGGAT
GGATAGATAGATAGATGGATGGATGGATGGACGGACAGATAGATAGACGGACGGATGGATGGATTGATAGATAGATAATATTAT
TATTATTATTATTTTTTAGGTATACATTTTGCATCCCTAATTTGCATGCTGTTGTTTT**GTACAGGTATGCAGTTGCAGCAAGC**CATCA
AGAAGCACTATTATAATCTACTGAACCTCAACGCTTATTTTGGAGAACCCAAACCGCCCAGACTGGAGATGATCTTCAGCAAGACCAA
ACCTCGCTTCTTCAAACGCACTAGTTCAGCAGTCTGGAATTCTCCACCACGTTTGGGAAATATGGGGCATTAGTAAACAGATGTATTT
TTTTTCCTTCTCTATTTTATCTGTCATCCTTTTTATGTGTTCTCTTTTATACTCTCTTTAATAAAGCCTTTTAAAAATCTACAGGTAA
GTAGATTACATAGAAAAAGATGGCCGTGGCATTTTGCCCACATAATTCACTTCACAAAGAGTGTCAAATTACTCTGGACTGTTAATAA
AAGGTAAAAATATTTAAATGACTCTGTGATGTCGTTCATTCATAGGGGGAAAAAACTGAAGCATTGTTGTTTTAGTGCAGAGATGCCC
AAACTGGGGCCCGCGGGCAAAAGTTGGCCCATGTTAACCTTTGATTTGGCCCTTCATCTCATCTGAGAAGAGGAGGAGAATGCTGGGG
AATTTCTTGGTTTTGCCTTGAACGGAGTCATTTCTAATTTAACATAATTATTTTTTAACTGTTGATCTACAAATAAGCAAACTTGAAC
TAAATGTTTTAATTAAATGTTGTGAGTGAATCAAGATTTTTCCCCAAAAAAATTTGTCTCTTATGAATAGCGGACATTGTTCATGTTT
TTAATTGTAAGATGTTTATTTTAAAATGTACAAAAATTTTAAATTATATAATGAATCCGGCCCAGCACCCTCAATCAAGTTCAGTTTT
TGACCCCTCGTAAGAAAAAGTTGGATATGAAGTAGTCAGAAACTCGTGGAACTTACTCTCCTGATGCTAAAATACAGGTTTCTCATAT
TACTGGTAGTTTGGTTAAATCGCAATGTATGCATGTGACTTTGGCTTATATTTGACATTTTACAAAAGGATTGAATGTTTTGAATTGA
GAAACCTGTAAACAGATAAACCTATCAGGGTTCAACACTAAGGATTTGTCCTACTAGTCTGATTGGGCCAGCAGTTTAGATTTTTACT
TGCCTGCCTATATTTTCACTGGCCCCACCCCAAAAAAAAAGTCAAAGTTAATAGCAATTTCTTAGGCACATGTGTTAAATATTGTGAC
AAAAATGTGTTCGAATCTAGATTTAAAATATTACTAAAATGTTAATAAATGTATAGTTTGCAAATCAGCGCTCTGTACCCATGTAAAC
ATCTGCTTCTAAGACATTCTTTTAGCCTCATAATTTGATGTTGCCGTCACTTCGCTGTACTGGTTTTTACCAGGTTACAAAACAACAT
CATGCTCACAACATGAAATCAGATAAGAAGAACCGAAAAGCAACAT**GGTGTTTACGACATCACTTAGAAGGCAGC**ATTTAAAGGCTTA
AAATCACAAACTGTTTTTTTCTCGATTCTAAGACTGTATTCATA**CACAACTGACAAATAGTCGCAGGCA**AATTTAACTTTTGTGACTA
GGGAGCATAGATCAGGCCAGTAACATTCCTGTCGATTGTATGTTATATATATATATGTATGTGTATATATATGTATGTATATATGTAT
ATGTATATATGTGTGTATATATATGTATGTATATATGTATATGTATATATGTGTGTATATGTGTGTGTGGGTTAGTGTGTATATATAT
ATATATATGAGCAATCCAGCATTACGGATGTGACATTTGCAGTAAAATCTCAAAACATAAATTCACAGTGAAAGTATTTAACATTGTA
TTAAAACATCTGTTTATATTTTCCAAAACAACTAACTACAGAGTTTATATGAGCAGAAAAATATCAACATGTTTATGGAATGTGACGA
AAACGTTTACAGTATACAACATACTTTGGATTATACTGCACAACCCAAAAGAAACAATGCTTAACAAAAGGAGAAGAGTTCTCTCTAT
AGAACAAAAACGACTGATTTTATTTTATTTGACCTGTTTTTTGGGGTTTTTGCATTTATGAGGAAAAATCTTTGTTATGGATGTGAAG
TAAAATTGGCACTTGTGATTCTGGTAAATCGATTATAACGGTTTGTAAACATTAACACATACATTTGTATTTTTAATACAGAACAGTA
TTTTTAAAGTGCTGTAAAATACCTGCCAACACAAAAAATGCATAAACGGTTTCGATATTTTGGTGAAAACCTTTTTTTCATGGCAAGG
TTGACATTTGCATGGAATTACTCATATATTCCTAAATGAACTGCTTAGCTATTTTATATAATAAAATCCAGCAAACAAGCCTATAAAA
GAGAAAGAAGGATCAATAAATATTAATTTTCTGCTATTGTGTCAATATTGTAATCCATAAAACATCTAATTTGAGTAATTAGGAACAT
TTCATTAGAAGTACTTCTTTTTTTTTTTTTGGAAATCTGATAACGTAATCTGGATTAAATGCAAATTAACCTGCATAAATCCTGTACCT
TCGGCGGGAAGGTGTCTCTTAATGGACAAAGGGTGTGTTGGTTTAAAAACGAACATCTGCTTAATTTAAAAATGAATTTAAATAGACA
TTAGCTGTGCCCGAATCCCCACGACATTTCTGGTAAATCGTGTCAGAAGAGATTACGACCAGTGCGCATGCGCATACGAATATTACTC
ATCTCGTGAGCGCGCACGGCTGTGGAAACTTCATCGTCACTAACGGC**GTCGCGCATCCAAGGGACAACAATC**GCC**CAAGAGAGGACTC**
GCTTAAAACAGCCCGAAGGTAGGTTAGCGATTAAAACACGTTTATTATTACAGTAAACACGATGTTCTGTTATGTCTGGTGTTTGATT
TACTGTAGCTGACCGTTTTGCTTTATAATAACAGGTGCAGTTGTCGGTCACACGCCTAATAATAGTCCCGCTATTTAAAGCCGTTGTG
AATTTACACGCGGCCTGTTTTTGTTTACATTTAATATAAACTTGAGTATCGAATATTTACCTATTATATAAGATTAAATCCTCCTCAT
GTTATGAAGGAGTGAATTATATAACTGATTAATATATAATAGGAAAATAAACAAGGATGTGTTGTTATTGATTATTTGATATTAAATT
TGGAGGCCATTTAGTTTGATATTTTGGATTTCTTACCGCTCAAACTGATGTATATAAGCGAAAATGGCCTCCGAACATTGACACACAC
TCACTCACTCATTGACTTTCATTCACACATTCATACTGTTGTTTGAAAGAGCATCTGTGGTCAATCATGCGCTAAGATGAACGTGATA
ACAGATTTGCTTTCCATTAAGTTAGCATGAATCTGAGCAGCACTATATGTGTGACTGGGGCTAGTTCTCATTTTGCAGAGGCATAAAT
TATTAAAGACTTAATTGCAATTTTGTTATGTGAGAAATAAGATTAACATAAAAAACTGTGAGAAGAAAGCACAAAAAAAGTACAATTT
TAATATTTGTGTATAAATTTTGTATTAACTGTAACATTTCACCTGTAATACACATCTAAAACATTTACTCAGATTTAAACAAATAAGT
TTGCAAGCCAGAAAACACTCTTTTAAAAAATAAAGTATATAAGTATGATATATACAAATAAAACATGAATTAAATGCATTTTTTTTGT
AAATGAATAACACATTTATGTATAAATAACTAGAAATGCAGGAGACATTTTAGCTTTACACAAATTGAGATTTGCATAAACAAAACTT
TGCATTAGATGTTGGATGGTGGGTGAAATTATGTTTTGATAAAATGAGGGGGTAACCTGTTTTTTTTTCAGCAGGGTGAATTAGTGGG
GTATATAATTAATTGATTATTTAATGAAACATGCTAAATGTGGCCTCTTTGTCATATACTACATTCGTTGCATTTATTAAAACAATTC
CTAATACCACACTACTAAATAATGTGATATGCATAGCAGAACCACCTTTACTTTGAGCTTGCTTTATTCCTCTGCTTTTCTTCTCTTC
CGTTTTTCGTCTCTGGGTTTGGATGCAGTTTCATTTCAAATTAAAATTCGATTAATATTGATTAAAAATAGAAACGACACTTGTGGCA
TTATATTCGTTAAGACTTTAAGACGCTTTCGGCTTTATATCCAACGTGAGCAATTTGCCTCAAACAAAATGTATATTTTCTTTCAGAA
ACTTCCTTCTATTGTTTAGATGTGTGATAATGTCTTTTAAAGTGTTTGCAACCTTTATAAAACATGAC**GATGCTGGAGATTTGTTCAG**
CCACAGAAATCACTAACCACATATTTCCAATGCAAATATTTTACAGTGTGTCTTAAAGCAGGGTTCATACACGTTTTTACAAATAAAA
TTCCATGACTTTTCCAAGACTTTTAAGTACACTTTCATGTAATGTGTACATATACAGTTGAAGTCAGAATTGTTAGCCACTCTGTTTA
TGTTTTCATTTATTTCTGTTTAATGGAAAGCAGATTTTCTCAACACATTTCTCATCATAATAGATTTAATAACTAATTTCTTATGAAT
AATTTATTTTATCTTTGCCATGATGACAGTAAATAATATTTGACCAGATATTTTTCAAAACACTTCTAAAGTGTTAAAGGCTTAACTA
GGTTAATTAGGTTAACTAGGCAGGTTATGGTAATTAGGCAAGTTATTGTATAACGATGGTTTGTTCTGTAGACGATTGAAAAAAATAT
AGCTTAAAGGGGCTAATATTATTAACCTTAAAATAGTGTTTAAAAAATAAAAGACTGCTTTTATTCTAGCCGTAATGAAACAAATAAG
ACTTTCTCAAGAAGAAAAAATATTATCAGACATACTGTGAACATTTCATTGCTCTAAACATCATTTGGGAAATATTTAAAAAAAGACT
TCAACTGTACATGATAAATGACAAGAAAAACATTGGCGTTGAAATTCATTACAGCATATCATAAAACACGCAACATTCTATTTTGTTT
AAATTTGTTTTTATACCTATGTAAAATTGATTTTGATAATGCTGACACAGCACTAACAATATTAGAGCGAGTTTTTGGCCAAGCAAAG

AAAATAACTAAAGACAACCAACCTATATTCAAGTTAGAGCTGCACAATATATCATTTCAGCATGGATATCGCAATGTGTGCATCCGCA
ATAGTCACATCGCAGGATATGCAATGTTGAGATTATAGTTTAATATGGAGTTTAATCATAATGGAGTAAAAGTTTATCATCCGCATGC
GTTTTTAAAGAGATAGTTCACCCAAAAATAACCTGCCTTTTTCATTACACCCTTCACTTGTCTTAAACATTTGATTTTTCTTTTTCAA
ATGAACACAAAACAAACATGATTTAATAACTCCTATAGTATTGAACAAGTCAAGTGTAAGTAAATGGTGAGTAAAATTTAATCTGTCC
TTTTAAAGCCTGTAACTGAACATTTTAAAATATCAGAGTTTAAAACATCCGGCCACAATTAATTTTTTGTAACTTTAATAAAGACTAA
ATAATATACAATGAAGACTGCAGTGTTGATTATTCATTATCTGTACCTGAATAGTGAATACCTGAAACTATTAACTCACTTTTATCTT
TACTATATTTGATTTATGTAATTTATTTGTTATATATTATTTGGGATTCACTTCCTTCTACCAAAATCCCCCCAACATCCAAATAAAC
CTGTTAAATCATCTGGTGAATTTGTATTCACATCGCAATATACATCAGAAATACAAAATATGGGAATATCAGATTTTTCCAATGTCGC
GCAGCCTGAATTCAAGTATACAGATATTTGTTTAACTAATTTCCATGACTTTTCCAAAACTTCGTGGGTTTTTCTCTTTTTTCAAAAC
TTTTCCAGGCCTGAAAAATGCCATGTCAATTCCATGACTTGTCCAGGTTTCCCATGACCGTATAAACCCTGTTAAAAGGAATAATTTA
CTGACACTTGACTTGTTCCAAGCCAGTTTGATGGTTTGTTTGTTTGTTCTGTTGAACAAGAAGATAATTTGAAGAATGATGGAAACCG
GAAACCACTGACATCCAGGAAGTGGGACATACTAATACCCAAAAATCAATATTCTGTCATCATTCCCTCTTTACTTGTTCAAAATTTA
TTTGAGTCTCTCTTCTGTTGATCACAGAATAACATATATTGAACGCTTGAAGCTGGCACCAATTTTATTCCATAGTCTTCTCCCCCTT
CTTTTGTATTCAATAAAAGAAAGAAACCTGTAAAGCTTTAAA**CCACATCTGGAAGATTAAATGATGAGG**TAATTATTCATTTTTGGGT
GAAATATCCCTTTAAAGAAATCTTGAAAGATGTTTTAATGCCAAAAGAGTGTTTATGACAGCCCTAAATCTTTTAATATAGTTTAATA
TTGATATATTTACATAGCAATTTTCCATTTAGTGAGTGTGTTTACATAATATGTTGGTCACCACATCCAGTTTAATTTACTGCTATGA
ATCAGTGTTTCCTATTATATGCAAAAGCTATGTACGGTATAAACTTAAATAAAAAAATTAATGATGTTGTCAGATAGTTAAATGTCTG
ATGGAAGTTTTCTCATGTTATTGAAACCATCTCTCTTTTTCCATGCAG*ATG*GGTCGCTTAACAATA**CTATTGCTCTGTCTGGCTCTTG**
TGGTGTCAGTTGTGGCCAAACGCGGGAGTGGATCCAAAGGTGGATTCGGCTGGGGTAAAACTAGTAGTAGTTCAAACAAAGGTGGAAG
CAGCTCCGGAAACAAAGGTGGCAGCAGCAGTAGTAGTAGCAGCAGCAGCAGCAGTAAAGGCACCTCATCACATGGGACACACTCATCC
CCTGGAAACTATCCTCGCCAACCTCAAGTACCAAACCAGAATCCACCTCCCTACCCTGGTGCTGGAGGTGGGTACCCTGGTCAAGGAA
GATATCCACCAGCAGGCTCTAACCCAGGATACCCAAACCAAGGGAGCTACCCAGGACGTGCTGGATACCCTAACCAAGGTGGGTACCC
AGCCCAAGGTGGATACCCAGCCCAAGGTGGCTATCCTGCGCAAGGTGGCTATCCTGCGCAAGGAGGTTACCCAGCACAAGGTGGATAC
CCAGCGCAAGGTGGATATCCACAAGGTAACTACCCAGGACGTAGTGGTTACCCAGGACAAGGTGGTTACCCAGCACAAGGTGGCTACC
CAGGCGGAGCATCATATCCTGGAGCAGGAGCAGGGTCGTACCCCAACAGGTACCCTGGTGGAAACCCTTACCCAGTTGGTGGGTCCTA
TCCAGGTTATCCAGTCCGAGGAGGTTCCAGTCCTAACCAGTTTGGTGGAGGTGTTGGAGGAGCAGGCGGATATCCTGGAGCGGCACAG
TATCCAAACTGGAACCCCAACAATAAAATCATGAGCCCAGCCTATGGCGGCAACTATGGTGGTTACGGGTATGGTGGCAGATCACCAT
TCGCCCAGTCAGTGCAGGGCATGGGTATGGCCCCTTCTTACAAATCAAAGGGATTCGGCAAGCAAGCAATTATAGCTGCTGGGGCTGG
GGCTGTGGCTGGAATGGCTCTGGGATATGGCCTGGGAAGTTTCCCCCGCCCCCGTTTCCACTTTCACAACCCTCAGGAGGAATACTAC
TATAATCACTACATGTACAGACGGTATGGCCAGACTCCACCTGACGGTAACATGAACGGGAACACTAACTCTCAAGGCGGCATTCCTG
GTGATGCAGGATCGGCTTCTGCAGGACAAGGAAATCTTAATCCTCATGAAATCTTCCAGAATCCTCCTCCTCAGTCCTATGACAAGTT
TATGGACACGTGTATGAAGAGGACAGATTTGCTCAGAGAGAAATCGGAAGGAAGATCGAGGAGATCCCCTGACCTCTTTGAGCCTCAG
GCAGCAGAAGTAGGCAATGATCCGAGTGAAATGCAAGAAAATGTTACAAACAACAACAGCACTTCTGAAAGAACCAACTCTGCAGCTA
GTACAGATGATTCTGTAACCACAAACCCACCAGAAACACCCAGCCAACCTCAAAAGCAGACCCAAGAAAGAGCAGCAGAAGCCAATGA
TGATAATGACGGCGACACTGTTAGCATTATGGAGATTGGCTATCCTATGCTAATCGAGCAAATGAAAGCTCGACGTTGTGTTGAGCTG
TACATGGTCTACGCAGAACAGCATGCAGAAAAACAGGCACAAGCTCAAGCTAAGATGGCGGATGAAGCCAGAAACGGTAGTGGCAACC
TTCCAGGACCCATAAGCCACGGAGGCATGTTACTTATCACTAGCATCCTGTCTCTTATCTTCAGTTCCCTTCTACAC*TGA*CATTCTCA
AGTAGATGACACGTAAAACCCTTTCATCATGGCGGAGAGACAGATGAGTGAACCAATCACGCCTACGTTACCTAACAAATTTATCTTT
GATTTTGGGCAGGACATTTTTGAAAGACTACTTGAAGATTCAACAGAAAGTACTGACATTTCTCACAATCACTTTTGTTCAGTAACGT
TACTAAACTGCAACATGAGCCACCTAACAACTTCACATGAGCCTGTTCTTGGAAGCATTATATATTATAATATCATTTTCCCCCTAGC
AAAGTTCACCTTCCATCACTGCCACCTTTCTTGCATGTAGTTTGGTGAACGTTAGTTTGGCTTCCGAGACAGCATGCTGCATGAACAT
TTCATTACCTCCAGGTTTTTTTGCATGGCATTGGCATTTGGCTAATCGTTTTCAGCTGGCAACAGTAAATGAATCAAAGATTTTAATG
ACATCAGGTGGTGGCTTCTTGTTGAATTTTTTTAATGGCTCTCCTGCTTGTGTAGTACTTACTGAAACATTTGTGTTGTAGTGTTTAT
TTTCCATTATTTGGACTGTTAACCGAATTAACTGAGCTATTTATTGTTGTATTAATTTATAATTTTGACCATTTTAA**CGTTCGTTAAA**
TGTCCTCCACTGTTGGTTTTCCTATTTTATTTCCTGATGGCACTTCATACAGAATTCTCCTTGTGTTTTAATCAGCTCTTTTGTAAAA
ATGATCTAGACTTTGCCCGTTTAATCCTCTGCAGTGAAAATACTGCATTTTAGGAACTAATGGCCTGTTTCCACTGAGTGGTACTGTA

6.1.3 zCREST and Hsp70

The sequences for the Islet-1 enhancers (zCREST1, -2, -3) and of the Hsp70 promoter can be viewed in the genbank NIH genetic sequence database (http://www.ncbi.nlm.nih.gov/) under the accession numbers mentioned in chapter 4.

Danksagung

Frau Prof. Dr. Claudia A. O. Stürmer danke ich für die Bereitstellung des Themas und der Mittel, sowie der Möglichkeit meine Diplomarbeit an ihrem Lehrstuhl durchzuführen. Ganz besonderer Dank gilt auch ihren positiven, stets motivierenden Worten und, dass sie es mir ermöglichte am Prion-Meeting 2006 in Turin teilzunehmen.

Herrn Prof. Dr. Martin Scheffner möchte ich für die bereitwillige Annahme der Zweitkorrektur danken.

Dr. Edward Málaga-Trillo gilt besonderer Dank für die Betreuung meiner Arbeit, die Einführung in die Prion- und Zebrabärblingforschung und, dass er stets bereit war auch außerhalb der Uni mit mir über die Arbeit zu diskutieren.

Dr. Gonzalo Solis möchte ich für die stete Hilfe im Labor, die vielen Tipps und Tricks bei molekularen Arbeiten und seiner immer offenen und ehrlichen Meinung danken. Danke, dass du ein guter Freund geworden bist!

Yvonne Schrock und Lydia Luncz danke ich, dass sie mich in meinem gesamten Studium unterstützend begleitet haben. Danke für die Hilfe beim Lernen und Schreiben und für die wundervollen Reisen und Feiern!

Ulrike Binkle möchte ich für die nette und unterhaltsame Gesellschaft beim Arbeiten im gemeinsamen Labor danken.

Dr. Eric Rivera-Milla und Dr. Matthias Langhorst danke ich dafür, dass sie jederzeit alle meine Fragen beantwortet haben. Besonderer Dank gilt Matthias, der mir mit seinem riesigen Wissensschatz über Mikroskope immer wieder aus dem „Dunkel“ geholfen hat.

Anette-Yvonne Loos möchte ich dafür danken, dass sie meine Arbeit immer bereitwillig mit unzähligen Zebrabärbling-Babys unterstützt hat.

Marcia Rocha, Alexandra Shypitsyna, Houari Abdesselem, Friedericke Jäger, Christina Munderloh, Silvia Hannbeck und Marianne Wiechers danke ich ganz für die schöne, angenehme und lehrreiche Zeit im Labor.

All meinen Freunden und Kommilitonen in und um Konstanz danke ich für die schönen Jahre „Studentenzeit“.

Andrea Schäfer danke ich für die Einladung zum Kaffee vor 6 Jahren, ohne sie wäre ich nie nach Konstanz gekommen.

Michael Matschiner bin ich sehr dankbar für die Durchsicht meines Manuskripts. Du bist jederzeit zum Essen eingeladen!

Nathi Feiner, der besten Mitbewohnerin die es gibt, danke ich dafür, dass sie mir immer zugehört, mich aufgebaut und zum Lachen gebracht hat. Danke für unsere vielen gemeinsamen Koch- und Tratschstunden!

Marc, ich danke dir für deine erfahrenen Worte und deine Liebe.

Zuletzt möchte ich ganz besonders meiner Mama Gerlinde und meinem Papa Klaus danken, die mich immer in all meinen Vorhaben mit Rat und Tat unterstützt haben und mich bestärkten meinem eigenen Weg zu folgen. Ich danke euch dafür, dass ihr mir mein Studium und Leben in Konstanz ermöglicht habt, für eure grenzenlose Unterstützung und Liebe!

VIELEN DANK,

MANY THANKS,

MUCHAS GRACIAS!

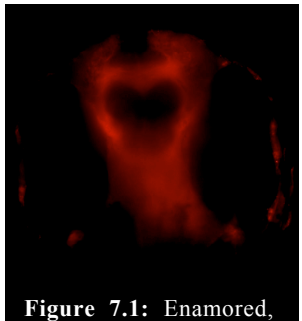


Figure 7.1: Enamored, anti-acetylated tubulin stained zebrafish embryo

Literature Cited

AGUZZI, A., and M. POLYMENIDOU, 2004 Mammalian prion biology: one century of evolving concepts. Cell **116:** 313-327.

ALPER, T., W. A. CRAMP, D. A. HAIG and M. C. CLARKE, 1967 Does the agent of scrapie replicate without nucleic acid? Nature **214:** 764-766.

ANDERSON, G. M., and S. O. FREYTAG, 1991 Synergistic activation of a human promoter in vivo by transcription factor Sp1. Mol Cell Biol **11:** 1935-1943.

BAILLY, Y., A. M. HAEBERLE, F. BLANQUET-GROSSARD, S. CHASSEROT-GOLAZ, N. GRANT *et al.*, 2004 Prion protein (PrPc) immunocytochemistry and expression of the green fluorescent protein reporter gene under control of the bovine PrP gene promoter in the mouse brain. J Comp Neurol **473:** 244-269.

BASLER, K., B. OESCH, M. SCOTT, D. WESTAWAY, M. WALCHLI *et al.*, 1986 Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. Cell **46:** 417-428.

BERNARDOS, R. L., and P. A. RAYMOND, 2006 GFAP transgenic zebrafish. Gene Expr Patterns **6:** 1007-1013.

BILLETER, M., R. RIEK, G. WIDER, S. HORNEMANN, R. GLOCKSHUBER *et al.*, 1997 Prion protein NMR structure and species barrier for prion diseases. Proc Natl Acad Sci U S A **94:** 7281-7285.

BIRNEY, E., T. D. ANDREWS, P. BEVAN, M. CACCAMO, Y. CHEN *et al.*, 2004 An overview of Ensembl. Genome Res **14:** 925-928.

BLECHINGER, S. R., T. G. EVANS, P. T. TANG, J. Y. KUWADA, J. T. WARREN, JR. *et al.*, 2002 The heat-inducible zebrafish hsp70 gene is expressed during normal lens development under non-stress conditions. Mech Dev **112:** 213-215.

BOLTON, D. C., M. P. MCKINLEY and S. B. PRUSINER, 1982 Identification of a protein that purifies with the scrapie prion. Science **218:** 1309-1311.

BROWN, D. R., K. QIN, J. W. HERMS, A. MADLUNG, J. MANSON *et al.*, 1997 The cellular prion protein binds copper in vivo. Nature **390:** 684-687.

BROWN, D. R., B. S. WONG, F. HAFIZ, C. CLIVE, S. J. HASWELL *et al.*, 1999 Normal prion protein has an activity like that of superoxide dismutase. Biochem J **344 Pt 1:** 1-5.

BUELER, H., A. AGUZZI, A. SAILER, R. A. GREINER, P. AUTENRIED *et al.*, 1993 Mice devoid of PrP are resistant to scrapie. Cell **73:** 1339-1347.

BUELER, H., M. FISCHER, Y. LANG, H. BLUETHMANN, H. P. LIPP *et al.*, 1992 Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature **356:** 577-582.

CALZOLAI, L., D. A. LYSEK, D. R. PEREZ, P. GUNTERT and K. WUTHRICH, 2005 Prion protein NMR structures of chickens, turtles, and frogs. Proc Natl Acad Sci U S A **102:** 651-655.

CAMBRAY-DEAKIN, M. A., and R. D. BURGOYNE, 1987 Posttranslational modifications of alpha-tubulin: acetylated and detyrosinated forms in axons of rat cerebellum. J Cell Biol **104:** 1569-1574.

CASHMAN, N. R., R. LOERTSCHER, J. NALBANTOGLU, I. SHAW, R. J. KASCSAK *et al.*, 1990 Cellular isoform of the scrapie agent protein participates in lymphocyte activation. Cell **61:** 185-192.

CHESEBRO, B., R. RACE, K. WEHRLY, J. NISHIO, M. BLOOM *et al.*, 1985 Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain. Nature **315:** 331-333.

CHESEBRO, B., M. TRIFILO, R. RACE, K. MEADE-WHITE, C. TENG *et al.*, 2005 Anchorless prion protein results in infectious amyloid disease without clinical scrapie. Science **308:** 1435-1439.

CHIARINI, L. B., A. R. FREITAS, S. M. ZANATA, R. R. BRENTANI, V. R. MARTINS *et al.*, 2002 Cellular prion protein transduces neuroprotective signals. Embo J **21:** 3317-3326.

CHITNIS, A. B., and J. Y. KUWADA, 1990 Axonogenesis in the brain of zebrafish embryos. J Neurosci **10:** 1892-1905.

COLLINGE, J., 1997 Human prion diseases and bovine spongiform encephalopathy (BSE). Hum Mol Genet **6:** 1699-1705.

COLLINGE, J., 2001 Prion diseases of humans and animals: their causes and molecular basis. Annu Rev Neurosci **24:** 519-550.

COLLINGE, J., M. A. WHITTINGTON, K. C. SIDLE, C. J. SMITH, M. S. PALMER *et al.*, 1994 Prion protein is necessary for normal synaptic function. Nature **370:** 295-297.

COTTO, E., M. ANDRE, J. FORGUE, H. J. FLEURY and P. J. BABIN, 2005 Molecular characterization, phylogenetic relationships, and developmental expression patterns of prion genes in zebrafish (Danio rerio). Febs J **272:** 500-513.

COULPIER, M., S. MESSIAEN, D. BOUCREAUX and M. ELOIT, 2006 Axotomy-induced motoneuron death is delayed in mice overexpressing PrPc. Neuroscience **141:** 1827-1834.

CREUTZFELDT, H. G., 1920 Über eine eigenartige herdförmige Erkrankung des Zentralnervensystems. Gesamte Neurol. Psychiatr. **57:** 1-19.

CUILLÉ, J., and P.-L. CHELLE, 1939 Experimental transmission of trembling to the goat. Comptes Rendus des Seances de'l Academie des Sciences **208:** 1058-1160.

DESLYS, J.-P., and A. PICOT, 2002 *Mad cow disease - The risk to humans*. Flammarion.

DIENER, T. O., M. P. MCKINLEY and S. B. PRUSINER, 1982 Viroids and prions. Proc Natl Acad Sci U S A **79:** 5220-5224.

DONNE, D. G., J. H. VILES, D. GROTH, I. MEHLHORN, T. L. JAMES *et al.*, 1997 Structure of the recombinant full-length hamster prion protein PrP(29-231): the N terminus is highly flexible. Proc Natl Acad Sci U S A **94:** 13452-13457.

FISCHER, M., T. RULICKE, A. RAEBER, A. SAILER, M. MOSER *et al.*, 1996 Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. Embo J **15:** 1255-1264.

FLECHSIG, E., I. HEGYI, R. LEIMEROTH, A. ZUNIGA, D. ROSSI *et al.*, 2003 Expression of truncated PrP targeted to Purkinje cells of PrP knockout mice causes Purkinje cell death and ataxia. Embo J **22:** 3095-3101.

FORCE, A., M. LYNCH, F. B. PICKETT, A. AMORES, Y. L. YAN *et al.*, 1999 Preservation of duplicate genes by complementary, degenerative mutations. Genetics **151:** 1531-1545.

GAJDUSEK, D. C., C. J. GIBBS and M. ALPERS, 1966 Experimental transmission of a Kuru-like syndrome to chimpanzees. Nature **209:** 794-796.

GIBBS, C. J., JR., D. M. ASHER, A. KOBRINE, H. L. AMYX, M. P. SULIMA *et al.*, 1994 Transmission of Creutzfeldt-Jakob disease to a chimpanzee by electrodes contaminated during neurosurgery. J Neurol Neurosurg Psychiatry **57:** 757-758.

HAFFTER, P., M. GRANATO, M. BRAND, M. C. MULLINS, M. HAMMERSCHMIDT *et al.*, 1996 The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. Development **123:** 1-36.

HAIGH, C. L., J. A. WRIGHT and D. R. BROWN, 2007 Regulation of Prion Protein Expression by Noncoding Regions of the Prnp Gene. J Mol Biol.

HALLORAN, M. C., M. SATO-MAEDA, J. T. WARREN, F. SU, Z. LELE *et al.*, 2000 Laser-induced gene expression in specific cells of transgenic zebrafish. Development **127:** 1953-1960.

HARRIS, D. A., 1999 Cellular biology of prion diseases. Clin Microbiol Rev **12:** 429-444.

HARRIS, D. A., P. LELE and W. D. SNIDER, 1993 Localization of the mRNA for a chicken prion protein by in situ hybridization. Proc Natl Acad Sci U S A **90:** 4309-4313.

HARRIS, D. A., and H. L. TRUE, 2006 New insights into prion structure and toxicity. Neuron **50:** 353-357.

HIGASHIJIMA, S., Y. HOTTA and H. OKAMOTO, 2000 Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. J Neurosci **20:** 206-218.

INBAL, A., J. TOPCZEWSKI and L. SOLNICA-KREZEL, 2006 Targeted gene expression in the zebrafish prechordal plate. Genesis **44:** 584-588.

INOUE, S., M. TANAKA, M. HORIUCHI, N. ISHIGURO and M. SHINAGAWA, 1997 Characterization of the bovine prion protein gene: the expression requires interaction between the promoter and intron. J Vet Med Sci **59:** 175-183.

JAKOB, A., 1921 Über eigenartige Erkrankungen des Zentralnervensystems mit bemerkenswertem anatomischem Befund. Spastische Pseudosklerose-Encephalomyelopathie mit disseminierten Degenerationsherden). Z. Gesamte Neurol. Psychiatr. **64:** 147-228.

KANE, D. A., and C. B. KIMMEL, 1993 The zebrafish midblastula transition. Development **119:** 447-456.

KARLSSON, O., S. THOR, T. NORBERG, H. OHLSSON and T. EDLUND, 1990 Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. Nature **344:** 879-882.

KIMMEL, C. B., W. W. BALLARD, S. R. KIMMEL, B. ULLMANN and T. F. SCHILLING, 1995 Stages of embryonic development of the zebrafish. Dev Dyn **203:** 253-310.

KOCISKO, D. A., J. H. COME, S. A. PRIOLA, B. CHESEBRO, G. J. RAYMOND *et al.*, 1994 Cell-free formation of protease-resistant prion protein. Nature **370:** 471-474.

KOSTER, R. W., and S. E. FRASER, 2001 Tracing transgene expression in living zebrafish embryos. Dev Biol **233:** 329-346.

KREGEL, K. C., 2002 Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol **92:** 2177-2186.

LEE, I. Y., D. WESTAWAY, A. F. SMIT, K. WANG, J. SETO *et al.*, 1998 Complete genomic sequence and analysis of the prion protein gene region from three mammalian species. Genome Res **8:** 1022-1037.

LI, G., and D. C. BOLTON, 1997 A novel hamster prion protein mRNA contains an extra exon: increased expression in scrapie. Brain Res **751:** 265-274.

LYSEK, D. A., C. SCHORN, L. G. NIVON, V. ESTEVE-MOYA, B. CHRISTEN *et al.*, 2005 Prion protein NMR structures of cats, dogs, pigs, and sheep. Proc Natl Acad Sci U S A **102:** 640-645.

MALLUCCI, G. R., M. D. WHITE, M. FARMER, A. DICKINSON, H. KHATUN *et al.*, 2007 Targeting cellular prion protein reverses early cognitive deficits and neurophysiological dysfunction in prion-infected mice. Neuron **53:** 325-335.

MANSON, J., J. D. WEST, V. THOMSON, P. MCBRIDE, M. H. KAUFMAN *et al.*, 1992 The prion protein gene: a role in mouse embryogenesis? Development **115:** 117-122.

MARTIN, C. C., P. TANG, G. BARNARDO and P. H. KRONE, 2001 Expression of the chaperonin 10 gene during zebrafish development. Cell Stress Chaperones **6:** 38-43.

MIESBAUER, M., T. BAMME, C. RIEMER, B. OIDTMANN, K. F. WINKLHOFER *et al.*, 2006 Prion protein-related proteins from zebrafish are complex glycosylated and contain a glycosylphosphatidylinositol anchor. Biochem Biophys Res Commun **341:** 218-224.

MO, H., R. C. MOORE, F. E. COHEN, D. WESTAWAY, S. B. PRUSINER *et al.*, 2001 Two different neurodegenerative diseases caused by proteins with similar structures. Proc Natl Acad Sci U S A **98:** 2352-2357.

MOUILLET-RICHARD, S., M. ERMONVAL, C. CHEBASSIER, J. L. LAPLANCHE, S. LEHMANN *et al.*, 2000 Signal transduction through prion protein. Science **289:** 1925-1928.

NÜSSLEIN-VOLHARD, C., and R. DAHM, 2002 *Zebrafish : a practical approach*. Oxford University Press, New York.

OESCH, B., D. WESTAWAY, M. WALCHLI, M. P. MCKINLEY, S. B. KENT *et al.*, 1985 A cellular gene encodes scrapie PrP 27-30 protein. Cell **40:** 735-746.

PAN, K. M., M. BALDWIN, J. NGUYEN, M. GASSET, A. SERBAN *et al.*, 1993 Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci U S A **90:** 10962-10966.

PAULY, P. C., and D. A. HARRIS, 1998 Copper stimulates endocytosis of the prion protein. J Biol Chem **273:** 33107-33110.

PEAKE, I., 1989 The polymerase chain reaction. J Clin Pathol **42:** 673-676.

PIPERNO, G., and M. T. FULLER, 1985 Monoclonal antibodies specific for an acetylated form of alpha-tubulin recognize the antigen in cilia and flagella from a variety of organisms. J Cell Biol **101:** 2085-2094.

PREMZL, M., M. DELBRIDGE, J. E. GREADY, P. WILSON, M. JOHNSON *et al.*, 2005 The prion protein gene: identifying regulatory signals using marsupial sequence. Gene **349:** 121-134.

PREMZL, M., J. E. GREADY, L. S. JERMIIN, T. SIMONIC and J. A. MARSHALL GRAVES, 2004 Evolution of vertebrate genes related to prion and Shadoo proteins--clues from comparative genomic analysis. Mol Biol Evol **21:** 2210-2231.

PRUSINER, S. B., 1982 Novel proteinaceous infectious particles cause scrapie. Science **216:** 136-144.

PRUSINER, S. B., 1989 Scrapie prions. Annu Rev Microbiol **43:** 345-374.

PRUSINER, S. B., 1991 Molecular biology of prion diseases. Science **252:** 1515-1522.

PRUSINER, S. B., 1997 Prion diseases and the BSE crisis. Science **278:** 245-251.

PRUSINER, S. B., 1998 Prions. Proc Natl Acad Sci U S A **95:** 13363-13383.

PUCKETT, C., P. CONCANNON, C. CASEY and L. HOOD, 1991 Genomic structure of the human prion protein gene. Am J Hum Genet **49:** 320-329.

RIVERA-MILLA, E., B. OIDTMANN, C. H. PANAGIOTIDIS, M. BAIER, T. SKLAVIADIS *et al.*, 2006 Disparate evolution of prion protein domains and the distinct origin of Doppel- and prion-related loci revealed by fish-to-mammal comparisons. Faseb J **20:** 317-319.

ROSS, L. S., T. PARRETT and S. S. EASTER, JR., 1992 Axonogenesis and morphogenesis in the embryonic zebrafish brain. J Neurosci **12:** 467-482.

ROTHENBERG, E. V., 2001 Mapping of complex regulatory elements by puffer-fish/zebrafish transgenesis. Proc Natl Acad Sci U S A **98:** 6540-6542.

RUDD, P. M., M. R. WORMALD, D. R. WING, S. B. PRUSINER and R. A. DWEK, 2001 Prion glycoprotein: structure, dynamics, and roles for the sugars. Biochemistry **40:** 3759-3766.

SAEKI, K., Y. MATSUMOTO and T. ONODERA, 1996 Identification of a promoter region in the rat prion protein gene. Biochem Biophys Res Commun **219:** 47-52.

SANCHEZ-GARCIA, I., H. OSADA, A. FORSTER and T. H. RABBITTS, 1993 The cysteine-rich LIM domains inhibit DNA binding by the associated homeodomain in Isl-1. Embo J **12:** 4243-4250.

SANTUCCIONE, A., V. SYTNYK, I. LESHCHYNS'KA and M. SCHACHNER, 2005 Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59fyn and to enhance neurite outgrowth. J Cell Biol **169:** 341-354.

SCHMITT-ULMS, G., G. LEGNAME, M. A. BALDWIN, H. L. BALL, N. BRADON *et al.*, 2001 Binding of neural cell adhesion molecules (N-CAMs) to the cellular prion protein. J Mol Biol **314:** 1209-1225.

SHYU, W. C., S. Z. LIN, M. F. CHIANG, D. C. DING, K. W. LI *et al.*, 2005 Overexpression of PrPC by adenovirus-mediated gene targeting reduces ischemic injury in a stroke rat model. J Neurosci **25:** 8967-8977.

SOLFOROSI, L., J. R. CRIADO, D. B. MCGAVERN, S. WIRZ, M. SANCHEZ-ALAVEZ *et al.*, 2004 Cross-linking cellular prion protein triggers neuronal apoptosis in vivo. Science **303:** 1514-1516.

SPIELHAUPTER, C., and H. M. SCHATZL, 2001 PrPC directly interacts with proteins involved in signaling pathways. J Biol Chem **276:** 44604-44612.

SPRAGUE, J., L. BAYRAKTAROGLU, D. CLEMENTS, T. CONLIN, D. FASHENA *et al.*, 2006 The Zebrafish Information Network: the zebrafish model organism database. Nucleic Acids Res **34:** D581-585.

STAHL, N., M. A. BALDWIN, D. B. TEPLOW, L. HOOD, B. W. GIBSON *et al.*, 1993 Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing. Biochemistry **32:** 1991-2002.

STEELE, A. D., J. G. EMSLEY, P. H. OZDINLER, S. LINDQUIST and J. D. MACKLIS, 2006 Prion protein (PrPc) positively regulates neural precursor proliferation during developmental and adult mammalian neurogenesis. Proc Natl Acad Sci U S A **103:** 3416-3421.

STUERMER, C. A., M. F. LANGHORST, M. F. WIECHERS, D. F. LEGLER, S. H. VON HANWEHR *et al.*, 2004 PrPc capping in T cells promotes its association with the lipid raft proteins reggie-1 and reggie-2 and leads to signal transduction. Faseb J **18:** 1731-1733.

THOR, S., J. ERICSON, T. BRANNSTROM and T. EDLUND, 1991 The homeodomain LIM protein Isl-1 is expressed in subsets of neurons and endocrine cells in the adult rat. Neuron **7:** 881-889.

UEMURA, O., Y. OKADA, H. ANDO, M. GUEDJ, S. HIGASHIJIMA *et al.*, 2005 Comparative functional genomics revealed conservation and diversification of three enhancers of the isl1 gene for motor and sensory neuron-specific expression. Dev Biol **278:** 587-606.

VAN ROSMALEN, J. W., and G. J. MARTENS, 2006a Cell type-specific transgene expression of the prion protein in Xenopus intermediate pituitary cells. Febs J **273:** 847-862.

VAN ROSMALEN, J. W., and G. J. MARTENS, 2006b Transgene expression of prion protein induces crinophagy in intermediate pituitary cells. J Neurobiol **67:** 81-96.

WEISSMANN, C., and H. BUELER, 2004 A mouse to remember. Cell **116:** S111-113, 112 p following S113.

WESTAWAY, D., C. COOPER, S. TURNER, M. DA COSTA, G. A. CARLSON *et al.*, 1994a Structure and polymorphism of the mouse prion protein gene. Proc Natl Acad Sci U S A **91:** 6418-6422.

WESTAWAY, D., S. J. DEARMOND, J. CAYETANO-CANLAS, D. GROTH, D. FOSTER *et al.*, 1994b Degeneration of skeletal muscle, peripheral nerves, and the central nervous system in transgenic mice overexpressing wild-type prion proteins. Cell **76:** 117-129.

WILLE, H., M. D. MICHELITSCH, V. GUENEBAUT, S. SUPATTAPONE, A. SERBAN *et al.*, 2002 Structural studies of the scrapie prion protein by electron crystallography. Proc Natl Acad Sci U S A **99:** 3563-3568.

WILSON, S. W., L. S. ROSS, T. PARRETT and S. S. EASTER, JR., 1990 The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, Brachydanio rerio. Development **108:** 121-145.

XIAO, T., T. ROESER, W. STAUB and H. BAIER, 2005 A GFP-based genetic screen reveals mutations that disrupt the architecture of the zebrafish retinotectal projection. Development **132:** 2955-2967.

ZAHN, R., A. LIU, T. LUHRS, R. RIEK, C. VON SCHROETTER *et al.*, 2000 NMR solution structure of the human prion protein. Proc Natl Acad Sci U S A **97:** 145-150.

ZEIDLER, M., and J. W. IRONSIDE, 2000 The new variant of Creutzfeldt-Jakob disease. Rev Sci Tech **19:** 98-120.

ZIGAS, V., and D. C. GAJDUSEK, 1957 Kuru: clinical study of a new syndrome resembling paralysis agitans in natives of the Eastern Highlands of Australian New Guinea. Med J Aust **44:** 745-754.

Web site Cited

ENSEMBL DATABASE 2007: Zebrafish genome browser. Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom. Zv6, Mar 2006; Database version 42.6c [cited 2007 Feb/Mar]. Available from: http://www.ensembl.org/Danio_rerio/index.html

GENBANK NIH GENETIC SEQUENCE DATABASE 2007: National library of medicine, National institutes of health [cited 2007 Feb/Mar]. Available from: (http://www.ncbi.nlm.nih.gov/)

PREMIER BIOSOFT NETPRIMER: Premier Biosoft International NetPrimer primer analysis software, Corina Way, Palo Alto, CA 94303-4504, USA [cited 2007 Feb/Mar]. Available from: http://www.premierbiosoft.com/netprimer/index.html

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Diplomarbeit mit dem Thema „Characterization of gene regulation and neuronal transgene expression of prion protein in zebrafish" selbständig angefertigt habe. Es wurden nur die in der Arbeit ausdrücklich benannten Quellen und Hilfsmittel benutzt. Wörtlich oder sinngemäß übernommenes Gedankengut habe ich als solches kenntlich gemacht.

Diese Arbeit wird nach Abschluss des Prüfungsverfahrens der Universitätsbibliothek Konstanz übergeben und ist durch Einsicht und Ausleihe somit der Öffentlichkeit zugänglich. Als Urheber der anliegenden Arbeit stimme ich diesem Verfahren zu.

Ort, Datum

Julia M.I. Barth